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# Organic and inorganic Pb affects autochthonous bacterial communities and phytoextraction potentials of plant species grown in a formerly industrial soil contaminated by tetraethyl-lead

S.S.D. AGR16

**Coordinatore:** Prof. Roberto Bassi

**Tutor:** Prof. Giovanni Vallini
**Co-Tutor**: Dott.ssa Silvia Lampis

**Dottoranda**: Dott.ssa Anita Ferrari

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# Abbreviations

| | |
|---|---|
| **ACC** | 1-aminocyclopropane-1-carboxylate |
| **DEL** | diethyl-lead |
| **EDTA** | ethylenediaminetetraacetic acid |
| **EPA** | Environmental Protection Agency |
| **IAA** | indole-3-acetic acid |
| **PAH** | polycyclic aromatic hydrocarbons |
| **PGPR** | Plant growth promoting rhizobacteria |
| **TAL** | tetraalkyl-lead |
| **TEL** | tetraethyl-lead |
| **TML** | tetramethyl-lead |
| **TREL** | triethyl-lead |
| **TRML** | trimethyl-lead |

# Abstract/Riassunto

# Abstract


## *Introduction*

Releasing of heavy metals into the environment, as a consequence of their wide use in anthropogenic activities, is worldwide of great impact to human health. Nowadays lead in particular is worldwide one of the pollutants of major concern due to its widespread diffusion, persistence and toxicity (Lu *et al.*, 2005; Hill, 2004). Lead in fact has been widely used since ancient time and plays a central role in the industrial economy. Besides, its spreading in the environment is also connected to both agricultural and urban activities, such as land application of sewage sludge, smelting operations and use of leaded petrol (Thornton *et al.*, 2001; Hill, 2004; International Lead association, 2009).

Indeed Tetraethyl-lead (TEL) was used since the beginning of the last century as a petrol additive because of its anti-knocking properties, which increase the fuel octane rating and efficiency. Despite organic Pb has been banned from petrol in most countries due to its high toxicity, its use for almost a century in automotive petrol has lead to an ubiquitous pollution with both organic and inorganic Pb and more severe soil-water contamination at oil refineries and petrol stations (Gallert *et* Winter, 2002; Lazzaro *et al.*, 2006). While inorganic Pb is an aspecific toxin interacting with metalloenzymes, TEL is essentially a severe neurotoxin affecting mainly the nervous system (Godwin, 2001; World Health Organization, 2007; Environmental and Occupational Medicine, 2007). Remediation of contaminated areas is therefore essential and imposed by law.

Although the mechanism is still not clear, in soil TEL is known to be degraded both biologically and chemically in sequential de-alchylations and a reduction of environmental risk is achieved by the complete mineralization to the much less toxic and mobile inorganic Pb (Teeling *et* Cypionka, 1997; Gallert *et* Winter, 2002; Ou *et al.*, 1994). Actually considering inorganic Pb bioavailability in soil, it generally decreases with increasing residence time, due to the reactions between metal ions and soils, including complexation, adsorption, and precipitation of metal ions - as carbonates, hydroxides and phosphates - in the soil particles surface or diffusion into the mesopores and micropores of soil (Lu *et al.*, 2005). However, as far as organic lead and its degradation are concerned, only few studies are available in literature.

Considering the physical-chemical remediation technologies, although they can be effective and applicable at high contamination levels, they are expensive and invasive - disrupting both soil structure and biological activity - and are not applicable for extensive areas (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 Phytoremediation approach enhanced by microorganisms - based on the use of plant in synergy with microorganisms – offers a low cost, eco friendly and *in-situ* applicable method to remediate and restore perturbed areas (McGuinness *et* Dowling, 2009; Manousaki *et* Nicolas, 2009; Shukla *et al.*, 2010).

In this context the present PhD study deals with a real case of a soil contaminated by both organic and inorganic lead in the former industrial area SLOI (*Società Lavorazioni Organiche ed Inorganiche*) in Trento. SLOI was one of the few leader companies in Europe manufacturing anti-knocking additives for automotive petrol for almost 40 years till the end of the '70s. Nowadays a high contamination is present in the entire former industrial area reaching values up to 23.000 mg/kg of inorganic Pb and reporting an esteem of approximately 168 tones of total lead, of which at least 62 tones of alkyl-lead. This area has been in fact declared site of national concern by the Environmental Department (DM 468/01) – i.e. implying high environmental risk and priority remediation.

### *Aims*

Dealing with a real case of the dismissed Ex-SLOI area, the present PhD study can be divided in two main sections; the first one is focused on the study and characterization of the soil autochthonous bacterial community selected by and acclimated to the contamination of both organic and inorganic Pb, present in the former industrial area for over half century. Three distinct sampling points were therefore chosen within the area with different contamination levels (point I,II,III), also including the most contaminated Hot Spot, reporting the highest Pb contamination detected within the entire area of 23.000 mg/kg inorganic Pb and over 1000 mg/kg organic Pb. This study included both culture-dependent and culture-independent techniques, and the primary objectives of this first part were:

- to study the biodiversity and composition of the soil autochthonous bacterial community at different contamination levels within the area, to evaluate the impact of a long-term exposure to organic and inorganic Pb and the resistance and bioremediation potential of the selected micro flora;
- to isolate and characterize members of the soil microbial community in relation to their resistance and plant growth promoting potential, even in the perspective of a Phytoremediation application in a biaugmentation protocol.

On the other side the second section of this PhD thesis focused on the interaction of the examined autochthonous soil micro flora and plants in relation to Pb contamination, in a Phytoremediation approach. Phytoextraction in particular specifically refers to the use of pollutant-accumulating plants that can extract and translocate contaminants to the harvestable portions, which can then be removed from the site. The efficiency of a phytoremediation/phytoextraction process depends directly on both biomass production and accumulation of the contaminant by the plant. However plants not only tolerant but also characterized by an extraordinary ability to accumulate the contaminants, known as hyperaccumulators, have usually a small above-ground biomass, slow growth and a long maturity phase, affecting therefore the process efficiency. Hence a proposed approach is the use of tolerant plants with relatively higher accumulation ability as compared to most other plants (but with lower ability as compared to hyperaccumulators) but higher biomass production (Marchiol *et al.*, 2004; Karami *et* Shamsuddin, 2010).
In particular two plants were included in the study: *Brassica juncea* and *Apocynum cannabinum*. While the first one is a crop plant known to accumulate various metals including inorganic Pb (Liu *et al.*, 2000; Kapourchal *et al.*, 2009; Sheng *et al.*, 2008a), the second was suggested for both organic and inorganic Pb phytoextraction, although only few data are reported in literature (Cunningham, 1994).
In this context the main objectives were:

- to evaluate the interaction of the stress adapted autochthonous micro flora established within the examined area and its possible synergistic role in a Plant-Rhizobacteria system, in relation to both inorganic and organic Pb;
- to study the tolerance and basic phytoremediation potential of *Brassica juncea* and *Apocynym cannabinum* in relation to the examined combined contamination of inorganic and organic Pb.

### *Materials and methods*

The three sampling points chosen within the Ex-SLOI area – along with the above mentioned Hot Spot - were identified as Point I: with a contamination >2000 mg/kg of inorganic lead and <50 mg/kg of organic Pb, and a co-contamination of about 20-30 mg/kg hydrocarbons; Point II: the most contaminated of the three, with levels >4000 mg/kg for inorganic Pb and >100 mg/kg of organic Pb; Point III: with the lowest content of both organic and inorganic Pb but co-contaminated by Hg in the order of tens of mg/kg.

For each examined point the culture-dependent approach involved the enumeration of the cultivable microflora, along with the direct isolation from soil and taxonomic identification of heterotrophic aerobic eubacterial components of the microbial community (from rich-medium Nutrient and low-nutrient medium Reasonar's2 agar). Considering the long-term high contamination present in the area, the direct isolation and identification of the microbial community members was of particular interest.
Besides, to push the selection further on most resistant and metabolically potential strains, enrichment cultures were performed supplementing soil, in minimal medium, with 125 mg/l TEL, respectively as sole carbon source (named DM), and with addiction of 0,1% yeast extract (named DMY). After 8 weeks of incubation in the dark, strains selected were isolated in pure cultures and Operational taxonomic units (OTUs) were identified.
Cultivation-based and molecular methods were therefore used to characterize the most represented OTUs obtained from direct isolation, and the OTUs selected through the strict conditions imposed in laboratory by the enrichment cultures, and by the highest contamination *in-situ* at the Hot Spot. The strains were characterized in relation to their metal resistance, degrading potential and Plant growth promoting (PGP) traits. Minimum inhibitory concentration (MIC) for organic and inorganic Pb were therefore determined, and a molecular study was performed targeting the resistance determinants for Pb and other heavy metals – which are often associated - namely *pbr, czc, chr*, *ncc* and *mer* genes, respectively responsible for resistance to Pb, Cd-Zn-Co, Cr, Ni and Hg (Abou-Shanab *et al.*, 2007; Borremans *et al.*, 2001).
Besides the study examined the determinants *nah* and *phn*, encoding the large (α)subunits of Polycyclic aromatic hydrocarbon (PAH) dioxygenases for the degradation of PAH – associated to petrol and detected in Point I – and *alk,* encoding an alkane hydroxylase involved in the first step of aliphatic alkanes' degradation, and potentially connected to a degrading potential towards organic lead (Smits *et al.*, 1999; Laurie *et* Lloyd-Jones, 1999).
Strains were moreover screened for the PGP characteristics of indoleacetic acid (IAA) production, and 1-amino-cyclopropane-1-carboxylic acid (ACC)-deaminase activity, of particular interest in the prospective of a phytoremediation approach.
In the culture-independent approach, in order to evaluate the potential and biodiversity of the soil bacterial community comprehensive of the uncultivable fraction, the above-described molecular study was also performed on the total DNA extracted from soil, and a Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis was performed on soil samples from each examined sampling point.

As far as the Phytoremediation section is concerned, a lab-scale and a field-scale trials were performed with the two plant species under study and the soil of the 3 sampling points (I,II,III) chosen and studied within the Ex-SLOI area.
In order to evaluate the actual role of the autochthonous microbial community on plant growth and on the phytoremediation process, for each sampling point (I,II,III) 1 kg lab-scale mesocosms were set up in parallel, respectively with the soil untreated and sterilized by thermal treatment. Cultivation experiments were carried out in triplicate, in a glasshouse at temperature $\geq$ 24°C and with a photoperiod of 12 hours.
In the case of *B.juncea* for untreated, sterilized and control mesocosms - set up with an uncontaminated agricultural soil - all plants from a mesocosm were collected at three different times:

- T1: after 2 months from sowing
- T2: after 3 months from sowing
- T3: after 4 months from sowing

For *Apocynum cannabinum* mesocosms, due to its slower growth, a unique sampling was carried out:

- Tf: after 6 months from sowing

Dry weight of shoot and root biomass and lead content were measured to evaluate plant growth and Pb uptake. Soil samples were also analysed for total Pb content. Moreover for *Brassica juncea* mesoscosms, the alkyl-lead content in soil explored by the roots was analysed.

Besides 3 different parameters were monitored: Lead phytoextracted in shoots tissues per plant - defined as µg Pb Accumulated/plant = tissue [Pb] x dried biomass – which gives a measure of the contaminant actually extracted and therefore of the system efficiency; the Bioaccumulation Factor (BF) that is the concentration of Pb in tissues divided by that in soil - defined as $C_{TISSUE}/C_{SOIL}$ – which measures the plant capability to bioconcentrate the toxic element (Pb) into its tissues; the Translocation factor (TF), which refers to the concentration of heavy metal in shoots divided by that in roots - defined as $C_{SHOOTS}/C_{ROOTS}$ - which gives a quantification of the plant capability of translocating Pb taken up from roots to the harvestable aerial part.

Besides a PCR-DGGE analyses was performed on *Brassica juncea* rhizosphere soil samples along the trial, in order to characterize the composition of the bacterial community along the phytoremediation process.

A scale up to field trial was also performed to test the plant in the uncontrolled conditions as those of a real scale process. In this case two cultivars of *Brassica juncea* were included in the trial (PI173874 - used in lab-scale – and PI426308), and at the 3 sampling points I,II and III parcels of respectively 60, 23 e 45 square meters were set up, depending on the size of accessible and available area.

In the case of *Brassica juncea* at least 4 plants for each cultivar were collected at each sampling time:

- T1: after 6 weeks from the sowing
- T2: after 14 weeks from the sowing
- T3: after 18 weeks from the sowing

For *A. cannabinum,* as in lab-scale mesocosms, a unique sampling of all plants was carried out:

- T1: after 16 months from sowing

Dry weight of shoot and root biomass and lead content were measured to evaluate plant growth and Pb uptake.

## *Results and discussion*

The characterization of the autochthonous soil microbial community was performed complementing both a culture-dependent and -independent approach. Actually while the first approach, relying on cultivation techniques of classical microbiology, allows the isolation, subsequent characterization and use of strains obtained in pure culture, the second - independent from strains' cultivability - is comprehensive of the unculturable fraction of a microbial community.

The results obtained by the culture analysis performed indicated the selection within the Ex-SLOI area of a tolerant soil bacterial community - selected by and acclimated to the contamination present in the area for over half century - and characterized by an heterogenic structure, a rich biodiversity - for a perturbed soil - and resistance potential. Actually all the isolated strains - relating respectively to 21 gram-negative and to 7 gram-positive distinct genera - displayed high homologies with bacteria diffused in contaminated soils, capable of degrading recalcitrant pollutants and hydrocarbons and reporting high resistances to heavy metals.

Moreover a detailed analysis pointed out the preponderance of gram-negative Proteobacteria. Actually this *phylum* included the majority of OTUs directly isolated from the most contaminated examined sampling points, including the Hot Spot. It also accounted for the totality of strains isolated in the strictest condition imposed in laboratory by the Enrichment culture set-up with TEL as sole carbon source - performed to isolate the strains most resistant and potentially able to degrade organic Pb.

The molecular analysis performed further indicated the selection of dominant members within the autochthonous bacterial community exerted by the long-term contamination. In particular a distinct

ribotype was observed in the most polluted Hot Spot, indicating the selection for a specific microbial community. It is interesting to notice as the results of the molecular analysis were consistent with those obtained by the culture approach. Indeed the sequencing results obtained for some of the major bands of the DGGE profiles indicated the predominance of the Proteobacteria *phylum* at the sampling points under study, even at the highest contaminated spot identified within the Ex-SLOI area. The molecular analysis also allowed identifying genera not detected within the culture study.

Besides, the molecular analysis targeting genetic determinants for heavy metal resistance and degrading potential towards hydrocarbons - along with the MIC determination - evidenced the heavy metal resistance within the autochthonous microbial community, with strains reporting genes for multiple resistances. A degrading potential towards hydrocarbons was identified by the metagenomic molecular analysis targeting the whole autochthonous microbial community, further confirming the value of a combined approach integrating molecular and culture techniques.

In particular 8 strains displayed at least one heavy metal resistance determinant, while 5 of them presented the concomitant presence of multiple resistances. It is worth noticing that heavy metal resistance determinants were exclusively detected within the most represented classes of Beta (*Cupriavidus, Ralstonia* and *Delftia* genera) and Gamma-proteobacteria (*Pseudomonas* and *Stenotrophomonas* genera), confirming the high resistance potential of the gram-negative population within the indigenous community. In particular a strain of *Cupriavidus campinensis* – isolated from the most contaminated Hot Spot - displayed at the same time 4 resistance determinants: for Pb, Hg, Cr and Cd-Zn-Co.

Considering also the microbial ability of promoting plant growth, the high represented class of Gamma-proteobacteria included the 2 genera - *Pseudomonas* and *Stenotrophomonas* - whose members within the autochthonous community demonstrated both examined PGP (Plant growth promoting) traits.

Moreover in the screening for PGP characteristics the 68% of examined OTUs possessed 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.

Besides to the Gamma-proteobacteria genera *Pseudomonas* and *Stenotrophomonas*, and to the *Delftia* genus of the Beta class, belong the 3 OTUs reporting both Heavy metal resistance determinants and PGP traits: namely OTU A5 *Delftia* sp., OTU A10 *Pseudomonas putida* and OTU A16 *Stenotrophomonas maltophilia,* therefore of particular interest even in the context of a Phytoremediation approach in a biaugmentation protocol. Actually tolerance to the concentration of heavy metal is 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).

The strain *Stenotrophomonas maltophilia* drew particular attention reporting at the same time three heavy metal resistance determinants - to Pb, Hg and Ni - and both analyzed potential PGP traits.

In other members of the Proteobacteria, such as *Cupriavidus, Delftia, Alcaligenes* and *Variovorax* of the Beta class and *Ochrobactrum* and *Agrobacterium* of the Alpha class, have been also detected heavy metal resistances or PGP traits and are therefore also interesting in a bioremediation/phytoremediation context.

The study performed indicated therefore the selection imposed by the contamination of a resistant microbial cenosis, with members of particular high resistance potential belonging to the most represented gram-negative Proteobacteria *phylum*, and at the same time characterized by a high PGP potential.

Considering the minor gram-positive population detected within the microbial community, the majority of the gram-positive Firmicutes and Actinobacteria were directly isolated from the less contaminated examined sampling point III. Nevertheless few member of the Actinobacteria were also detected in enrichment cultures DMY and two genera *Microbacterium* and *Arthrobacter* were part of the indigenous

culturable community selected within the most contaminated Hot Spot. Besides, members of these genera also displayed PGP traits. This indicated that also the gram-positive Actinobacteria *phylum,* although less represented within the cultivable fraction of the indigenous community, includes community members of high resistance and bioremediation potential.

Considering the interaction of indigenous micro flora with the plants studied in a Phytoremediation approach, the results of the lab-scale study showed, at all the three sampling points under study, in both species a higher growth in presence of the examined indigenous micro flora. Moreover in *B.juncea* higher Pb concentrations were also detected in shoot tissues in untreated mesocoms, pointing out the synergistic effect on both plants growth and on Phytoextraction efficiency exerted by the autochtonous micro flora selected by and adapted to the long-term Pb contamination.
Actually, comparing untreated and sterilized *B. juncea* lab-scale mesocosms, total Pb accumulated in shoots per plant - defined as µg Pb/plant - generally increased in the range of 120%-400%, reaching an 1700% increase in the most contaminated point II. As far as *A. cannabinum* is concerned, total Pb accumulated in shoots per plant increased from about 80% to 260% in presence of the soil autochthonous micro flora.
Indeed, the molecular analysis performed on the rhizosphere soil of the *Brassica juncea* mesocosms, allowed the detection along all the trial of members of the indigenous bacterial community for which are reported plant growth promoting activities, degrading potential and also phytoextraction assistance. The results are also in agreement with those above reported on the characterization of the autochthonous micro flora, and with literature evidences on promotion of plant growth and metal uptake in hyperaccumulator or non-hyperaccumulator plants by heavy metal-resistant bacteria (Sheng *et al.*, 2008a). Moreover, the analyses performed on the speciation of the organic Pb compounds along the trial in soil of *B.juncea* mesocosms, pointed out a progressive degradation of organic lead during the phytoremediation process, suggesting a promotion by the plant-micro flora system of the mineralization of organic Pb - lowering therefore the environmental risk to it associated.
As far as the examined plants are concerned, obtained data indicated tolerance and resistance of both plants towards this kind of contamination combining inorganic and, even if in lower levels, organic Pb.
For the two tested cultivars of *B.juncea* no significant differences were detected in either Pb content or biomass production, reaching almost the same results. On the other side comparing the 2 plant species, *A. cannabinum* showed a lower shoot biomass production and a much slower growth.
Although *A. cannabinum* reached the highest detected Pb concentration within its tissues, it displayed a sharp preferential accumulation within roots, while accumulation in the harvestable part of the plant is the main objective of the Phytoextraction process.
Despite Pb is a challenging metal tightly bound in most soils, data obtained in lab-scale and field trial showed for *Brassica juncea* quite high Pb concentrations in its shoots tissues up to 270 mg/kg d.w. On the other side even if *Apocynum cannabinum* was able to reach slightly higher shoots concentration in lab-conditions, almost one order of magnitude decrease was detected in field trial.
However in comparison to the lab-scale trial, for both species a much higher growth was observed in open field conditions, with a biomass production of at least one order of magnitude higher.
It has moreover to be considered that a high TF value in plant is important in practical phytoremediation of heavy metal-contaminated soils, as a preferential accumulation in the harvestable part of the plant can enable phytoremediation by its only harvesting, thus simplifying the agricultural practices. In the present study, both *B. juncea* cultivars showed 0,2-0,7 TF values, comparing well with those reported in literature (Kumar *et al.*, 1995; Xiong, 1998; Marchiol *et al.*, 2004). It has in fact to be mentioned that although TF value >1 would be desirable, for lead hyperaccumulating plants, which usually have a higher shoot/root

ratio of lead content in plant than the non-hyperaccumulators, are reported values of 0.04-0.1 TF (Kumar *et al.*, 1995; Xiong, 1998). Considering *A.cannabinum,* despite the higher TF values detected in lab-scale mesocosms, values <0,15 were detected in field condition, as expected due to the low shoot concentrations reached in open field.

Especially for *B.juncea*, the results pointed out a positive correlation between the lab-scale experiment performed in the controlled conditions of a glasshouse and the field trial set up directly at the Ex-SLOI area.

Moreover the increase in biomass production scaling up from 1kg mesocosms pots to open field was reflected in the values of Pb phytoextracted - defined as µg Pb/plant - which evaluates the actual Phytoremediation efficiency.

Actually, higher phytoextraction efficiency was obtained for both plants in field-scale experiment, accumulating a comparable amount of Pb in their harvestable part. However, taking moreover into account the lower time of growth of *B.juncea* trial, an about 4 times shorter time, results obtained indicated therefore a higher Phytoextraction potential associated to this plant in relation to the examined contamination.

It has to be mentioned that not only no chelating agents, but also no fertilizer or any agricultural practice were performed during the field trial; improvements to phytoextraction could hence be obtained by simply implementing specific agronomic practices that may have a positive influence on the global efficiency of the process (Quartacci *et al.*, 2006).

It has however to be considered that Biaccumulation factors values – evaluated for lab-scale mesocosms - were very low and plant growth was highly affected in the most contaminated sampling point II, stopping from performing the *A.cannabinum* field trial. This indicates the presence of contamination in the area too high for a direct application of a Phytoremediation approach with the examined plants at a global scale. Nevertheless, in moderate and shallow contamination levels and/or in combined approach with physical-chemical techniques - a phytoremediation process with *B.juncea* could be interesting to remediate and restore soil contaminated by both inorganic and organic Pb.

In any case values obtained indicated, accordingly to literature, the need of several crop seasons and of increasing the Phytoremediation performance, whose main drawbacks are the need of time and low efficiency associated to pollutant fixation by soil particles and low absorption and/or transportation by plants (Kapourchal *et al.*, 2009; Neugschwandtner *et al.*, 2008). Therefore growing interest has been drawn by microorganisms characterized by PGP activity - able to increase plant biomass and/or increase metal uptake - and by the use of higher plants such as Hybrid poplar under study (Glick, 2003; Di Lonardo *et al.*, 2010; Karami *et* Shamsuddin, 2010).

Indeed in this perspective a lab-scale trial in a biaugmentation protocol is in progress with the arboreal plant Hybrid poplar - characterized by extensive root system and great biomass production- set up with the soil of the less contaminated Point III. Strains amended were chosen from the isolated components of the culturable indigenous micro flora, among the Proteobacteria population reporting PGP traits. Results of this trial will allow determining the effect of the Proteobacteria consortium under study on the phytoextraction process and the efficiency of the plant/rhizoflora system in a Phytoremediation process applied on a contamination combining organic and inorganic Pb.

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 considerations:

- The high Pb contamination present in the Ex-SLOI 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 predominance of gram-negative Proteobacteria has been detected at the higher contamination levels examined within the area, including strains with multiple Heavy metal resistances and/or PGP traits;
- Among the community members isolated in pure culture, strains of *Deltia* sp., *Pseudomonas putida* and *Stenotrophomonas maltophilia* - reporting both Heavy metal resistance determinants and PGP traits – are of particular interest, even in a Phytoremediation perspective with a biaugmentation protocol.

As far as the study in the context of a Phytoremediation approach with the 2 plants *Brassica juncea* and *Apocynum cannabinum* is concerned, it is interesting to point out the following considerations:

- The examined autochthonous micro flora, selected by and adapted to Pb contamination at the EX-SLOI area, exerted a positive influence on both plant species, improving plant growth and the Phytoremediation efficiency. In *B.juncea* it also positively affected Pb uptake;
- Both plants showed tolerance and resistance towards inorganic and organic Pb, reaching good Pb concentrations in their tissues, despite Pb is a challenging metal which tightly bind to soils particles and plant materials;
- Comparing the 2 plant species, *B.juncea* showed a higher Phytoextraction efficiency reaching a comparable shoot accumulation – most important parameter in a Phytoextraction process – both in lab-scale mesocosms and in open field conditions, but in a much shorter time;
- Despite it is not applicable at the high contamination as those detected at the examined points within the Ex-SLOI, a Phytoremediation process with *B.juncea* could be interesting at lower/shallow contamination and in a combined approach with physical-chemical techniques;
- Interesting prospective - and trial in progress - is the application of the arboreal plant Hybrid poplar – with extensive root system and great biomass production - in a biaugmentation protocol with strains isolated from the examined indigenous micro flora selected within the Ex-SLOI area.

# Riassunto


## *Introduzione*

Il rilascio di metalli pesanti nell'ambiente, come conseguenza del loro ampio utilizzo in attività antropiche, costituisce oggigiorno una delle piu' severe problematiche ambientali a livello mondiale. Il piombo, in particolare, costituisce uno degli inquinanti di maggiore preoccupazione per la sua ampia diffusione, persistenza nell'ambiente ed elevata tossicità (Lu *et al.*, 2005; Hill, 2004).

Del piombo, infatti, è stato fatto un uso esteso e continuato fin dall'antichità e svolge un ruolo centrale nell'economia industriale. Inoltre, tra i principali responsabili della sua diffusione nell'ambiente si annoverano sia attività industriali, che agricole ed urbane, tra quest'ultime di particolare impatto l'utilizzo di piombo nelle benzine (Thornton *et al.*, 2001; Hill, 2004; International Lead association, 2009).

Il piombo tetraetile (TEL) infatti, è stato utilizzato fin dall'inizio del secolo scorso come additivo antidetonante per benzine, per aumentarne il numero di ottano ed efficienza. Nonostante il suo utilizzo sia stato bandito dalle benzine nella maggior parte dei paesi a causa della sua elevata tossicità, l'uso per quasi un secolo su scala globale di benzine addizionate di piombo ha provocato attraverso le emissioni dei gas di scarico un inquinamento ambientale diffuso ed ubiquitario, sia in termini di ioni piombo che dei suoi composti piu' tossici alchilati. Inoltre, perdite di composti tetra-alchilati del piombo durante la produzione, il trasporto o la miscelazione nelle raffinerie e nelle stazioni di servizio, hanno causato contaminazioni di acque e suoli molto più gravi (Gallert *et* Winter, 2002; Lazzaro *et al.*, 2006).

Mentre il Pb inorganico è una tossina aspecifica, che interagendo con metallo-enzimi inibisce molteplici attività enzimatiche alla base delle normali funzioni biologiche, il TEL presenta una tossicità superiore ed è essenzialmente una neurotossina, con target principale il sistema nervoso (Godwin, 2001; World Health Organization, 2007; Environmental and Occupational Medicine, 2007). La bonifica di aree contaminate da piombo inorganico e/o organico risulta pertanto essenziale ed imposta per legge.

Nonostante l'esatto meccanismo della degradazione del TEL non sia stato ancora completamente delucidato, è noto che nei suoli contaminati può essere degradato sia biologicamente che chimicamente in dealchilazioni successive (Teeling *et* Cypionka, 1997; Gallert *et* Winter, 2002; Ou *et al.*, 1994). La completa degradazione e mineralizzazione delle specie organiche a piombo inorganico, meno tossico e scarsamente solubile, comporta una riduzione della tossicità, biodisponibilità e quindi pericolosità del contaminante. Nel suolo inoltre, la biodisponibilità del Pb inorganico generalmente diminuisce nel tempo, in quanto esso può venire immobilizzato attraverso adsorbimento alla frazione organica e/o precipitando in composti insolubili quali fosfati, idrossidi e carbonati (Lu *et al.*, 2005). Tuttavia, per quanto riguarda il piombo organico e la sua degradazione, pochi studi sono riportati in letteratura.

Nel contesto della bonifica, considerando i metodi fisici e chimici tradizionalmente utilizzati, anche se efficaci ed applicabili ad elevati livelli di contaminazione, risultano tuttavia non solo molto costosi e complessi, ma anche distruttivi rispetto alla struttura ed attività biologica del suolo e alla sua fertilità (Kirpichtchikova *et al.*, 2006).

A tal riguardo un'interessante risposta è fornita dalla bonifica biologica *(Bioremediation*), basata sull'impiego di microrganismi e/o piante in grado non solo di resistere ad elevate concentrazioni ma al contempo di degradare, detossificare e/o accumulare il contaminante. Essa può costituire un'alternativa economica e meno invasiva ovvero un complemento in una seconda fase di finissaggio ai trattamenti chimico-fisici, contribuendo non solo alla decontaminazione ma anche al ripristino della struttura ed attività di una matrice biologica complessa come il suolo. In particolare l'approccio *Phytoremediation* coadiuvata da microrganismi, basato sull'utilizzo di piante in sinergia con i microrganismi, è economico, eco-compatibile ed applicabile *in situ* sia per il risanamento che ripristino di suoli contaminati (McGuinness *et* Dowling, 2009; Manousaki *et* Nicolas, 2009; Shukla *et al.*, 2010).

Inserendosi in questo contesto, nel presente dottorato di ricerca è stato preso in esame il caso reale di un suolo contaminato da piombo organico ed inorganico, nella zona industriale dismessa Ex-SLOI (Società Lavorazioni Organiche Inorganiche) a Trento. SLOI, azienda *leader* in Europa, produsse per quasi 40 anni sino alla fine degli anni '70 miscele antidetonanti per benzine contenenti TEL. Oggigiorno, l'intera area ex-industriale presenta un'elevata contaminazione in piombo organico ed inorganico, raggiungendo concentrazioni fino a 23.000 mg/kg di piombo inorganico e valori superiori a 1000 mg/kg in piombo organico nel punto a maggiore contaminazione (definito *Hot spot*); è inoltre stimata la presenza nell'intera area di circa 168 tonnellate di piombo totale, di cui almeno 62 tonnellate di Pb alchile. Quest'area è stata infatti dichiarata sito di interesse nazionale dal Ministero dell'Ambiente (DM 468/01), alla quale è associato pertanto un alto rischio ambientali e priorità di bonifica.

## *Obiettivi*

Prendendo in esame il caso reale dell'area industriale dismessa Ex-SLOI, il presente studio di Dottorato può essere suddiviso in due parti principali. La prima parte è focalizzata sullo studio e caratterizzazione della comunità batterica autoctona, selezionatasi e acclimatatasi alla contaminazione da piombo organico ed inorganico presente nell'area da oltre mezzo secolo. Tre distinti punti di indagine sono stati quindi individuati all'interno dell'area, caratterizzati da diversi livelli di contaminazione (Punto I,II,III), includendo inoltre nello studio il suddetto *Hot Spot*, caratterizzato dalla concentrazione più elevata registrata nell'area. I principali obiettivi in questo primo studio, svolto integrando sia un approccio *culture-dependent* che *culture-independent*, riguardano:

- Lo studio della biodiversità e composizione della comunità batterica autoctona a diversi livelli di contaminazione all'interno dell'area, per valutare da un lato l'impatto della contaminazione sulla comunità pedologica, dall'altro la risposta, resistenza e potenzialità della stessa in relazione alla contaminazione presente;
- Isolamento e caratterizzazione dei componenti della comunità microbica in esame, in relazione alla loro resistenza e capacità di promuovere la crescita vegetale - esaminando caratteri *Plant Growth Promoting (PGP)* - nell'ulteriore prospettiva di un approccio di *Phytoremediation* secondo un protocollo di *biaugmentation.*

Nella seconda parte di questo Dottorato di ricerca, lo studio si e' incentrata sull'interazione tra la microflora autoctona del suolo in esame e specie vegetali, in relazione alla contaminazione da piombo organico ed inorganico presente nell'area e nel contesto di un approccio di *Phytoremediation*. Il termine *Phytoextraction* in particolare, si riferisce all'utilizzo di piante in grado non solo di tollerare ma anche di accumulare e traslocare il contaminante nella propria porzione area, che può essere quindi facilmente raccolta e rimossa dal sito. Per quanto riguarda l'efficienza di un processo di *Phytoremediation/Phytoextraction*, essa risulta direttamente proporzionale sia alla produzione di biomassa che all'accumulo di contaminante da parte della pianta. Tuttavia piante caratterizzate da una straordinaria capacità di accumulare metalli nei loro tessuti, note come iperaccumulatrici, sono generalmente caratterizzate da una lenta e scarsa produzione di biomassa, influenzando pertanto negativamente l'efficienza del processo. E' stato quindi proposto e studiato l'utilizzo di piante tolleranti, con capacità di accumulo, seppur inferiore rispetto alle iperaccumulatrici, superiori alla maggior parte delle piante e caratterizzate al contempo da una buona produzione di biomassa (Marchiol *et al.*, 2004; Karami *et* Shamsuddin, 2010).

In questo studio sono state pertanto prese in esame due specie vegetali: *Brassica juncea* ed *Apocynum cannabinum*. Mentre la prima è una specie coltivata, ad elevata produzione di biomassa e nota per la capacita' di accumulare vari metalli tra cui Pb inorganico (Liu *et al.*, 2000; Kapourchal *et al.*, 2009; Sheng

*et al.*, 2008a), la seconda è stata proposta per la bonifica fito-assistita in relazione a contaminazioni da Pb organico ed inorganico, sebbene solo pochi dati ne siano riportati in letteratura (Cunningham, 1994).
In questa seconda parte, sono stati quindi posti i seguenti obiettivi:

- Valutare l'interazione della microflora autoctona, selezionata ed acclimatasi nel tempo all'elevata contaminazione presente nell'area in esame, e suo possibile ruolo sinergico in un sistema Pianta-microflora, in relazione alla contaminazione da Pb organico ed inorganico;
- Studiare la tolleranza, potenzialità ed efficienza di *Phytoremediation* di *Brassica juncea* e *Apocynym cannabinum,* in relazione alla contaminazione da Pb inorganico ed organico.

## *Materiali e metodi*

I tre punti di indagine individuati all'interno dell'area Ex-SLOI, presi in esame assieme all'*Hot Spot* sopracitato, sono i seguenti: Punto I, caratterizzato da una contaminazione >2000 mg/kg in piombo inorganico e <50 mg/kg in piombo organico, con una co-contaminazione di 20-30mg/kg in idrocarburi; Punto II: con un livello di contaminazione >4000 mg/kg in piombo inorganico e >100 mg/kg in piombo organico, esso risulta essere il più contaminato dei 3; Punto III: presenta la minore contaminazione sia in piombo organico che inorganico, ma al contempo una co-contaminazione da mercurio nell'ordine delle decine di mg/kg.
Per ciascun punto d'indagine, nel contesto dello studio *culture-dependent*, è stata valutata la carica microbica totale enumerando la microflora coltivabile, e condotto l'isolamento diretto dei componenti della comunità microbica, isolando in coltura pura ed identificando tassonomicamente gli eubatteri eterotrofi aerobi (da terreno ricco Nutrient ed oligotrofico Reasonar's 2 agar). Infatti, considerando l'elevata contaminazione presente da oltre mezzo secolo nell'area, l'isolamento diretto ed identificazione dei componenti della comunità microbica risulta pertanto di particolare interesse.
Sono state inoltre allestite delle colture di arricchimento, in terreno minimo, addizionando alla sospensione del suolo in esame TEL (125 mg/l), in modo da spingere ulteriormente la selezione verso ceppi particolarmente resistenti al piombo organico e potenzialmente interessanti nell'ottica di un approccio di *bioremediation*.
Tali colture sono state condotte secondo due diverse modalità: in presenza di un'ulteriore fonte di carbonio, addizionando 0,1% *yeast extract* (denominate DMY), ovvero fornendo TEL come unica fonte di carbonio (denominate DM). Dopo 8 settimane di incubazione al buio, i ceppi selezionatisi sono stati isolati in coltura pura ed identificate le *Operational taxonomic units* (OTUs).
Metodi *culture-dependent* e *culture-independent* sono stati quindi utilizzati per condurre la caratterizzazione delle OTUs maggiormente rappresentate tra i ceppi da isolamento diretto, e per tutte le OTUs selezionate nelle condizioni più restrittive, ovvero mediante colture di arricchimento allestite in laboratorio ed *in situ* a livello dell'*Hot Spot* maggiormente contaminato.
La caratterizzazione è stata condotta in relazione alla resistenza a metalli, alla capacità degradativa ovvero in ralazione a caratteri *PGP*. E' stata quindi determinata la *Minimum inhibitory concentration* (MIC) per piombo organico ed inorganico, e condotto uno studio molecolare a livello genotipico, per identificare la presenza di determinanti genetici codificanti per la resistenza a piombo e ad altri metalli pesanti, resistenze infatti spesso associate. In particolare, oggetto dell'analisi sono stati i determinanti genetici per la resistenza a metalli pesanti *pbr*, *czc*, *chr, ncc* e *mer,* codificanti rispettivamente per le resistenze a Pb, Zn-Cd-Co, Cr, Ni e Hg (Abou-Shanab *et al.*, 2007; Borremans *et al.*, 2001). Questo studio molecolare è stato inoltre condotto relativamente ai determinanti genetici *phn* e *nah*, codificante la subunità α della diossigenasi per la degradazione degli Idrocarburi policiclici aromatici (IPA), associati alle benzine e

presenti nel punto I, e per il gene *alk,* codificante l'idrossilasi coinvolta nella degradazione degli alcani alifatici, potenziale indice della capacità di mineralizzare la componente organica dei composti alchilati del piombo (Smits *et al.*, 1999; Laurie *et* Lloyd-Jones, 1999).
E' stato inoltre condotto uno *screening* in relazione a tratti *PGP*, mediante i saggi per l'attività acido 1-amminociclopropano-1-carbossilico(ACC)-deamminasica e per la produzione di acido indolacetico (IAA).
Nell'ambito dello studio *culture-independent*, al fine di valutare la potenzialità e biodiversità della microflora batterica comprensiva della frazione non coltivabile, il suddetto studio molecolare è stato inoltre applicato direttamente al DNA genomico estratto da suolo, mentre un'analisi *Denaturing Gradient Gel Electrophoresis* (PCR-DGGE) è stata condotta su tutti i punti d'indagine in esame.

Per quanto riguarda lo studio secondo un approccio di *Phytoremediation*, sono state condotte due prove: *lab-scale* e *field-scale*, allestite con entrambe le specie vegetali in esame e con il suolo dei tre punti di indagine (I, II,III) individuati all'interno del'area Ex-SLOI.
La prova *lab-scale* è stata condotta in serra, alla temperature $\geq$ 24°C and con un fotoperiodo di 12 ore, allestendo in parallelo ed in triplicato mesocosmi di 1 kg con il suolo di ciascun punto di prelievo tal quale (mesocosmi tal quale), e con il medesimo terreno sterilizzato termicamente (mesocosmi sterilizzati), in modo da valutare il contributo fornito dalla microflora autoctona sia allo sviluppo delle due specie vegetali che alla complessiva efficienza del processo.
Per *B. juncea,* sia per i mesocosmi tal quale che sterilizzati e per i controlli (allestiti con suolo agricolo non contaminato) sono state prelevate tutte le piante di un mesocosmo per ciascuno dei seguenti prelievi:

- T1: a 2 mesi dalla semina
- T2: a 3 mesi dalla semina
- T3: a 4 mesi dalla semina

Per quanto riguarda *Apocynum cannabinum,* a causa della sua crescita più lenta, è stato eseguito un unico prelievo:

- Tf: a 6 mesi dalla semina

La porzione vegetale prelevata ad ogni campionamento è stata suddivisa nelle parti epigea ed ipogea, sulle quali è stata effettuata la determinazione del peso secco e del contenuto totale in piombo. E' stato inoltre determinato il contenuto di piombo totale nel suolo, con la speciazione del piombo alchile nei mesocosmi allestiti con *B.juncea.*
Sono stati quindi calcolati 3 diversi parametri: Pb fitoestratto nei tessuti aerei – definito come µg Pb /pianta =[Pb] x biomassa – indice del contaminante effettivamente estratto e quindi dell'efficienza del processo; Fattore di Traslocazione (TF), definito come il rapporto tra la concentrazione di Pb presente nei tessuti vegetali aerei e quella presente nelle radici, indice della capacità della pianta di traslocare il Pb dalle radici alla parte aerea; Fattore di Bioaccumulo (BF), definito come il rapporto tra la concentrazione di Pb presente nel tessuto vegetale e quella nel suolo, indice della capacità di bioconcentrare il contaminante nei propri tessuti.
E' stata inoltre analizzata la composizione della comunità microbica della rizosfera di *B. juncea,* mediante la tecnica molecolare PCR-DGGE, per fornire un quadro generale della composizione della microflora rizosferica, comprensivo della componente non coltivabile, lungo la prova.

La prova *field-scale* è stata quindi condotta in pieno campo, allestendo in corrispondenza dei punti indagine I,II,III, delle parcelle rispettivamente di 60$m^2$, 23$m^2$ e 45$m^2$, e condotta nelle condizioni non

controllate di un processo di scala reale. Per quanto riguarda *Brassica juncea*, in questa prova sono state incluse nello studio 2 *cultivar*: PI173874, utilizzata nella prova *lab-scale*, e PI426308.
Nella prova in campo con *Brassica juncea* è stato effettuato il campionamento di almeno 4 piante per ciacuna *cultivar* ai seguenti prelievi:

- T1: a 6 settimane dalla semina
- T2: a 14 settimane dalla semina
- T3: a 18 settimane dalla semina

Per *A. cannabinum,* come nella prova *lab-scale*, tutte le piante sono state raccolte in un unico campionamento:

- T1: a 16 mesi dalla semina

La porzione vegetale prelevata ad ogni campionamento è stata quindi suddivisa nelle porzioni epigea ed ipogea, con la determinazione del peso secco e del contenuto totale in piombo.

## *Risultati e discussione*

La caratterizzazione della comunità microbica autoctona è stata condotta integrando due diversi approcci: il primo *culture-dependent,* che utilizzando tecniche di coltura classiche consente di ottenere in coltura pura, caratterizzare ed utilizzare ceppi batterici presenti nel suolo in esame; il secondo *culture-independent* che consente, tramite l'applicazione di tecniche molecolari, una caratterizzazione microbiologica del sito indipendentemente dalla coltivabilità dei ceppi microbici presenti in esso e comprensiva della frazione non coltivabile.
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 oltre mezzo secolo, e caratterizzata da una struttura eterogenica e, per un suolo perturbato, da una ricca biodiversità.
I ceppi ottenuti in coltura pura si riconducono, infatti, a 21 gram-negativi e 7 gram-positivi generi diversi, mostrando un'alta omologia con genere batterici associati a suoli contaminati, resistenti a metalli pesanti ovvero caratterizzati da capacità degradative verso composti organici e recalcitranti.
Prendendo quindi in considerazione la distribuzione filogenetica dei ceppi identificati, è evidente una prevalenza dei Proteobacteria. A questo *phylum* infatti, si riconducono la maggioranza delle OTUs isolate direttamente dai punti di indagine maggiormente contaminati, incluso l'*Hot spot*. Inoltre questo *phylum* racchiude la totalità dei ceppi isolati nelle condizioni più restrittive imposte in laboratorio dalle colture di arricchimento con TEL come unica fonte di carbonio, allestite per isolare ceppi maggiormente resistenti e metabolicamente interessanti.
L'analisi molecolare condotta, ha inoltre indicato la selezione di componenti dominanti all'interno della comunità batterica, esercitata dalla contaminazione nel tempo. In particolare uno specifico ribotipo è stato osservato a livello dell'*Hot spot* maggiormente contaminato, ad indice della selezione di una specifica comunità microbica.
E' interessante inoltre notare come i risultati dei due approcci *culture-dependent* e *-independent* siano concordanti. Infatti, il sequenziamento ed identificazione di bande maggiormente rappresentate ottenute nei proli elettroforetici dell'analisi molecolare DGGE, hanno a sua volta indicato la predominanza del *phylum* dei Proteoabatteri nella microflora in esame, anche a livello dell'*Hot spot*. L'analisi DGGE ha inoltre permesso l'identificazione di generi non individuati nello studio *culture-dependent.*

Considerando l'analisi molecolare condotta in relazione ai determinati genetici per la resistenza ai metalli pesanti e capacità degradative verso gli idrocarburi, essa ha evidenziato, in accordo con i valori di *MIC* ottenuti, la resistenza a Pb e metalli pesanti all'interno della comunità microbica in esame, con ceppi riportanti resistenze multiple. A livello metagenomico è stata inoltre identificata una potenzialità degradativa verso gli idrocarburi, insita nella microflora autoctona. Ciò per tanto avvalora ulteriormente l'integrazione dei due approcci adottati in questo studio di ricerca.
In particolare questo studio ha evidenziato in 8 OTUs almeno un determinante genetico per la resistenza a metalli pesanti, con la presenza in 5 di esse di resistenze multiple. E' interessante anche notare come tali OTUs si riconducano esclusivamente alle classi maggiormente rappresentate dei Beta (generi *Cupriavidus, Ralstonia* e *Delftia*) e Gamma-proteobatteri (generi *Pseudomonas* e *Stenotrophomonas*), confermando l'elevata resistenza associata alla popolazione gram-negativa della microflora autoctona. In particolare in un ceppo appartenente alla specie *Cupriavidus campinensis*, isolato dall'*Hot spot*, sono stati evidenziati 4 determinanti genetici, rispettivamente codificanti per le resistenze a Pb, Hg, Cr e Cd-Zn-Co.
Considerando inoltre lo studio in relazione a tratti *PGP*, tutti e 3 i ceppi che presentano entrambi i caratteri *PGP* in esame, appartengano alla classe dei Gamma-proteobatteri, ed in particolare ai generi *Pseudomonas* e *Stenotrophomonas.*
Inoltre il 68% delle OTUs esaminete risulta presentare tratti *PGP*, ad indice di una potenzialità sinergica della comunità batterica e dei suoi componenti in un una approccio di *Phytoremediation.*
Ai generi *Pseudomonas* e *Stenotrophomonas* della classe Gamma e *Deltia* della classe dei Beta-proteobatteri*,* appartengono inoltre le OTUs che presentano al contempo sia determinanti per la resistenza a metalli pesanti che tratti *PGP*, ovvero OTU A5 *Delftia* sp., OTU A10 *Pseudomonas putida* e OTU A16 *Stenotrophomonas maltophilia.* Tali ceppi risultano pertanto di particolare interesse anche nel contesto di uno processo di *Phytoremediation* secondo un protocollo di *biaugmentation*. Nell'applicazione di ceppi *PGPR (Plant growth promoting rhizobacteria)* per la promozione della crescita vegetale, uno dei maggiori fattori limitanti risulta infatti essere la concentrazione di metalli pesanti, che ne limita l'uso in suoli a bassa contaminazione (Wu *et al.*, 2006).
Particolare attenzione volge inoltre al ceppo *Stenotrophomonas maltophilia,* in quanto riporta al contempo 3 determinanti per le resistenze a Pb,Hg e Ni, ed entrambi i tratti *PGP* esaminati.
In altri Proteobatteri, appartenenti ai generi *Cupriavidus, Delftia, Alcaligenes* e *Variovorax* della classe Beta, e ad *Ochrobactrum* e *Agrobacterium* della classe Alfa, sono state identificate resistenze a metalli pesanti o tratti *PGP*, pertanto anch'essi sono potenzialmente interessanti in una prospettiva di *Bioremediation/Phytoremediation.*
Lo studio condotto ha indicato quindi la selezione imposta dall'elevata contaminazione presente nell'area di una cenosi microbica resistente, con componenti di particolare potenzialità, sia nell'ambito della resistenza a metalli pesanti che per capacità di promozione della crescita vegetale*,* appartenenti al *phylum* maggiormente rappresentato dei Proteobatteri.
Considerando la componente meno rappresentata dei gram-positivi, la maggioranza di Firmicutes e Actinobacteria è stata identificata in corrispondenza del punto III a minore contaminazione. Tuttavia, alcuni membri del *phylum* degli Actinobacteria sono stati selezionati tramite colture di arricchimento DMY, mentre due generi, *Microbacterium* e *Arthrobacter,* risultano membri della comunità microbica speciatasi a livello dell'*Hot spot* a maggiore contaminazione. A questi 2 generi si riconducono inoltre ceppi riportanti tratti *PGP*. Pertanto seppur meno rappresentato, questo *phylum* racchiude componenti della microflora autoctona di alta resistenza e potenzialità nel contesto della *Bioremediation*.

Per quanto riguarda la seconda area di studio, inerente all'interazione della microflora autoctona con le piante in esame nel contesto di un approccio di *Phytoremediation*, i risultati della prova *lab-scale* hanno

mostrato, a livello di tutti e 3 i punti di indagine in esame (I,II,III), una maggiore crescita vegetale in presenza della microflora autoctona. In *B.juncea* è stato inoltre osservato un incremento nelle concentrazioni di Pb raggiunte nei tessuti aerei, attestando pertanto effetto sinergico, sia sulla crescita vegetale di entrambe le specie vegetali che sull'efficienza del processo, da parte della microflora autoctona selezionata dalla contaminazione di Pb organico ed inorganico protratta nel tempo. Confrontando inoltre i mesocosmi allestiti col suolo tal quale e sterilizzato, per quanto riguarda l'accumulo totale di Pb nella parte aerea della pianta (µg Pb/pianta), è riscontrabile un incremento generale nel *range* 80%-260% in *A.cannabinum*, e nel *range* 120%-400% in *B.juncea*, quest'ultima raggiungendo inoltre un incremento di 1700% a livello del punto II a maggiore contaminazione.

Infatti, l'analisi molecolare mediante PCR-DGGE condotta sul suolo della rizosfera di *B.juncea*, ha evidenziato la presenza di componenti della comunità batterica a cui si riconducono attività *PGP*, potenzialità degradative e di promozione dell'assorbimento di contaminanti nelle piante (*phytoextraction assistance*).

I dati ottenuti nella prova *lab-scale* sono quindi in accordo con i risultati inerenti alla caratterizzazione della microflora in esame, e con studi in letteratura che riportano la promozione della crescita ed assorbimento di metalli nelle piante da parte di batteri resistenti ai metalli (Sheng *et al.*, 2008a).

Inoltre, le analisi condotte sulla speciazione del piombo nel suolo nell'arco della prova allestita con *B.juncea,* attestano la progressiva degradazione dei composti organici del piombo a maggiore alchilazione durante la prova di *Phytoremediation,* suggerendo la promozione da parte del sistema pianta-microflora del processo di mineralizzazione del piombo organico, con riduzione del rischio ambientale ad esso associato.

Per quanto riguarda le piante in esame, i dati indicano una buona tolleranza e resistenza per entrambe le specie alla tipologia di contaminazione in esame. In particolare per le due *cultivar* di *B.juncea* non sono state riscontrate differenze significative né nella produzione di biomassa né nell'accumulo di Pb, riportando pertanto una simile efficienza.

Comparando invece le due specie vegetali, *A.cannabinum* mostra una crescita più lenta e una minore produzione di biomassa. Nonostante questa specie vegetale abbia raggiunto nei suoi tessuti le concentrazioni di Pb più elevate registrate nelle prove, si riscontra tuttavia un netto accumulo preferenziale nell'apparato radicale. L'accumulo nella parte aerea, quindi facilmente asportabile della pianta, è invece il principale obiettivo in un processo di *Phytoextraction.*

Seppure il Pb è un metallo scarsamente mobile e biodisponibile nei suoli, dati ottenuti sia nella prova *lab-scale* che *field-scale* hanno evidenziato per *B.juncea* la capacità di accumulare concentrazioni piuttosto elevate nei tessuti epigei, sino a 270 mg Pb/Kg d.w..

Nonostante in *lab-scale Apocynum cannabinum* abbia raggiunto concentrazioni in Pb leggermente superiori a *B.juncea* nei suoi tessuti epigei, in pieno campo ha riportato concentrazioni nettamente inferiori, con la diminuzione di un ordine di grandezza nei valori raggiunti nei suoi tessuti aerei.

Tuttavia, entrambe le specie vegetali hanno sviluppato in pieno campo una biomassa superire di un ordine di grandezza rispetto all'esperimento condotto in serra, nelle ridotte dimensioni di mesocomi da 1kg di suolo.

Considerando inoltre il fattore di traslocazione (TF), indice della capacità della pianta di traslocare il contaminante nella parte aerea, entrambe le *cultivar* di *B juncea* hanno mostrato valori nel *range* 0.2-0.7, superiori o comparabili a valori riportati in letteratura (Kumar *et al.*, 1995; Marchiol *et al.*, 2004; Xiong, 1998). Nonostante un valore TF >1 sarebbe auspicabile, è tuttavia da considerare come valori nel *range* 0.04-0.1 siano riportati per piante iperaccumulatrici, che mostrano solitamente una maggiore capacità di traslocazione ed accumulo nella parte aerea rispetto alle piante non iperaccumulatrici (Kumar *et al.*, 1995; Xiong, 1998).

Per *A.cannabinum* inoltre, nonostante abbia ottenuto valori superiori nella prova *lab-scale*, bassi valori TF<0,15 si sono riscontrati nella prova in campo, riflettendo le basse concentrazioni raggiunte nei suoi tessuti epigei in pieno campo.
Pertanto in particolare per *B.juncea,* i risultati ottenuti attestano una correlazione e riscontro positivo della prova in campo allestita secondo uno *scale-up* della prova *lab-scale,* condotta in condizioni controllate, dando un quadro non solo in linea, per quanto riguarda le concentrazioni raggiunte nei tessuti, ma mostrando un'efficienza superiore, come µg Pb/pianta, a seguito della maggiore crescita e biomassa prodotta dalla stessa pianta posta in campo.
In effetti per entrambe le piante si è ottenuto una maggiore efficienza di fitoestrazione in pieno campo, accumulando comparabili quantità di Pb nella loro parte area. Una maggiore efficienza è tuttavia associata a *B.juncea*, in quanto ha ottenuto un accumulo comparabile nella porzione aerea, ma in un tempo nettamente inferiore rispetto ad *A.cannabinum*, in particolare 4 volte inferiore comparando la durata delle rispettive prove in pieno campo.
Inoltre nessun agente chelante, fertilizzante o pratica agricola è stato fornito od effettuata nell'arco della prova, pertanto un incremento dell'efficienza del processo potrebbe essere ottenuta mediante la semplice applicazione di pratiche agronomiche (Quartacci *et al.*, 2006).
E' tuttavia da considerare come, sia i bassi valori del fattore di Biaccumulo (BF) determinato nella prova in serra, sia la crescita stentata e morte vegetale riscontrata nel punto II a maggiore contaminazione, attestino la presenza di una contaminazione troppo elevata nell'area in esame per l'applicazione di una bonifica fitoassistica con le piante in studio. Un processo di *Phytoremediatio*n promosso da *Brassica juncea* in relazioni a contaminazione di Pb organico/inorganico potrebbe risultare tuttavia interessante nel contesto di contaminazioni moderate e non profonde, ovvero a valle in un approccio combinato con trattamenti chimico-fisici.
In ogni caso, i valori ottenuti in accordo con dati in letteratura, indicano la necessità di effettuare numerosi cicli di coltivazione, con incremento del tempo conseguentemente necessario per il processo di bonifica. La durata e bassa efficienza, legate alla bassa biodisponibilità del contaminante nel suolo e alla ridotta traslocazione nelle piante, costituiscono infatti i principali svantaggi della *Phytoremediation* (Kapourchal *et al.*, 2009; Neugschwandtner *et al.*, 2008). Crescente attenzione è volta pertanto all'applicazione di microorganismi *PGP,* in grado di promuovere la crescita ovvero l'assorbimento del contaminante nelle piante, e all'utilizzo di piante arboree quali il pioppo (*Populus sp.*), caratterizzato da un'elevata produzione di biomassa ed esteso sistema radicale (Glick, 2003; Di Lonardo *et al.*, 2010; Karami *et* Shamsuddin, 2010).
Con quest'ultima pianta è stata pertanto allestita una prova *lab-scale*, tuttora in corso, col suolo del punto III a minore contaminazione, secondo un protocollo di *biaugmentation* con ceppi isolati dalla microflora in esame e riportanti caratteri *PGP*. I risultati permetteranno quindi di valutare l'effetto del consorzio di Proteobatteri ammendato e l'efficienza del sistema pianta/microflora così costituito in relazione alla contaminazione da piombo inorganico ed organico.

Sulla base dei risultati ottenuti, nel contesto della caratterizzazione della microflora autoctona del suolo in esame è possibile fare le seguenti considerazioni finali:

- L'elevata contaminazione presente nell'area Ex-SLOI ha esercitato una pressione selettiva sulla cenosi batterica autoctona verso una comunità più tollerante, caratterizzata da un'elevata biodiversità, resistenza e potenzialita biodegradativa;

- La prevalenza del *phylum* dei Proteobatteri gram-negativi è stata riscontrata in corrispondenza dei livelli di maggiore contaminazione esaminati all'interno dell'area; ad esso si riconducono inoltre ceppi con resistenze multiple a metalli pesanti e/o tratti PGP;
- Tra i membri della comunità isolati in coltura pura, ceppi di *Deltia* sp., *Pseudomonas putida* e *Stenotrophomonas maltophilia*, che presentano al contempo determinanti per la resistenza a metalli pesanti e tratti *PGP*, risultano di particolare interesse, anche in una prospettiva di Phytoremediation secondo un protocollo biaugmentation.

Per quanto riguarda lo studio nel contesto di un approccio *Phytoremediation* con le due specie vegetali *Brassica juncea* e *Apocynum cannabinum*, è interessante evidenziare i seguenti aspetti:

- La microflora autoctona in esame, selezionata e acclimatata alla contaminazione da Pb presente nell'area Ex-SLOI, ha esercitato un'influenza positiva su entrambe le specie vegetali, incrementando la crescita vegetale e l'efficienza del processo di *Phytoremediation*. In *B.juncea* ha inoltre influenzato positivamente anche l'assorbimento del Pb nei tessuti epigei;
- Entrambe le specie vegetali hanno mostrato tolleranza e resistenza verso il Pb inorganico ed organico, raggiungendo concentrazione piuttosto elevate di Pb nei loro tessuti, nonostante esso sia un metallo scarsamente biodisponibile, che si lega fortemente alle particelle del suolo ed al materiale vegetale;
- Confrontando le due specie vegetali, *B.juncea* ha mostrato una maggiore efficienza di fitoestrazione, raggiungendo comparabili valori di accumulo di Pb nella porzione aerea - parametro di maggiore importanza in un processo di fitoestrazione - sia in *lab-scale* che in *field-scale*, ma in un tempo molto più breve;
- Nonostante non sia applicabile a contaminazioni elevate quali quelle rilevate nei punti di indagine nell'area Ex-SLOI, un processo *Phytoremediation* con la specie vegetale *B.juncea* risulta interessante per contaminazioni moderate/poco profonde e in un approccio combinato con metodiche chimico-fisiche;
- Interessante prospettiva, e sperimentazione in corso, è l'applicazione della pianta arborea *Populus* sp., caratterizzata da un sistema radicale esteso ed elevata produzione di biomassa, in un protocollo di biaugmentation con ceppi isolati dalla microflora autoctona selezionatasi all'interno nell'area Ex-SLOI.

# 1. Introduction

# 1.1 Lead in the environment

## 1.1.1 Pb general characteristics

Lead is a metallic element belonging to Group IV A of the Periodic Table and classified as a heavy metal – i.e. elements with metallic properties and an atomic number >20.

Pure lead is in fact a silvery-white metal that oxidizes and turns blue-grey when exposed to air, with metallic luster and a particularly high density (Tab.1.1). Compared with other metals such as copper and aluminium it is a poor conductor of electricity. On the other hand, in comparison to most metals it has a very low melting point of 327°C and is therefore readily fusible. Lead is soft, highly malleable, ductile, easy to produce, work and cast, characterized by low strength and high resistance to corrosion in most common environments (Adriano, 2001; Thornton *et al.*, 2001; Brown *et al.*, 2010). In table 1.1 are presented some of its physical and chemical properties.

| **Physical and chemical properties of lead** | |
|---|---|
| Atomic number | 82 |
| Atomic mass | 207.2 g $mol^{-1}$ |
| Density | 11.34 g $cm^{-3}$ at 20°C |
| Melting point | 327°C |
| Boiling point | 1755°C |
| Vanderwaals radius | 0.154 nm |
| Ionic radius | 0.132 nm (+2) ; 0.084 nm (+4) |
| Stable isotopes | 4 |
| Oxidation states | 0;+2;+4 |

**Tab.1.1** Physical and chemical data of lead

Several different isotopes of lead exist in nature. Their relative concentrations in different mineral deposits can vary and this can be used as a diagnostic tool in identifying sources of lead in soils and sediments (Thornton *et al.*, 2001).

Lead exists in three oxidation states: Pb(0) - the elemental form -, Pb(II) and Pb(IV), and in three chemical forms: metallic lead, inorganic lead compounds and organic lead compounds, the latter containing at least one lead-carbon bond.

Pure Pb(0) is insoluble in water; however its chloride and bromide salts are slightly soluble (≈1%) in cold water, whereas carbonates and hydroxides salts are almost insoluble (Adriano, 2001). In most inorganic compounds Pb is in the II oxidation state. Actually while the divalent oxidation state prevails in inorganic compounds, the tetravalent oxidation state prevails in organic lead chemistry (Manceau *et al.*, 1996; Adriano, 2001).

### 1.1.2 Pb natural occurrence and ore extraction

Metallic lead Pb(0) exists in nature but its occurrence is rare. Lead, like all other metals, occurs naturally in small concentrations in all rocks and soils and its average concentration in the Earth's crust is estimated to be approximately 16mg/kg, though it is not evenly distributed (Adriano, 2001).

Lead is usually extracted from sulphide ores in which it is found in association with other metallic sulphide minerals, most frequently those of zinc and copper, but also precious metals such as silver. Actually lead occurs most commonly as the mineral galena (lead sulfide, PbS), and it is sometimes found in other mineral forms, which are of lesser commercial importance, such as anglesite (lead sulphate, $PbSO_4$) and cerussite (lead carbonate, $PbCO_3$). Lead concentrates can be easily extracted from the ore and winning the metal from the concentrate does not need much energy (Environmental protection agency, 1998; Brown *et al.*, 2010).

In terms of mine output, lead is almost always a co-product with other base minerals. World reserves of lead are 79 million tones, of which Australia holds 24 million tones, China 11 million tones and USA 7,7 millions tones (U.S. Geological Survey; U.S. Department of the Interior, 2009; Brown *et al.*, 2010).

### 1.1.3 Use of Inorganic lead

Lead important properties, in particular malleability, ease of production, ease of melting and joining and good corrosion resistance has led to its extended use since ancient times (Thornton *et al.*, 2001).

Indeed lead was known to man as early as 4.000 BC, when dated the earliest known example of metallic lead, which is a metal figure recovered from the Temple of Abydus in Upper Egypt. The Romans also produced an average of 60.000 tons of lead per year for 400 years. In fact they used Pb on a large-scale for plumbing, tank lining and domestic articles (Thornton *et al.*, 2001).

Lead use has continued to grow and in the present era it is a very vital metal in any industrial economy; with a multitude of valuable uses it is encountered by everybody in virtually every aspect of their daily lives. The only nonferrous metals that are used in greater amounts than Pb are Al, Cu, and Zn (Adriano, 2001).

Certain compounds of lead, particularly brightly colored lead oxides, have been used for millennia. Lead chromate (yellow) and lead molybdate (red/orange) are used as coloring pigments for ceramic glazes, plastics and, to a lesser extent under current legislation, in paints e.g. in road paints. Other minor uses include weights and molten Pb is used as a coolant in fast reactions (Thornton *et al.*, 2001).

Due to its toxicity, in the late 1970s lead use in drinking-water pipes, for sealing food-cans and in paint for domestic purposes was banned. Nevertheless leaded paint remains in place for many years and virtually any US house built before 1980 has some (Hill, 2004).

Nowadays the major use of Pb is in lead-acid batteries, which account for 80% of the reported current global use. It is primarily within starter batteries in motor vehicles and in emergency backup power supplies, mostly for computer and telecommunication systems. Other major application areas are rolled extrusions (6% of the total), pigments (5%), ammunition (3%), alloys (2%) and cable sheathing (2%), used to protect underwater and some underground power cables (International Lead association, 2009; Brown *et al.*, 2010).

Its high density has proved effective for weights and anchors for fishing lines, boats, and later for munitions. This property is now utilized in lead radiation shielding in medical applications, in the nuclear industry and in soundproofing (Thornton *et al.*, 2001).

### 1.1.4 Organic lead

When the metal atom is bound to other elements it behaves as a different substance with specific properties dissimilar from those of the parental metal. Organo-metallic compounds - in which a metal

atom is bound through carbon to an organic radical 'R' - are fundamentally different from the parental metal and its ionic compounds. Indeed the organic lead compounds are colourless liquids that are insoluble in water but soluble in organic solvents like petrol (Hill, 2004; Collins *et al.*, 2004).

### 1.1.4.1 Use of Organic lead

Since the 1920s organic lead compounds Tetraethyl-lead (TEL) and Tetramethyl-lead (TML) were produced worldwide as anti-knocking additives for petrol to improve its efficiency at low cost (Ou *et al.*, 1994). While TEL - $Pb(C_2H_5)_4$ - was first introduced by General Motors for use as an anti-knocking agent in petrol in 1923, its analogous chemical TML was subsequently introduced in 1960 and the two chemicals were added to petrol either singly or as mixtures to increase the octane numbers (Ou *et al.*, 1994; Environmental and Occupational Medicine, 2007).

The octane number is a measure of the burn rate of the fuel, and while petrol of higher octane number burns well and smoothly, low octane number petrol can cause "knocking" during combustion, giving poorer performance and causing some engine damage. Thus the addition of lead to petrol was hailed as a great success at the time, as it allowed good performance from fuel, without the need for expensive sophisticated refining (Thornton *et al.*, 2001).

Normally most exposure of lead is to its inorganic form as a lead salt. Nevertheless since the beginning of the last century the introduction of alkyl-lead derivates in petrol and the use of leaded petrol over almost one century have caused a massive increase and ubiquitous pollution of the environment with both lead ions and organic compounds (Gallert *et* Winter, 2002). For years it has been established that the use of tetraalkyl-lead (TAL) compounds as anti-knocking additives in petrol has been the major worldwide source of anthropogenic lead (Mikac *et* Marko, 1994).

Actually, from one side car emissions led to a ubiquitous distribution of low concentrations of lead compounds in the upper layers of soil; on the other side spillages of highly toxic TAL compounds during production, transportation or blending at oil refineries and petrol stations caused more severe soil and groundwater contaminations (Gallert *et* Winter, 2002).

In the USA production of leaded petrol peaked in early 1970 and until the mid-1970s motor-vehicle exhaust was a major source of lead to the environment and of exposure. After banning lead from petrol in 1996 - due to its toxicity - emissions to air fell more than 90% and blood levels in US children fell concurrently (Hill, 2004). With the advent of more sensitive analytical techniques in the late 1970 and early 1980, alkyl-lead compounds were found to be ubiquitously present in the environment. They were detected in aerosol dusts, in rainwater surface water, snow, sediments, soils, fish and leaves (Ou *et al.*, 1994).

In Europe due to the increasing concern that lead dispersed in the environment was causing damage to human health and the environment, a series of regulations and directives have been adopted since the 1980s, in order to phase out the use of leaded petrol. Leaded gasoline was withdrawn entirely from the European Union market on 1 January 2000 (Thornton *et al.*, 2001).

Subsequently the majority of countries have banned the use of leaded petrol. Since Sub-Saharan Africa completely eliminated the import and production of leaded petrol in January 2006, as of January 2011 there are only 3 countries worldwide using leaded petrol - North Korea, Afghanistan and Myanmar - and 3 countries using both leaded and unleaded petrol - Algeria, Yemen and Iraq, as reported by the Partnership for Clean Fuels and Vehicles (UNEP).

TEL however is still widely added to special types of petrol used by vintage and race cars, small piston-engine aeroplanes and in recreational marine vehicles (Gallert *et* Winter, 2004; Collins *et al.*, 2004).

Most available long-term measurements of lead background concentrations and depositions demonstrate significant reduction of lead pollution levels in the environment after the phase-out of leaded petrol in many countries (EMEP).

### 1.1.4.2 Tetraethyl-lead (TEL)

#### *Properties*

Tetraethyl-lead (TEL) - $Pb(C_2H_5)_4$ - is a lead atom bonded to a tetrahedral arrangement of ethyl groups and the molecule can be thought as a metal atom surrounded by a hydrocarbon cage (Fig. 1.1) (Collins *et al.*, 2004).

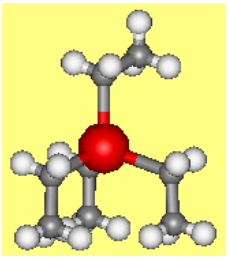


**Fig. 1.1**: Structure of a molecule of TEL

TEL is therefore a hydrophobic compound with very low water solubility, while it is highly soluble in hydrophobic solvents such as petrol, benzene, and hexane (Ou *et al.*, 1994). Its physiochemical properties are summarised hereafter (tab. 1.2).

| **Physical and chemical properties of TEL** | |
|---|---|
| Appearance | Colourless to yellow viscous liquid with characteristic sweet odour |
| Melting Point | -136.8 °C |
| Boiling Point | Above 200°C (decomposes) |
| Vapour Density | 8.6 (air=1) |
| Relative Density | 1.653 at 20°C (water=1) |
| Vapour Pressure | 2.25 $E^{-01}$ mm Hg at 20°C<br>2.7 $E^{-02}$ Kpa at20.0°C |
| Henry's Law constant | 30 |
| $K_{oc}$ | 10,580 |
| $Log(K_{ow})$ | 5.27 |
| $Log(P_{ow})$ | 4.15 |
| Diffusion Coefficient | 9.92 $E^{-02}$ $cm^2$ $s^{-1}$ in air<br>1.56 $E^{-05}$ $cm^2$ $s^{-1}$ in water |

| | |
|---|---|
| Specific Gravity | 1.653 |
| Flash Point | 93.3 °C |
| Auto-ignition | above 110.0°C |
| Explosion Limits | 1.8 vol% in air |
| Water Solubility | 1.2 $E^{-04}$ $gl^{-1}$ |
| Molecular Weight | 323.45 g $mol^{-1}$ |
| Solubility | Soluble in benzene, ethyl alcohol petrol ether, gasoline and ethyl ether<br>0.2 mg $l^{-1}$ in water |
| Conversion Factors | 1ppm=13.4 mg $m^{-3}$<br>1mg $m^{-3}$=0.07 ppm |

**Tab. 1.2**: Physical and chemical data for tetraethyl-lead (Collins *et al.*, 2004)

TEL is known to reacts violently with strong oxidising agents, concentrated acids and fats causing fire and explosion hazards. The International Programme of Chemical Safety (IPCS) together with European Environment Agency (EEA) report that this chemical compound can quickly decompose and volatilise to form dangerous gaseous phases if exposed to ultra-violet light and trace chemicals in air such as acids or oxidizing agents (INCHEM).

It is also able to dissolve some types of rubber, plastics and coatings and decomposes on heating above 200°C. According to IPCS and CEC (Commission for Environmental Cooperation), harmful contamination of the air can be reached rather quickly on evaporation of this substance at 20°C, hence the strong advice (Recommendation by International Labour Organisation) not to let the chemical enter into the environment (INCHEM).

Transformation of TEL to ionic forms triethyl-lead (TREL) and diethyl-lead (DEL) occur in soil both biologically and chemically, and the ionic trialkyl-lead compounds – triethyl-lead (TREL) and trimethyl-lead (TRML) - were reported to be the major species found in the environment through analytical techniques (Ou *et al.*, 1994).

TREL and DEL - very little considered in literature - are not soluble in hydrophobic solvents but are highly water soluble (Table 1.3) (Ou *et al.*, 1994).

| **Parameter** | **Triethyl-lead** | **Diethyl-lead** |
|---|---|---|
| Molecular Weight* | 326.0 g $mol^{-1}$ | 326.0 g $mol^{-1}$ |
| Henry's Law Constant** | 1.0 $E^{-07}$ | 1.0 $E^{-07}$ |
| $K_{oc}$ *** | 1.0 $E^{+02}$ | 1.0 $E^{+02}$ |
| Log($K_{ow}$)**** | -1 | -1 |
| Solubility | 2.0 $E^{+04}$ mg $l^{-1}$ in water | 2.0 $E^{+04}$ mg $l^{-1}$ in water |

| | | |
|---|---|---|
| Vapour Pressure | 1.0 $E^{-08}$ mm Hg | 1.0 $E^{-08}$ mm Hg |
| Diffusion Coefficient | 0.0 $cm^2$ $s^{-1}$ in air | 0.0 $cm^2$ $s^{-1}$ in air |
| | 1.0 $E^{-05}$ cm2 $s^{-1}$ in water | 1.0 $E^{-05}$ $cm^2$ $s^{-1}$ in water |

**Tab. 1.3**: Chemical and physical parameters for triethyl and diethyl-lead (Collins *et al.*, 2004) Notes: *, these represent the values for the cations; **, estimated on the basis of good solubility in water and low volatility; ***, estimated after experimental analysis which have proved that organic forms of lead are mobilised: good (10% $NH_4H_2PO_4$, 5% EDTA sat. $KPO_3$), medium (10% $NH_4HCO_3$ 10% $Na_2CO_3$), low (sat. $CaCO_3$); ****, estimated on the basis of good solubility (refers to $K_{ow}$ =0.1)

### *TEL in the environment*

In the environment TEL is known to undergo chemical or biological degradation to other organic forms before finally degrading to inorganic lead; nevertheless alkyl-lead is persistent over decades.

As TEL is normally added to petrol together with organic halogen such as ethylene di-bromide - the latter acting as a scavenger for the removal of lead after combustion - from vehicle exhausts lead is generally emitted as halogenated compounds (chlorides, bromides). These are highly soluble in water and fairly rapidly react to produce more stable compounds such as lead sulphate (Thornton *et al.*, 2001).

Although from motor vehicles lead is emitted mainly as inorganic salts, 0.3-3% of emitted total lead is organic, mostly as TAL, but also as ionic alkyl-lead species. It has to be mentioned that approximately 1% of TAL in petrol is lost to the atmosphere during fuel transport and handling (Mikac *et* Marko, 1994).

In the atmosphere the organic Pb compounds undergo fairly rapid degradation and they form persistent inorganic lead compounds in water and soil. In fact in the atmosphere TAL is decomposed photocatalytically by UV irradiation, by ozone or by hydroxyl radicals to the more stable, water-soluble ionic tri- and dialkylated species. Monoalkyl-lead cations are assumed to be very unstable and exist only as a transient intermediate in the breakdown to inorganic lead, which is the final product of alkyl-lead emissions (Manceau *et al.*, 1996; Ou *et al.*, 1994).

In addition to photodecomposition or oxidative decay in the presence of ozone or hydroxyl radicals, TAL can be decomposed into trialkylated lead species by catalysis of $Cu^{2+}$or $Fe^{2+}$ ions, whereas ionic trialkyl- and dialkyl-lead species are stable in aqueous solution and cannot be decomposed by the catalysis of these cations (Gallert *et* Winter, 2002).

Although degradation pathways of TAL compounds in soil, including TEL, have barely been studied and are not completely known, degradations pathways for TEL in aqueous solutions is believed to proceed through a series of sequential dealkylation, eventually to inorganic lead (Teeling *et* Cypionka, 1997; Ou *et al.*, 1994; Collins *et al.*, 2004; Rhue *et al.*, 1992). Thus TEL- $Et_4Pb$ - is first degraded to monoionic TREL - $Et_3Pb^+$ - which is in turn degraded to di-ionic DEL - $Et_2Pb^{2+}$ - and finally to $Pb^{2+}$:

$Et_4Pb \rightarrow Et_3Pb^+ \rightarrow Et_2Pb^{2+} \rightarrow Pb^{2+} \rightarrow PbS\ PbO\ PbCO_3$

Consequently highly hydrophobic TAL compounds are transformed into water soluble ionic species. Owing to their water-solubility, ionic alkyl-lead compounds represent a major fraction of organo-lead compounds in natural waters, where hydrophobic TAL compounds are only occasionally detected (Mikac *et* Marko, 1994).

In soil after chemical decay and/or biological degradation of organic lead compounds, the inorganic and less toxic $Pb^{2+}$ is immobilized by precipitation as PbO, PbS ,$PbCO_3$ or $Pb_5(PO_4)_3Cl$ (Teeling *et* Cypionka, 1997; Gallert *et* Winter, 2002; Rhue *et al.*, 1992).

Moreover $Pb^{2+}$ is complexed by organic matter, in fact in soil contaminated by alkyl-tetravalent lead compounds, lead was found to be divalent and complexed to salicylate and catechol-type functional groups of humic substances (Manceau *et al.*, 1996).
Besides in presence of sulfide, trialkyl lead cations can be chemically converted into tetraalkyl-lead and dialkyl lead di-cations, in a sulfide-mediated transalkylation process (Teeling *et* Cypionka, 1997).
As an environmental pollutant in soil and waste sites, it has been shown that TEL may cause transalkylation of other metals. Indeed in laboratory experiments and in field studies with soil from an industrial site contaminated with organo-lead and inorganic mercury, inorganic mercury was alkylated to ethylmercury by tetraethyllead (Gallert *et* Winter, 2002).

### 1.1.5 Pb releases to the environment

Lead occurs naturally in the environment and can be released to the biosphere through mobilization of naturally-occurring lead in the Earth's crust and mantle, due to wind resuspension, weathering of parent rocks and volcanic activity.
However most lead concentrations that are found in the environment are a result of human activities and its biogeochemical cycle has been affected by man to a greater extent than that of most other toxic metals, as through time, enormous amounts of anthropogenic lead have been stored in the biosphere (Thornton *et al.*, 2001; Adriano, 2001).
Actually used since ancient time, its accumulation in the environment has risen rapidly since the Industrial Revolution as a result of activities such as mining and smelting, acid battery recycling, synthesis of tetraalkyl-lead and lead paints, and combustion of leaded gasoline (Manceau *et al.*, 1996; Thornton *et al.*, 2001). Lead concentration in recently formed Antarctic ice is indeed four times greater than those formed prior to the industrial age. Pb concentration in recent coral shells is also 15 times greater than in shells deposited a century ago, while lead concentration in household dusts can be 500 times greater than the background level in the Earth's crust (Hill, 2004).
In connection to the replacement of leaded by unleaded fuel, to installation of filters in incinerators and metal smelters, emissions into the atmosphere have considerably decreased over the past few decades (Lazzaro *et al.*, 2006; Johnson *et al.*, 1995). Modern techniques have also minimized industrial emissions to meet statutory requirements. However due to its ubiquity, the large build-up of loads in the past and its toxicity, lead is the most problematic metal pollutant in the environment in industrialized and urbanized areas and their surroundings.
Nevertheless, as lead is strongly retained in most soils, in particular if pH and organic matter quantities are high, accumulated loads in soils and sediments are still increasing in many places and continue to pose a potential long-term environmental risk (Adriano, 2001; Lazzaro *et al.*, 2006).
Atmospheric deposition is generally the major input in the biogeochemical cycle of Pb, which is transported as particulates. Small lead particles emitted to air can remain in the atmosphere for over three weeks and in that time they may travel many hundreds of kilometers, though larger particles, which may constitute up to 95% of the emission, settle out within very short distances of the source (Adriano, 2001; Lazzaro *et al.*, 2006; Thornton *et al.*, 2001).
Deposition from atmosphere is a major contributor to lead inputs also to water, where Pb can be carried either dissolved or as waterborne particles. However, few compounds of lead dissolve readily in water, though most of this lead is then precipitated as a solid and becomes incorporated in the sediments at the base of the watercourse or ocean (Thornton *et al.*, 2001).

### 1.1.5.1 Pb in soil

Lead concentration in normal field soil is in the range of 10 to 100 mg/kg, but in contaminated soils especially near mines or by sewage sludge applications, its concentration as high as 1000 mg/Kg has been reported (Akmal *et* Jianming, 2009).

Indeed soil constitutes a sink for lead which has a strong tendency to be adsorbed on particles of clay or organic matter, which has been recognized as a critical component in the retention of heavy metals in soils (Lu *et al.*, 2005).

In these adsorbed forms Pb is largely immobile and biologically inert. Lead is in fact one of the most persistent metals with a soil retention time of about 150-5000 years in the environment, and it usually mainly accumulates in the surface of soils. Actually very high levels of lead (up to 1% or more) in top soils - usually within the top few centimeters - occur in some urban areas but usually very little migrates to lower horizons (Thornton *et al.*, 2001; Kambhampati *et al.*, 2003; Adriano, 2001).

Considering Pb bioavailability in soil, it generally decreases with increasing residence time, due to the reactions between metal ions and soils, including complexation, adsorption, and precipitation of metal ions - as carbonates hydroxides and phosphates - in the soil particles surface or diffusion into the mesopores and micropores of soil (Lu *et al.*, 2005).

Actually total metal concentration does not necessarily reflect the amount of metal that is biologically toxic or bioavailable. There is a large consensus among the scientific community to believe that the risks for living organisms associated to the presence of heavy metals (HM) in our environment is determined for a large part by the solubility of the various HM-bearing phases present rather than by the total elemental concentration (Manceau *et al.*, 1996). Therefore soluble or bioavailable metal may be a more accurate predictor of ecosystem toxicity and, more specifically, a predictor of microbial metal resistance and bioremediation potential (Roane, 1999).

Pb is known to be extremely insoluble in the normal range of soil pH (Sharma *et* Dubey, 2005). Pb solubility is controlled by phosphate or carbonate precipitates in soils with ph 5.5-7.5, while extremes of soil above 7.5 and below 5.5 will respectively either decrese or increase Pb solubility (Kambhampati *et al.*, 2003). More acidic conditions - which can occur in mine wastes or from landfill leachate - in fact not only increase the solubility of lead, but also of other heavy metals (Thornton *et al.*, 2001). Acidic condition favors the metals added to soil as soluble in soil solution or weakly adsorbed on soil particle, and also restricts the metal ions to diffuse into the micropores of soil or complex with soil minerals or organic matter (Lu *et al.*, 2005).

Investigations on changes of speciation of Pb and Cd in soil at various pH values with different time of incubation showed that the increasing pH and incubation time resulted in the increase of adsorption and co-precipitation of heavy metals (Lu *et al.*, 2005).

Both changes in pH and oxidation-reduction potential may result in changes in the chemical availability of lead. In equilibrium solubility experiments conducted on Pb, Cd and Zn under three different pH values (3.3, 5.0, 8.0) and three redox potential (325, 0,-100 mV), results showed that metals were sparingly soluble under alkaline conditions (pH=8.0). Metal solubilities were higher under slightly acidic conditions (pH=5.0), and increased drastically when pH was kept at 3.3. When solubilities were compared under same pH values, it was observed that metal solubility increased as redox potential decreased. Hence acidic and reducing conditions were most favorable for metal solubilization, and the effect of pH was more significant than that of redox potential. In this study the pH-dependent metal adsorption reaction and the dissolution of metals on Fe-Mn oxyhydroxides under reducing conditions, was proposed as the mechanism controlling the release of heavy metals from soils (Chuan *et al.*, 1995).

Actually both pH and redox also affect the retention and release of metals by clay minerals, organic matter, iron oxides and, for coastal wetlands, sulfides.

The Eh-pH diagram, shown in Fig.1.2, draws a parallelogram (dashed lines), whose contour corresponds to the limit values of pH (from 4 to 9) and redox potential (from -0.059 to 1.22). Within these limits species in which lead is present in oxidation state +2 occur in nature. This suggests that the redox potential values that are found in most environments do not change the Pb oxidation state of +2 (Gambrell, 1994). It is interesting to notice as Pb compounds containing sulfur - very common forms in nature – are characterized by a thermodynamic stability and poor solubility in a wide range of pH at a low redox potential (Erten-Unal *et al.*, 1998). However the galena (lead sulfide, PbS) is unstable in oxidizing conditions and reacts with atmospheric oxygen to form anglesite (lead sulphate, $PbSO_4$). Therefore in high redox potential conditions the mobility of Pb increases due to the oxidation of the insoluble sulfides. Besides this process has the secondary effect of reducing the pH.
The formed lead sulphate is stable in a wide fluctuation of pH values from 1 to 6 (Erten-Unal *et al.*, 1998). On the other side at low values of redox potential - under anaerobic conditions - the sulphate group is not stable and is reconverted to sulfide.
A variety of oxides of lead including PbO and $PbO_2$ could also be formed. This set of compounds is easily converted to the hydrated form $Pb(OH)_2$ (Davis *et al.*, 1993). In particular the lead dioxide - the form Pb is present in the tetravalent state - accumulates in high oxidant conditions and pH greater than 8 (Erten-Unal *et al.*, 1998; Gambrell, 1994).

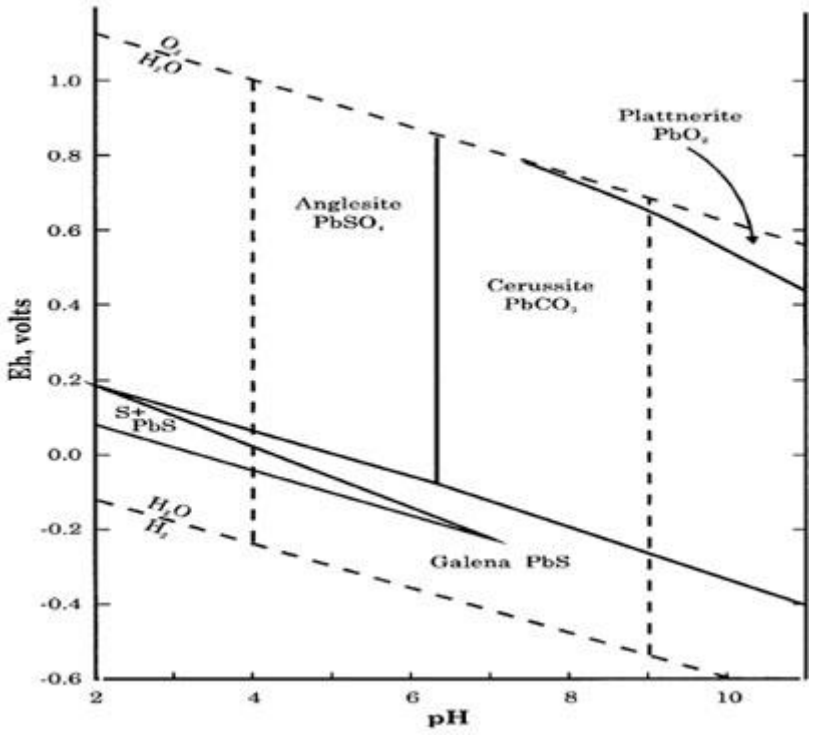



**Fig.1.2** Lead Eh-pH diagram

## 1.1.6 Human toxicity of lead

The US EPA ranks lead among the agents posing the greatest hazard to humans and among the ten most-common pollutants at hazardous waste sites. Lead is indeed in the meantime one of the most persistent and one of the most toxic metals in the environment (Shen *et al.*, 2002; Hill, 2004).

### 1.1.6.1 Toxicity of Inorganic lead

Inorganic lead is undoubtedly one of the oldest occupational toxins with the earliest written accounts of lead toxicity found in Egyptian papyrus scrolls (Hernberg, 2000; Gidlow, 2004).

Actually Pb is an aspecific toxin, whose targets are essential metalloenzymes, affecting therefore many metabolic processes. As other heavy metals it has high affinity for sulfur-containing ligands, therefore entering the cell it interacts with SH-groups inactivating many enzymes. Detailed biophysical studies have revealed that lead binds tightly to both zinc and calcium sites in proteins and alters their activity. However, the biology of lead is more complex and the multitude of symptoms associated with lead poisoning suggests that no class of targets will explain all of lead's effects (Godwin, 2001).

Exposure to inorganic lead occurs from breathing air, drinking water, and eating foods that contain the metal. Inhalation of lead fumes or of fine lead particles is the most important route of absorption in the working environment and general atmosphere. Chronic low-dose Pb exposure exerts subtle neuropsychiatric, reproductive and renal effects and children are particularly susceptible. The primary symptoms of acute poisoning are related to local irritation of the gastrointestinal tract and include vomiting and abdominal colic (Gidlow, 2004; INCHEM).

Besides lead is a neurotoxic metal, affecting visual/motor performance, memory, attention and verbal comprehension. Subtle changes in neuropsychological function have in fact been seen in inorganic lead workers with blood lead levels as low as 40µg/100ml. Moreover chronic workplace exposure increases the likelihood of high blood pressure, can damage the nervous system and kidneys, and sometimes leads to anemia and infertility (Hill, 2004; Gidlow, 2004).

Because the body treats Pb much as it does with calcium, lead accumulates in the skeleton and can remain in the bodes for decades. While blood level can show recent exposure, bone lead level reflects exposure over a life time (Collins *et al.*, 2004). Actually about 90% of a person's lead intake is eventually stored in the skeleton and lead levels in modern human skeletons and teeth are hundreds of times greater than those found in pre-industrial-age skeletons (Hill, 2004).

Lead is only weakly mutagenic, but in vitro it inhibits DNA repair and acts synergistically with other mutagens. Nevertheless there are at present insufficient data for suggesting that lead compounds are carcinogenic in humans (Gidlow, 2004).

### 1.1.6.2 Toxicity of Organic lead

The toxicity of organic lead compounds was recognized soon after they were first employed, in fact in 1920s several cases of severe poisonings were already described (Hernberg, 2000).

As reported by the International Programme on Chemical Safety (IPCS), indirect exposure arises from environmental contamination and generally poisoning due to organic compounds is a consequence of industrial exposure. Actually exposure to organic lead compounds occurs principally during synthesis, transport and mixing with petrol, in fuel terminal and in cleaning petrol tanks (Environmental and Occupational Medicine, 2007; Collins *et al.*, 2004).

The toxicity of organic lead compounds differs markedly from that of inorganic lead compounds. Indeed they are much more toxic than inorganic lead and their toxicity decreases with a decreasing number of ethyl- or methyl-moieties and from ethyl-lead to methyl-lead (Gallert *et* Winter, 2002; Gallert *et* Winter, 2004).

The highly hydrophobic TAL compounds easily penetrate through skin and biological membranes. Therefore in contrast to inorganic lead, organic lead respiratory and percutaneous absorption represent the main routes of exposure. Although inhalation is the most important route of absorption in the working environment, poisoning may result from the absorption of a sufficient quantity of lead, whether absorbed briefly at a high rate or for prolonged periods at a lower rate; whereas ingestion does not represent a

significant occupational hazard (World Health Organization, 2007). Therefore toxic properties of organic compounds require precautions against both their percutaneous and respiratory absorption (Collins *et al.*, 2004).

In particular TEL is essentially a central nervous system toxin. In fact because of the solubility in fat, accumulation occurs in Central Nervous System and symptoms of intoxication refer primarily to this organ (Environmental and Occupational Medicine, 2007).

One of the early symptoms is insomnia, and it can be accompanied by headache, anxiety, restlessness. The most severe responses include complete disorientation with hallucinations, and facial contortions (Collins *et al.*, 2004). While the early signs and symptoms are subtle and non-specific and may be easily missed, in those with continuing exposure or after a massive single exposure, florid symptoms of a toxic psychosis or even coma and death may occur (Gidlow, 2004).

Besides a greatest concern is for children, who experience symptoms at significantly lower blood lead levels than adults. In addition whereas many of the symptoms experienced by adults are reversed when exposure is ceased, children tend to develop permanent developmental and neurological problems when exposed chronically to lead (Hill, 2004).

# 1.2 Pb interaction with biological systems

## 1.2.1 Heavy metals and microorganisms

Interactions between bacteria and heavy metal ions are of great interest both as a fundamental process and potential bioremedial technology (Ianeva, 2009).

Although trace concentrations of some heavy metals, such as zinc and copper, are essential for microorganisms' growth, in high concentrations all heavy metals are toxic to most of them (Hynninen *et al.*, 2009). On the other side nonessential heavy metals, such as lead, mercury and cadmium are considered toxic at any concentration; they are therefore termed 'toxic metals' (Janssen *et al.*, 2010).

Non essential metals normally enter the cell through normal nutrient transport systems. At a molecular level, toxicity seems to occur through the displacement of essential metals (such as calcium, potassium and magnesium) from their native binding sites or through ligand interactions. Indeed nonessential metals, such as lead, bind with greater affinity to thiol-containing groups and oxygen sites than do essential metals. Moreover Pb shows particular high affinity to calcium and zinc binding sites. These interactions can cause alterations in the conformational structure of nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance, inhibiting enzymatic activities and disrupting membrane functions (Bruins *et al.*, 2000).

The toxicity of a metal to microorganisms is dependent upon metal bioavailability and, in general, as pH decreases and the solubility of the metal increases, metal toxicity increases due to enhanced bioavailability and mobility across cell membranes. However bacteria have adapted to the presence of heavy metal ions in their habitats and resistance systems are present in nearly all bacterial types (Ianeva, 2009; Bruins *et al.*, 2000). They arose because bacteria exist in an environment that has always contained metals, moreover human activities have created environments of high selection for metals (Bruins *et al.*, 2000).

For endurance under metal-stressed environment, microorganisms have evolved several mechanisms by which they can immobilize, mobilize or transform metals, rendering them inactive to tolerate the uptake of heavy metal ions (Shukla *et al.*, 2010). Actually microorganisms can possess one or a combination of several resistance mechanisms, even though the ability to resist the toxicity is not yet well known (Hu *et al.*, 2006).

As far as the bacterial soil community is concerned, the detection of heavy metals resistant strains has been reported in different studies on contaminated soils. Research carried out since the early 1970s identified several heavy metals resistant microorganisms including mostly aerobic microorganisms, with prominent examples being resistance in *Staphylococcaus* sp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus* sp. (Bruins *et al.*, 2000). For example bacteria able to survive in soils with high Pb level, in the amount of 204 µg Pb/g of soil, were identified in an area around a zinc and lead mine (Hu *et al.*, 2006).

Genetic determinants responsible for heavy metal resistance can be localized both on bacterial chromosomes and on extrachromosomal elements such as plasmids and transposons (Bruins *et al.*, 2000). In this context horizontal gene transfer plays an important role in the spread of heavy metal resistance in nature, which is abundant and widespread, with frequencies ranging from a few per cent in pristine environments to nearly 100 % in heavily polluted environments (Gadd, 2010).

Five mechanisms are postulated to be involved in resistance to heavy metals (Janssen *et al.*, 2010; Bruins *et al.*, 2000; Shukla *et al.*, 2010), although other mechanisms may possibly exist:

- metal exclusion by extracellular barrier
- active transport by efflux system
- intracellular sequestration

- extracellular sequestration
- enzymatic detoxification of the metal to a less toxic form.

Generally, the primary mechanism of resistance to heavy metal ions in prokaryotes is a reduced accumulation based on the active efflux to export toxic metals outside the cell (Hynninen *et al.*, 2009). Active transport (efflux systems) represents in fact the largest category of metal resistance systems (Bruins *et al.*, 2000).

Moreover preventing metal entrance in the cell, the formation of an intracellular complex with the toxic metal ion (mechanism mainly used in eukaryotes), binding factors and enzymatic reactions - such as methylation and demethylation, oxidation and reduction - also play a role of defence against toxic metals in bacteria (Hynninen *et al.*, 2009).

#### 1.2.1.1 Lead resistance strategies by microorganisms

Lead (II) resistance has been reported for bacteria representing different genera and in both gram-negative and gram-positive bacteria from lead-contaminated soil (Borremans *et al.*, 2001; Taghavi *et al.*, 2009b).

Although in the current literature the mechanism of resistance to tetraethyl-lead is still unknown and relatively little is known about lead resistance in bacteria, bacterial Pb II resistance strategies have been identified and belong to the first fourth of the previous list.

In particular active ion efflux, frequent resistance strategy employed by bacteria against heavy metals, and lead precipitation in an insoluble form, are the most detected in studies of prokaryotic tolerance and resistance to soluble lead (Mire *et al.*, 2004).

Indeed Pb-resistance mechanisms strategies include ATP-dependent efflux pumps, which transport ions against the concentration gradient using energy by ATP hydrolysis, and intracellular sequestration, which may be more effective at detoxifying the increased metal penetrating cell membranes under high bioavailable metal conditions (Roane, 1999). Pb-detoxifying systems also involve the exclusion by permeability barrier, based on the interaction with cell wall and envelope elements, and the production of metal binding proteins or other cell components in the cell for Pb complexation (Hynninen *et al.*, 2009).

##### *Metal exclusion by permeability barrier*

This mechanism allows the exclusion of metals from the cell and prevents them from interacting with vital cellular components, through the production of an extracellular polymeric coating which provides sites for the attachment of metal cations and protect metal-sensitive and essential cellular components (Bruins *et al.*, 2000).

Extracellular polymers, composed by proteins, humic acids, uronic acids, polysaccharides, acid nucleic and lipid, create a biofilm - involved in nutrient storage and adhesion - but that also acts as a barrier against environmental toxicants, including metals (Roane, 1999; Guildbaud *et al.*, 2003).

Actually many bacteria have a cell wall or envelope that is capable of passively adsorbing high levels of dissolved metals, usually via a charge-mediated attraction (Mohamed, 2001). Reactions involved in metal biosorption onto these polymers are ion exchange, complex with negatively charged functional groups, adsorption and precipitation (Guildbaud *et al.*, 2003). Indeed binding of heavy metals by these organisms takes place mainly through exopolysaccharides (EPSs) in a metabolism-independent process, and it is attributed to interaction between metal cations and negative charges of acidic functional groups of EPS (Perez *et al.*, 2008; Loaec *et al.*, 1997).

Adsorption of various heavy metals including Pb has been detected in exopolysaccharide-producing Cyanobacteria, in *Bacillus firmus* strains, and in exopolysaccharide-producing *Pseudomonas* sp.. A

particular heavy metal binding capacity has been observed in uronic acid rich EPSs of *Gloeothece magna*, a non-toxic freshwater Cyanobacterium, and in *Paenibacillus jamilae* (Perez *et al.*, 2008; Mohamed, 2001). In the latter the biosorption of several toxic heavy metals (Pb, Cd, Co, Ni, Zn and Cu) was observed, along with a tenfold higher affinity for Pb in comparison with the other five metals, with a Pb biosorption of 303.03 mg $g^{-1}$ (Perez *et al.*, 2008).

### *Efflux system*

Active transport or efflux systems represent the largest category of metal microbial resistance strategies. Microorganisms use active transport mechanisms to export toxic metals from their cytoplasm in chromosomal or plasmid-encoded mechanisms.

Non-essential metals normally enter the cell through normal nutrient transport systems but are rapidly exported. These efflux systems can be ATPases and chemiosmotic ion/proton exchangers and are highly specific for the cation or anion they export (Bruins *et al.*, 2000; Silver *et* Phung, 2009).

P-type ATPases is a superfamily of transport proteins whose members occur in all three kingdoms of life. They are single-subunit systems located in the cytoplasmic membrane that use energy provided by ATP hydrolysis to pump metal ions against the concentration gradient out of the cytoplasm, thus detoxifying microbial cells (Janssen *et al.*, 2010; Nies, 2003).

Members of the huge P-type ATPase superfamily that transport heavy metal cations carry a conserved proline residue, preceded and/or followed by a cysteine residue, and substrates are inorganic cations such as $Pb^{2+}$, $H^{+}$,$Na^{+}$, $Mg^{2+}$,$Ca^{2+}$,$Cu^{+}$, $Ag^{+}$, $Zn^{2+}$ and $Cd^{2+}$ (Nies, 2003).

Pb efflux resistence systems involving P-type ATPase have been detected in *Staphylococcus aureus*, in *Pseudomonas putida* and *Escherichia coli*. These systems constitute a highly conserved and widely distributed family of efflux ATPases, which contain heavy metal-associated metal binding domains that are also found in other proteins interacting with heavy metals (Borremans *et al.*, 2001; Hynninen *et al.*, 2009).

P-type ATPase involved in heavy metal resistance can be further divided into the $Cu^{+}/Ag^{+}$ translocating and $Zn^{2+}/Cd^{2+}/Pb^{2+}$-translocating P-type ATPase subgroups, based on substrate specificity. The latter group contains ZntA ATPase from *Escherichia coli*, CadA ATPase from *Staphylococcus aureus* and CadA2 ATPase from *Pseudomonas putida,* which are all relatively non-specific and confer resistance even to zinc and cadmium ( (Bruins *et al.*, 2000; Taghavi *et al.*, 2009b; Hynninen *et al.*, 2009).

Another example of microorganism using this active Pb transport is *Cupriavidus metallidurans* strain CH34, formerly known as *Alcaligenes eutrophus, Ralstonia metallidurans* and *Wautersia metallidurans,* the most deeply investigated metal resistant bacterium (Borremans *et al.*, 2001; Janssen *et al.*, 2010). Its resistance to lead is mediated by P-type ATPase which can transport lead outside the cell (Borremans *et al.*, 2001; Mergeay *et al.*, 2003). Moreover this strain accomplishes detoxification to various metals by the concerted action of efflux systems including at least eight P-type ATPases and a wide variety of systems for RND (Resistance, Nodulation, cell Division family of permeases) and CDF (Cation Diffusion Facilitator). Altogether, there are at least 30 heavy-metal-efflux systems in *C. metallidurans* CH34 encoded by 155 genes in 25 loci (Janssen *et al.*, 2010). It is worth mentioning that RND systems for Heavy Metal Efflux (HME-RND) were first discovered in *C. metallidurans* CH34 with the archytypes czcCBA, which confers resistance to Co, Zn and Cd located on its megaplasmids pMOL30.

RND are complexes which comprise three components spanning the complete cell wall and mediating cation/proton antiporter efflux, via a chemiosmotic gradient, from the cytoplasm towards the periplasm and into the exterior of the cell. In contrast, the cation diffusion facilitator CDF family transporters, which act as chemiosmotic ion-proton exchangers of the cytoplasm, are single-subunit systems, as the P-type ATPases, located in the cytoplasmic membrane (Janssen *et al.*, 2010).

## The case of *Cupriavidus metallidurans* CH34

The facultative chemolithoautotrophic Beta-proteobacterium *Cupriavidus* (formerly *Wautersia, Ralstonia, Alcaligenes*) *metallidurans* strain CH34 was originally isolated in 1976 from a decantation tank at a metal processing factory in Belgium. It is considered a major model organism for metal resistance, as it withstands millimolar range concentrations of over 20 different heavy metal ions. This tolerance is mostly achieved by rapid ion efflux but also by metal-complexation, sequestration and reduction (Janssen *et al.*, 2010). However metal detoxification in *C. metallidurans* CH34 mainly occurs via ion efflux including chemiosmotic efflux of cations with proton antiporters (HME-RND family) encoded by *czcCBA*, *cnrCBA*, and *silCBA*, cation diffusion facilitators such as CzcD and CnrT, and P-type ATPase family of ion pumps for cytoplasmic detoxification (*pbrA*, *copF*, and *czcP*) (Janssen *et al.*, 2010; Monchy *et al.*, 2007). These export systems may act on a range of cations, including $Cu^{+}$,$Ag^{+}$,$Cu^{2+}Cd^{2+}$,$Ni^{2+}$,$Pb^{2+}$and $Zn^{2+}$ (Janssen *et al.*, 2010). The genes involved in these heavy metal resistances can be found on its two megaplasmids but it also has chromosomally encoded resistance mechanisms (Monchy *et al.*, 2007; Mergeay *et al.*, 2003). The plasmid-encoded lead-resistance gene cluster pbrTRABCD from *Cupriavidus metallidurans* CH34 is thought to encode a unique, specific resistance mechanism for lead. However, the exact functions of these genes are unknown (Hynninen *et al.*, 2009). This gene cluster consists of six genes that code for: PbrR, a transcription factor belonging to the MerR family; PbrT, a putative $Pb^{2+}$uptake protein; PbrA, a P-type ATPase; PbrB/PbrC, a predicted integral membrane protein and a putative signal peptidase; and PbrD, a putative intracellular Pb-binding protein (Borremans *et al.*, 2001; Hynninen *et al.*, 2009).

Lead resistance in *C. metallidurans* is achieved through the cooperation of the Zn/Cd/Pb-translocating ATPase PbrA and the undecaprenyl pyrophosphate phosphatase PbrB. While PbrA non-specifically exported $Pb^{2+}$, $Zn^{2+}$and $Cd^{2+}$, a specific increase in lead resistance was observed when PbrA and PbrB were coexpressed (Hynninen *et al.*, 2009). As a model of action for PbrA and PbrB, Hynninen *et al.* (2009) proposed a mechanism where $Pb^{2+}$ is exported from the cytoplasm by PbrA and then sequestered as a phosphate salt with the inorganic phosphate produced by PbrB (fig 1.3). Similar operons containing genes for heavy metal translocating ATPases and phosphatases were found in several different bacterial species, suggesting that lead detoxification through active efflux and sequestration is a common lead resistance mechanism (Hynninen *et al.*, 2009).

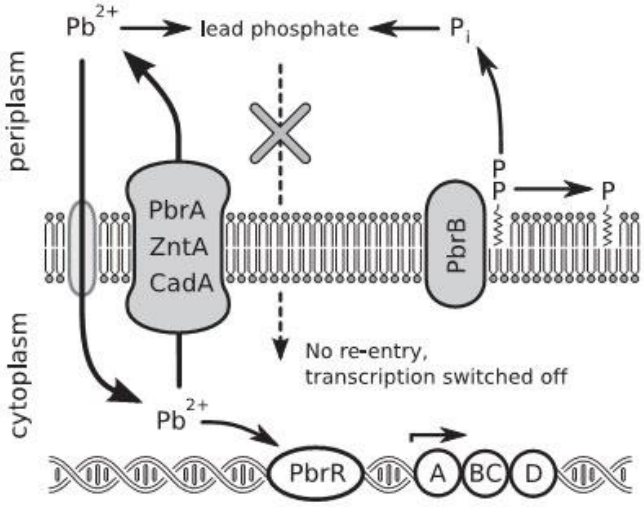



**Fig. 1.3** Model of Pbr protein action. Upon entrance of Pb into the cell, the synthesis of Pbr proteins is initiated. PbrA, as well as p-TYPE ATPases ZntA and CadA, begin to pump metal ions to the periplasm, where Pb is precipitated with phosphates produced by PbrB. The sequestration of lead discontinues the expression of the pbr operon, avoiding potentially harmful overexpression of PbrB. Also, synthesis of PbrC and PbrD is initiated; however, these are not essential for lead resistance (Hynninen *et al.*, 2009).

### *Pb sequestration*

Some bacterial lead-resistance mechanisms involve the intra- and extracellular binding of Pb and both mechanisms of extracellular exclusion and intracellular accumulation thus result in the sequestration and immobilization of lead in an insoluble form (Roane, 1999).

Actually several bacterial species use intra- and extracellular binding of $Pb^{2+}$ to avoid toxicity and a number of studies have shown Pb sequestration by bacterial strains.

In particular a variety of bacteria precipitate lead, most typically as a lead phosphate in the few cases in which the compound has been examined. Depending on the strain and the growth conditions, the precipitate can either accumulate at the cell membranes or be expelled (Mire *et al.*, 2004).

### *I) Intracellular sequestration*

Intracellular sequestration is a strategy performed by some bacteria able to accumulate metals within the cytoplasm to prevent exposure to essential cellular components, and a number of studies have shown that various bacterial strains sequester lead intracellularly (Bruins *et al.*, 2000; Mire *et al.*, 2004).

As with many other organisms, resistance to heavy-metal toxicity in bacteria has been related to their ability to over express Metallothioneins (MT) genes after exposure to metal ions. Metallothioneins (MTs) are low-molecular-mass cysteine rich metal-binding proteins, with a high affinity for essential ($Zn^{2+}$ and $Cu^{+}$) and non-essential ($Pb^{2+}$and $Hg^{2+}$) heavy metal ions and are found in a large variety of organisms including bacteria, fungi and all eukaryotic plant and animal species. Although the biological functions of MTs have not been fully elucidated, they are thought to play an important role in detoxification of toxic metals, as cysteine residues act as a sink for excess toxic cations. MT gene transcription is in fact induced by heavy metals such as Pb, as indicated for a *Streptomyces* strain able to resist to high concentration of the heavy metals Zn, Cu and Pb (Bruins *et al.*, 2000; Rifaat *et al.*, 2009).

In relation to heavy metal such as Cd and Zn examples of intracellular sequestration by binding proteins have also been reported in a *Synechococcus* sp. strain - able to produce MT proteins as a form of resistance – and in a *Pseudomonas* sp. strain, induced by heavy metals for the transcription of these proteins (Silver *et* Phung, 1996; Bruins *et al.*, 2000) (Bruins *et al.*, 2000).

Besides, although the molecular mechanism remains to be elucidated, resistant strains of *Bacillus megaterium* (Roane, 1999), *Staphylococcus aureus* (Levinson *et al.*, 1996)*, Citrobacter freundii* (Levinson *et* Mahler, 1998) and *Vibrio harveyi* (Mire *et al.*, 2004) have been reported to lower the concentration of free lead ions by precipitating lead and accumulating the metal as an intracellular cytoplasmic phosphate salt.

In particular the *Vibrio harveyi* strain was capable of precipitating lead as an unusual phosphate compound, $Pb_9(PO_4)_6$, and the process resulted to be regulated at least in part by quorum sensing (Mire *et al.*, 2004). The precipitate produced by a *Citrobacter sp.* was identified as $PbHPO_4$, whereas $Pb_3(PO_4)_2$ was suggested as the precipitate produced by *Staphylococcus aureus* strains (Levinson *et al.*, 1996; Levinson *et* Mahler, 1998).

In the study on *Staphylococcus aureus* strains, both Pb-resistant and Pb-sensitive strains were able to initially bound lead, but only the resistant ones accumulated the metal as an intracellular lead-phosphate in electron-dense inclusions. Actually metal accumulation by microorganisms has been characterized as generally comprising two phases: a rapid binding of cations to negatively charged groups on the cell surface, and progressive, usually metabolism-dependent intracellular cation uptake. Although Pb-sensitive cells also bind Pb(II) initially, Pb-phosphate crystals were not found in sections of these sensitive strains as probably lacking the system for precipitating the metal as Pb-phosphate. In Pb-resistant strains crystals were deposited as the non-toxic, negligibly soluble phosphate and the mechanism was supposed to continue until the Pb(II) concentration overwhelms the binding capacity of the cell (Levinson *et al.*, 1996).

PbS precipitation has been also suggested in a *Klebsiella* strain cultured in phosphate-limited medium. This bacterium was in fact able to precipitate $PbHP0_4$ granules on the cellular surface - as reported for a *Citrobacter* species grown in the presence of lead (Levinson *et* Mahler, 1998) - while it accumulates PbS in electron-dense granules in the cells in phosphate-limited cultures (Aiking *et al.*, 1985; Mire *et al.*, 2004).

***II) Extracellular sequestration***

Metal resistance based on extracellular sequestration result from the binding of the toxic metal in a complex so thus it cannot enter the cell membrane. This mechanism has been found in bacteria and even in several species of yeast and fungi.

*Saccharomyces cerevisiae* in fact may reduce absorption of Ni (II) by excreting large amounts of glutathione, which binds with great affinity to heavy metals. A similar mechanism exists in Cu(II)-resistant fungi, that secrete oxalate to form a metal-oxalate complex. Other organisms such as yeast or *Citrobacter* sp. form insoluble complexes of cadmium phosphate to confer resistance (Joho *et al.*, 1995; Bruins *et al.*, 2000)

A lead-resistant *Pseudomonas marginalis* strain, isolated from a soil with a neutral pH and undetectable levels of soluble lead, has been reported to avoid lead toxicity by precipitating it as an extracellular polymer. Without lead present in the medium, *P. marginalis* still produced the polymer, indicative of metal-independent resistance. Extracellular polymer production is not unique and is frequently encountered with other metals in polluted natural settings (Roane, 1999).

Detailed analyses of microbial precipitated lead compounds are rare. The precipitate produced by *Pseudomonas fluorescens* is known to contain lead and phosphorus, but the stoichiometry has not been reported. Moreover both phosphate-replete and phosphate-starved *Pseudomonas fluorescens* cultures have been reported to generate an insoluble material containing both lead and phosphorus, although phosphate-replete cultures are apparently more efficient at expelling the material (Al-Aoukaty *et al.*, 1991).

## *The case of Sulfate reducing bacteria, SRB*

Sulfate reducing bacteria (SRB) are gram-negative bacteria that use sulfate as terminal electron acceptor and constitute a unique physiological group of microorganisms that couple anaerobic electron transport to ATP synthesis. These bacteria (220 species of 60 genera) can use a large variety of compounds as electron donors and display a certain degree of metal tolerance as a secondary outcome of their metabolism (Lin *et al.*, 2010; Barton *et* Fauque, 2009).

Actually under anaerobic conditions, SRB utilize the sulfate ion as an electron acceptor during the oxidation of organic material, forming hydrogen sulfide which interacting with metals leads to the formation of stable metal sulfide and reduce metals' availability.

The formation of metal sulfide mediated by SRB is an important pathway for heavy metal stabilization in anoxic soil and they also play an important role in precipitation of heavy metals in natural waters and some wastewaters (Lin *et al.*, 2010; Kumar *et al.*, 2001).

In particular the anaerobic sulfate-reducing bacteria (SRB) play a significant role in coastal ecosystems where more than 50% of biodegradation is through sulfate-reducing activity (Harithsa *et al.*, 2002).

Sulfate reducers also occur in mine tailings where anaerobic conditions persist below aerobic surface layers and they have been employed among biological methods of mine water treatment, to precipitate metal ions as insoluble sulfides. In this context anglesite ($PbSO_4$) was tested as electron acceptor for sulfate-reducing bacteria, to form PbS as insoluble product. The biological formation of sulfide was demonstrated with no evidence of inhibitory effect and toxicity of $Pb^{2+}$ to the test bacteria (Karnachuk *et al.*, 2002). Accordingly, previous results showed no growth inhibition of *Desulfosarcina* sp. growth until

Pb concentration reached 125 mg $Pb^{2+}$/l (added as Pb-nitrate), although it is not clear whether some Pb precipitated during growth, thereby reducing the bioavailability to the bacteria (Bharathi *et al.*, 1990).

### 1.2.1.2 Microorganisms and Organic lead

#### *Organic lead degradation*

Environment TAL compounds might undergo both chemical and biological degradation, and microbial degradation has been detected both in groundwater and soil samples.

Although both field and laboratory studies have reported evidence of biological dealkylation of alkyl-lead, in current literature very little is reported on the biodegradative pathway of TEL and the exact mechanism is still not completely known (Macaskie *et* Dean, 1990; Ou *et al.*, 1994; DuPont, 1994).

The conversion of tetraalkyl-lead into trialkyl-lead can exclusively be a chemical reaction, enhanced by light, oxygen, $Fe^{2+}$ or $Cu^{2+}$ ions. However in the dark no decomposition of trialkyl-lead species in the absence or presence of these ions can occur. Degradation of trialkyl-lead compounds without sunlight (conditions as in groundwater or deep soil) must therefore be catalyzed by microorganisms to get a complete remediation of a tetra- and trialkyl-lead contaminated sites (Gallert *et* Winter, 2002).

In addition to photochemical or chemical transformation of alkyl-lead compounds, microbial degradation in soil is reported to occur by multiple dealkylations resulting finally in the formation of inorganic lead (Gallert *et* Winter, 2002; Gallert *et* Winter, 2004).

An experiment with Ethyl-1-$^{14}$C-labeled TEL was performed to determine the mineralization rates of TEL and mass balance, showing that $^{14}$C-TEL both in non-sterile and autoclaved soils disappeared rapidly while ionic ethyl-lead products, water soluble non-lead organic products and bound residues were rapidly formed. A small fraction (7.74%) of m$^{14}$C-TEL in non-sterile soil samples was mineralized to$^{14}CO_2$ in 28 days. Triethyl-lead (TREL) was the major ionic ethyl-lead product detected in both non-sterile and autoclaved soils; diethyl-lead (DEL) was occasionally detected. Based on the observations of more rapid initial disappearance of $^{14}$C-TEL, more rapid formation and more rapid disappearance of $^{14}$C-DEL, and occurrence of $^{14}CO_2$ production in non-sterile soils, it was concluded that both biological and chemical degradation contributed to the degradation of TEL in soils (Ou *et al.*, 1994).

Besides, even though there have been few investigations on the microbial degradation of alkyl-lead compounds, in soil spiked with trimethyl-lead chloride and dimethyl-lead dichloride, the degradation was reported to be faster in comparison to autoclaved control samples, thus confirming a microbia-promoted degradation. Furthermore, an *Arthrobacter* sp. and the fungus *Phaeolus schweinitzii* were found to degrade a small percentage of added trimethyl-lead cations during growth in complex media (Teeling *et* Cypionka, 1997; Macaskie *et* Dean, 1990; Teeling *et* Cypionka, 1997).

Moreover accelerated rates of organo-lead transformation were observed following the nutrient enrichment of contaminated ground water. Examining the effects of nutrient stimulation of microbial populations on the rates of alkyl-lead transformation to $Pb^{2+}$ - in microcosms containing contaminated groundwater from two former tetraethyl-lead manufacturing sites in New Jersey - results suggested that simply increasing the level of microbial activity organic lead transformation was accelerated. Therefore the accelerated decomposition of organo-lead compound following nutrient additions indicated a co-metabolic microbial attack, i.e. a transformation by non-specific enzymes (DuPont, 1994).

Further confirm was given by monitoring micro-calorimetry along the degradation of tetraethyl lead in soil (Teeling *et* Cypionka, 1997). Sieved agricultural soil samples were treated with tetraethyl lead. These additions resulted in an increase of the heat production rate, provided that oxygen was present and that the soil was not autoclaved. The increased heat production rate was accompanied by degradation of TEL, as verified by speciation analysis of the remaining TEL and its ionic degradation products triethyl-lead and

diethyl-lead. Therefore conclusive evidence was obtained that these transformations were mediated mainly by Microorganisms. Besides the biodegradation rate was not influenced by the addition of glucose, thus not confirming in this case a co-metabolic attack on TEL (Teeling *et* Cypionka, 1997).

### *Methylation of Pb to Organic lead*

Biological methylation designates processes in which a methyl group undergoes transfer by enzymes (methyltransferases) onto a metal or metalloid atom. The biological methylation in the environment has been proven for metals and metalloids such as S, Cl, Ge, As, Se, Br, Sn, Sb, I, Te, Cd, Hg, Tl, Bi and Pb; and it mostly commonly occurs in sediments from bacterial action, however, fungi and algae are also known to cause biomethylation (Thayer, 2010). Biomethylation of inorganic lead or of organo-lead cations to tetramethyl-lead were reported to be apparently also possible (Gallert *et* Winter, 2002), even if few data are available in literature. The biological methylation of lead by bacteria to produce TML in lacustrine sediments was first reported by Wong *et al.* in 1975 and then it was next proved that Pb(II) and Pb(IV) can both undergo biologically mediated methylation (Thompson *et* Crearar, 1980; Wong *et al.*, 1975).
Early reports also suggested that some species of *Pseudomonas, Acinetobacter, Flavobacterium* and *Aeromonas* can convert lead nitrate or trimethyl-lead acetate to tetramethyl-lead (Roane, 1999).

## 1.2.2 Pb in higher plants

Although lead is among those heavy metals which have no known biological function, numerous investigations show that it can be taken up by plants roots and be accumulated via roots and shoots, reaching concentrations in plant tissues which are significantly related to Pb levels and biodisponibility in environment and negatively affecting and inhibiting most basic plant physiological processes (Xiong, 1998; Baghour *et al.*, 2001).

### 1.2.2.1 Pb toxicity in higher plants

Excessive Pb accumulated in plant tissue can be toxic to most plants, as after entering the cell it inhibits activities of many enzymes, upsets mineral nutrition and water balance, changes the hormonal status and affects membrane structure and its permeability. In most cases inhibition of enzyme activities results from the interaction of the metal with enzyme –SH groups or displacement of an essential metal from metalloenzyme active sites (Sharma *et* Dubey, 2005; Manousaki *et* Nicolas, 2009).
Moreover Pb causes the imbalance of the minerals K, Ca, Mg, Mn, Zn, Cu, Fe within the tissues by physically blocking the access of these ions to the absorption sites of the roots and decreases photosynthetic rate, by distorting chloroplast ultrastructure, diminishing chlorophyll synthesis, obstructing electron transport and inhibiting activities of Calvin cycle enzymes (Sharma *et* Dubey, 2005).
As other heavy metals, Pb cause oxidative stress by overproducing reactive oxygen species (ROS) which can cause damage to various biomolecules such as lipids, proteins, nucleic acids, and possibly engender cell death (Bidar *et al.*, 2009). To resist oxidative damage, plants have an antioxidant defense system comprising of antioxidant enzymes such as peroxidases, superoxide dismutases and catalases, non-enzymatic constituents such as ascorbate and reduced glutathione which remove, neutralize and/or scavenge ROS (Bidar *et al.*, 2009; Manousaki *et* Nicolas, 2009). Considering visual non-specific symptoms of Pb toxicity, they include stunted growth, chlorosis and blackening of the root system (Manousaki *et* Nicolas, 2009). High concentrations of Pb (1 mM) have also been reported to cause 14 to 30 % decreased germination in rice seeds and a reduction by more than 13 to 45% in seedlings' growth (Verma *et* Dubey, 2003). Moreover in lead-contaminated sites, vegetation structure and biodiversity can be reduced, barren patches of soil occur, and trees can be sparse or absent (Xiong, 1998).

### 1.2.2.2 Pb uptake and transport in plants

Pb is taken up by plants, via passive uptake, mainly through the root system and partly in minor amounts through the leaves (Sharma *et* Dubey, 2005; Liao *et al.*, 2006). Although Pb uptake studies in plants have demonstrated that roots have an ability to take up significant quantities of Pb, its translocation from roots to shoots is limited and it accumulates primarily in the root. Indeed Pb binding at root surfaces and cell walls limits its translocation from roots to aerial shoots (Manousaki *et* Nicolas, 2009).

Pb retention in the roots is based on binding of Pb to ion exchangeable sites on the cell wall and extracellular precipitation, mainly in the form of Pb carbonate deposited in the cell wall. Actually after being taken up by roots, Pb binds strongly to the carboxyl groups of the carbohydrates galacturonic acid and glucuronic acid in the cell wall, which restricts its transportation via apoplast. Although one possible transport pathway of Pb across the plasma membrane appears to be through cation channels, such as Ca-channels, Pb moves predominantly into the root apoplast and thereby in a radial manner across the cortex and accumulates near the endodermis. The endodermis acts therefore as a partial barrier to the movement of Pb between the root and shoot, this may in part account for the reports of higher accumulation of Pb in roots compared to shoots (Verma *et* Dubey, 2003; Sharma *et* Dubey, 2005).

While at lower concentrations Pb ions predominantly flow in the apoplast, at higher concentrations the barrier function of plasmalemma is damaged and a greater amount of Pb enters into the cells and in the vascular tissues.

In general, concentration of Pb in aerial parts of the plant decreases as the distance from the root increases, due to greater localization of Pb in cell walls of the root than in other parts of the plant. The content of Pb in various plant organs tends to decrease in the following order: roots>leaves>stem>inflorescence>seeds. However, this order can vary with plant species. Further, binding of Pb occurs more in lignified rather than non-lignified tissues and ultrastructural studies have revealed Pb deposits of various size mainly in the intercellular spaces, cell walls and vacuoles (Sharma *et* Dubey, 2005).

### 1.2.2.3 Pb tolerance in plants

Despite Pb plant toxicity, soils strongly enriched in heavy metals, either naturally or due to contamination, often support characteristic plant species that thrive in these metal-enriched environments and known as hyperaccumulators. Whereas many species avoid the uptake of heavy metals from these soils, other species can accumulate significantly high concentrations of toxic metals, to levels which by far exceed the soil levels, without dramatically being impacted in their growth and development (Xiong, 1998; Memon *et* Schröder, 2009).

Three main different types of plants can be identified with three basic strategies for growing on contaminated and metalliferous soils: metal excluders, metal indicators and metal accumulators - usually referred to as hyperaccumulators. Metal excluders effectively prevent metal from entering their aerial parts over a broad range of metal concentrations in the soil; however, they can still contain amounts of metals in their roots. Metal indicators accumulate metals in their above-ground tissues and the metal concentrations in their tissues generally reflect metal levels in the soil. Metal hyperaccumulators concentrate metals in their above-ground tissues to levels far exceeding those present in the soil or in non-accumulating species growing nearby. In particular a species that can accumulate 0.1% of Pb, Ni, Co, Cu and Cr or 1% of Zn on a dry weight basis is defined a hyperaccumulator, irrespective of the metal concentration in the soil (Memon *et* Schröder, 2009; Shah *et* Nongkynrih, 2007; Baker *et* Brooks, 1989).

Nearly 450 hyperaccumulator plants, also known as metallophytes, have been described belonging to a wide range of taxa, ranging from annual herbs to perennial, geographically distributed in all continents.

Around 25% of the reported hyperaccumulator species are members of *Brassicaceae* family - such as *Alyssum sp.* and *Thlaspi sp.* (Memon *et* Schröder, 2009; Shah *et* Nongkynrih, 2007).
As far as lead is concerned, *Thlaspi rotundifolium* ssp. Cepaeifolium*, Thlaspi caerulescens* and *Sesbania drummondii* are reported as hyperaccumulator (Memon *et* Schröder, 2009). In particular *Thlaspi rotundifolium*, the first natural hyperaccumulator reported for Pb, is able to accumulate 0.13-8.2 g Pb/kg d.w. in leaves. Also *Helianthus annuus* is known to concentrate Pb both in its leaf and stem (Shah *et* Nongkynrih, 2007).
Besides for some cultivars of *Brassica juncea* - species able to accumulate various metals and metalloids, although not a hyperaccumulator - is reported a strong ability to accumulate Pb in roots and to transport Pb to the shoots, accumulating as high as 1.5 % Pb in the shoot when grown in nutrient solution containing 760 µM Pb (Sharma *et* Dubey, 2005; Kumar *et al.*, 1995).
Ragweed (*Ambrosia artemisiifolia*) and Indian Hemp (*Apocynum cannabinum*) have been also reported in literature for Pb accumulation and suggested for Pb phytoremediation of soils contaminated by both organic and inorganic Pb (Shukla *et al.*, 2010; Cunningham, 1994).

### *Pb tolerance biochemistry in plants*

Two basic strategies of metal uptake are related to tolerance in plants, as suggested by Baker (1981) (Baker, 1981). One involves an exclusion strategy in which the concentration of heavy metals is maintained at a constant low level, until critical soil concentration is reached when toxicity ensues and unrestricted metal transport results. The second is an accumulating strategy, in which metals are actively concentrated within the plant tissues over the full range of soil concentration implying a highly specialized physiology. These responses include exclusion, detoxification mechanisms and non-specific defense systems (Fig 1.4) (Sharma *et* Dubey, 2005; Memon *et* Schröder, 2009; Baker, 1981).

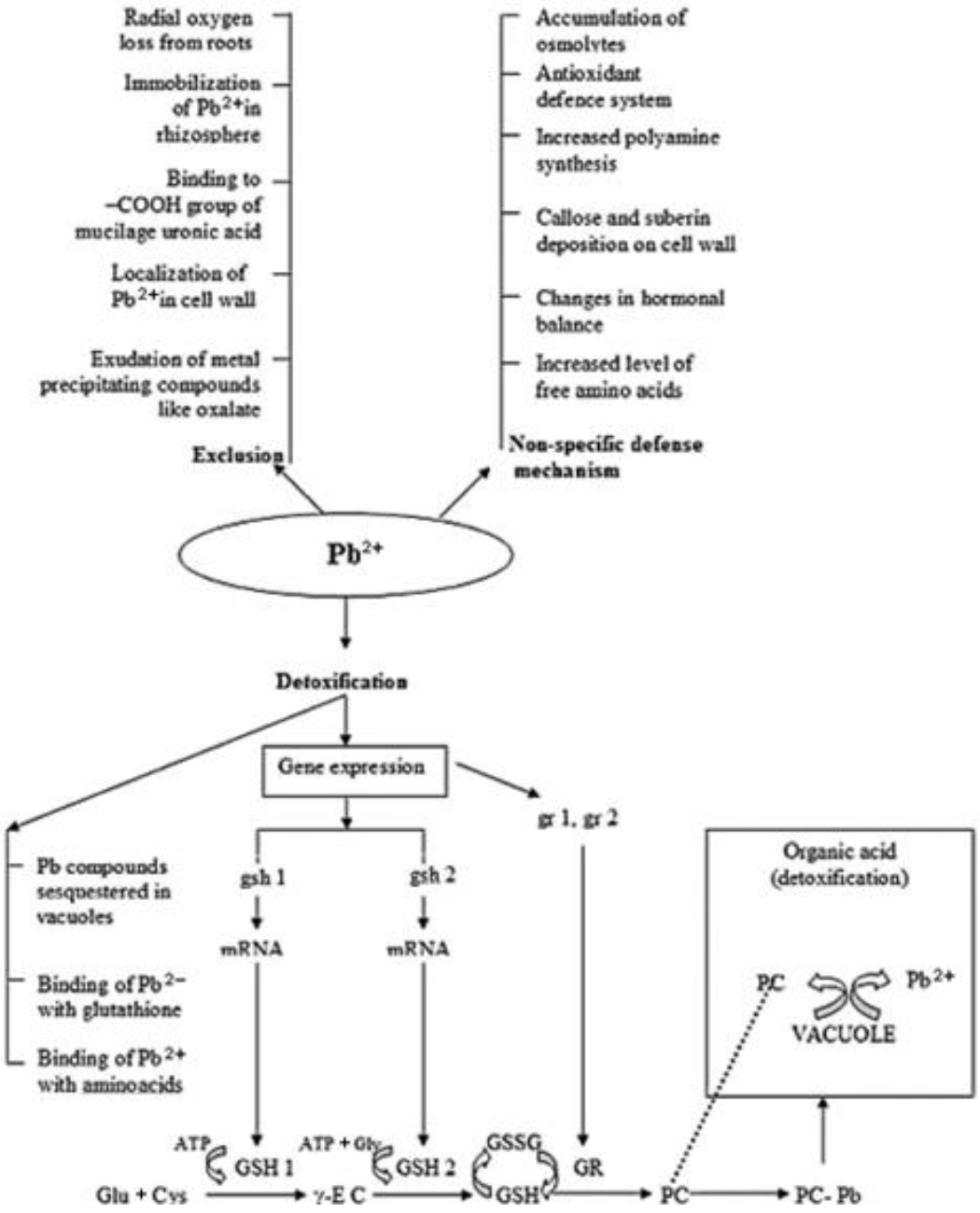



**Fig.1.4** Response of plant cell to toxic levels of lead (Sharma *et* Dubey, 2005)

Pb exclusion capacity of plants is related to oxygen transport ability, radial oxygen loss from the root, efficiency to immobilize Pb in the rhizosphere, binding capacity to -COOH groups of mucilage uronic acid and its precipitation by various compounds such as oxalate and aminoacids (Sharma *et* Dubey, 2005).
It was shown by Ye and coworkers (1997), that in the wetland plant *Typha latifolia* the concentrations of Pb in the leaves are maintained at low levels both in uncontaminated and metal-polluted areas, and metal tolerance and metal exclusion ability of this species appears to be related to its oxygen transport capability and radial oxygen loss from the roots, resulting in a greater ability to modify the rhizosphere and immobilize metals in it (Ye *et al.*, 1997).
Biochemical tolerance to Pb is related to the capacity of the plants to restrict Pb to the cell wall, actually binding of Pb to the carboxyl groups of mucilage uronic acids and of carbohydrate in cell walls also restricts respectively the uptake of Pb into the root and the transport via apoplast. Moreover tolerant rice

verities are reported to up-regulate the synthesis and secretion of oxalate, thereby precipitating and reducing Pb uptake by root (Sharma *et* Dubey, 2005; Yang *et al.*, 2000).

The Pb hyperaccumulator *Sesbania drummondii* has also been shown to accumulate Pb as lead acetate in roots and leaves, lead sulfate in leaves and lead sulfide in root and shoot as part of Pb-detoxification strategy (Sharma *et al.*, 2004; Shah *et* Nongkynrih, 2007).

Mechanisms of Pb detoxification include sequestration of Pb in the vacuole in the form of complexes as the major strategy, and binding of Pb by chelators such as phytochelatins, glutathione and amino acids. Chelators contribute to metal detoxification by buffering cytosolic metal concentrations, whereas chaperones specifically deliver metal ions to organelles and metal requiring proteins. In plants the principal classes of metal chelators include phytochelatins, metallothioneins, organic acids and amino acids (Sharma *et* Dubey, 2005).

Phytochelatins (PCs) and Metallothioneins (MTs) are different classes of cysteine-rich, heavy metal-binding protein molecules, that can effectively bind a wide range of heavy metals with high affinity. Phytochelatins (PCs) in plants form a family of structures with increasing repetitions of the γ-Glu-Cys dipeptide followed by a terminal Gly, their structure can be represented by (γ-Glu–Cys)n–Gly. Due to the repeating Glu–Cys moieties, PCs offer a higher metal-binding capacity than MTs resulting more attractive to heavy metals (Sharma *et* Dubey, 2005; Shah *et* Nongkynrih, 2007; Cobbett, 2000).

Metallothioneins (MTs) are ubiquitous low molecular mass cysteine-rich proteins and bind metal ions in metal-thiolate clusters. Actually they are a family of small proteins characterized by a high percentage of cysteine (Cys) residues, and recent reports show their involvement also in scavenging of reactive oxygen species (ROS) (Hassinen *et al.*, 2011; Cobbett *et* Goldsbrough, 2002). More than 50 MTs are reported in different plants (Cobbett *et* Goldsbrough, 2002).

Whereas MTs are gene-encoded polypeptides, PCs are enzymatically synthesized peptides. PCs are in fact synthesized from glutathione, through the transpeptidation of a γ-Glu-Cys moiety from a donor to an acceptor molecule by the action of PC synthase, a constitutive enzyme requiring post-translational activation by heavy metals and/or metalloids. Plants exposed to Pb and other heavy metal pollutants like Cd, Zn, Cu, Hg synthesize in fact phytochelatins, which complex the metal ions to inactivate and store them in the vacuole (Cobbett, 2000; Sharma *et* Dubey, 2005; Shah *et* Nongkynrih, 2007).

Besides organic acids and amino acids are potential ligands for chelation, owing to the capacity of metal ions to react with S, N and O. Accumulation of excess total amino acid in response to Pb can in fact be regarded as an important non-specific adaptive response of plants to avoid Pb toxicity (Sharma *et* Dubey, 2005). Citrate, malate, and oxalate have also been implicated in a range of processes, including differential metal tolerance, metal transport through xylem and vacuolar metal sequestration (Shah *et* Nongkynrih, 2007).

Several non-specific defense systems are also activated when plants are exposed to Pb. These include synthesis of osmolytes (like proline) and polyamines (putrescine), changes in the chemical composition of the cell wall (callose and suberin deposition), and changes in hormonal balance (primarily that of ethylene and abscisic acid ABA). There are different opinions regarding mechanisms by which osmolytes (proline) alleviate metal toxicity effects within the cell, and it has been shown that free proline acts as an osmoprotector, protein stabilizer, metal chelator, inhibitor of lipid peroxidation and free radical scavenger (Sharma *et* Dubey, 2005).

On the other hand as far as organic Pb compounds in plants are concerned, to the best of our knowledge no data are reported in literature.

### 1.2.2.4 Interactions plant-microorganisms

Symbiosis between plants and microbes in the rhizosphere has long been studied by microbial ecologists. The surface of plant roots and the surrounding soil area constitute a complex environment, referred to as the rhizosphere, where microbial activity is high and bacterial population density is one to two orders of magnitude higher than in bulk soil (Matilla *et al.*, 2007; Maier *et al.*, 2009).

Indeed with high concentration of nutrients exuded from the roots, such as organic acids, enzymes, amino acids, and complex carbohydrates, the rhizosphere attracts more bacteria than in the bulk soils leading to an enhanced microbial population. Moreover, the plant root system aerates the soil, distributes bacteria through soil and penetrates otherwise-impermeable soil layers (Belimov *et al.*, 2001).

The bacteria - including Plant growth promoting rhizobacteria (PGPR) - in reverse facilitate the growth of the plant - even in stress conditions such as flooding, drought or contaminated soils - and are considered potential tools for sustainable agriculture and the trend for future (Zhuang *et al.*, 2007; Erturk *et al.*, 2010; Taghavi *et al.*, 2009a; Khan *et al.*, 2009; Jing *et al.*, 2007). For example, maize and lettuce inoculated with *Rhizobium leguminosarum* were demonstrated to have increased growth through enhanced solubilization of phosphate, and sunflowers inoculated with *Rhizobium* sp. exhibited increased nitrogen uptake (Belimov *et al.*, 2001). Besides PGPR facilitated and increased the growth of Poplars in perturbed soils, of *Phragmites australis* - the common reed - in the presence of metals and organic compounds, and of *Lupinus luteus* (lupines) on heavy metal polluted soils (Reed *et al.*, 2005; Dary *et al.*, 2010).

#### *Plant growth promoting rhizobacteria (PGPR)*

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, capable of promoting the extent or quality of plant growth by colonizing the plant root. In the last few decades a large array of bacteria - including species of *Pseudomonas*, *Azospirillum, Azotobacter*, *Klebsiella*, *Enterobacter, Alcaligens*, *Arthobacter, Burkholderia*, *Bacillus* and *Serratia* - have been reported to enhance plant growth (Ahmad *et al.*, 2008; Zhuang *et al.*, 2007; Taghavi *et al.*, 2009a; Khan *et al.*, 2009).

The mechanisms involved in plant growth promotion by PGPR include direct and indirect effects. Indeed growth promotion can occur indirectly by the reduction or prevention of the action of plant pathogens, or directly either providing the plant with plant growth promoting substances that are synthesized by the bacterium, or facilitating the uptake of certain plant nutrients from the environment (Zhuang *et al.*, 2007; Erturk *et al.*, 2010).

Actually, even though the exact mechanisms by which PGPR promote plant growth are not fully understood, they include the ability to produce or change the concentration of plant growth regulators - like indole-3-acetic acid (IAA), gibberellic acid, cytokinins and ethylene -, $N_2$ fixation, solubilization of mineral phosphates and other nutrients, and antagonism against phytopathogenic microorganisms by production of siderophores, antibiotics and cyanide (Ahmad *et al.*, 2008; Rajkumar *et al.*, 2010; Banerjee *et al.*, 2010).

In particular some soil microorganisms are able to solubilize insoluble P forms through the process of organic acid production, chelation, and ion exchange reactions and make them available to plants - which take up inorganic phosphate in the two soluble forms of monobasic ($H_2PO_4^-$) and dibasic ($HPO_4^{2-}$) ions (Banerjee *et al.*, 2010). Seed or soil inoculations with phosphate solubilizing microbes have largely been used to improve crop growth and production by solubilizing of fixed and applied phosphates (Rajkumar *et al.*, 2010).

As far as ethylene is concerned, it plays an important role in plant growth but excessive ethylene promoted by stresses as heavy metals can depress growth (Zhuang *et al.*, 2007). Therefore of particular

interest in relation to heavy metal contamination is the PGPR ability to lower ethylene levels in plants through bacterial enzymatic cleavage of the immediate precursor 1-aminocyclopropane-1-carboxylate (ACC) to α-ketobutyrate and ammonia, by the ACC deaminase enzyme. It is postulated that following binding of plant growth promoting bacteria to the plant root and/or seed coat, a sufficient population of bacteria are able to establish a sink for ACC and thereby lower endogenous ethylene levels; as a consequence, root elongation is enhanced. This particular group of bacteria therefore facilitates the formation of longer roots, may enhance seedling survival and plant root growth, and also reduce the deleterious effects of flooding and phytopathogens (Penrose *et* Glick, 2003; Zhuang *et al.*, 2007; Husen *et al.*, 2009).

Moreover it should be mentioned that indole-3-acetic acid (IAA) production by rhizobacteria is believed to play an important role in plant-bacterial interactions, and any direct influence on IAA production by bacteria may in turn affect their phytostimulating efficiency. It has in fact been well documented that the biosynthesis of auxins with their excretion into soil makes a major contribution to the bacterial plant growth-promoting effect and bacterial supply of IAA may be important for successful growth in contaminated environments (Erturk *et al.*, 2010; Kuffner *et al.*, 2010; Jing *et al.*, 2007).

It is also well known that heavy metals can even be toxic for metal-accumulating and metal-tolerant plants, if the concentration of metals in the environment is sufficiently high. In a range of different plant species this is partly attributable to iron deficiency detected in heavy metal contaminated soil. Consequently, the low iron content of plants grown in the presence of high levels of heavy metals generally results in these plants becoming chlorotic, since iron deficiency inhibits both chloroplast development and chlorophyll biosynthesis. However iron deficiency may be prevented by the import of bacterial siderophore-iron complexes, which can serve as an iron source for plant. In fact bacterial siderophores are high-affinity Fe(III) chelators for the acquisition of iron under iron limiting conditions, which under certain conditions can be taken up by plant roots (Kuffner *et al.*, 2010; Jing *et al.*, 2007).

# 1.3 Bioremediation

## 1.3.1 Remediation Background and Bioremediation

Soils are the major sink for metal contaminants released into the environment by anthropogenic activities. Unlike many organic contaminants, heavy metals cannot be destroyed by bio-geochemical processes, but are only transformed from one oxidation state or organic complex to another. Thus remediation of heavy metal contamination in soils and site restoration relies on their removal, immobilization in a non-bioavailable form, or their re-speciation into less toxic forms. Although the latter approach does not solve the problem altogether, it help to reduce noxious effects (Garbisu *et* Alkorta, 2001; Kirpichtchikova *et al.*, 2006; Jing *et al.*, 2007).

Nowadays the remediation of heavy metal and Pb-contaminated soils represents a significant challenge to many industries and government agencies. Various *in-situ* and *ex-situ* soil clean-up technologies have been employed, of which the most common are excavation and disposal in landfill, thermal treatment, electroremediation and soil washing (Kirpichtchikova *et al.*, 2006).

Actually, usually contaminated sites are treated with traditional methods like physical, chemical and thermal processes, with huge costs of excavating and transporting large quantities of contaminated materials for *ex-situ* treatment. The cost of removal of 1 $m^3$ soil from a 1-acre contaminated site is in fact estimated as US $0.6-2.5 million (Shukla *et al.*, 2010). These processes are in fact expensive and also require additional site restoration.

In particular incineration and landfill, which account today for a large proportion of soil cleanup operations, are not environmentally acceptable and applicable for large volumes to be treated, as they simply transfer the pollutants, creating a new waste such as incineration residues and do not eliminate the problem. On the other hand electroremediation can be used to treat clayey and organic soils of low permeability, and soil washing - which is efficient in term of metal solubilization - is usually performed *ex-situ* in reactors with strong mineral acids and bases, destroying irreversibly the texture, biogeochemistry and fertility of the original soil, leaving essentially an inorganic matrix that will not support revegetation (Kirpichtchikova *et al.*, 2006; Mulligan *et al.*, 2001).

Moreover, while a conventional approach can be effective and applicable in small areas, they are not applicable for larger areas (Manousaki *et* Nicolas, 2009). Therefore this has led to an increasing interest in alternative technologies for *in-situ* applications, in particular those based on biological remediation capability of plants and microorganisms (Chaudhry *et al.*, 2005).

### *Bioremediation*

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). Moreover they generally have a high level of public acceptance and are aesthetically pleasing (Zhuang *et al.*, 2007; McGuinness *et* Dowling, 2009).

This technology includes **Microbe-induced bioremediation** based on microbial activities, **Phytoremediation** which uses green plants and **Rhizoremediation** (also called Phytoremediation assisted by soil rhizobacteria) that relies on mutual interaction of plant roots and suitable microbial flora

and which can be considered the most evolved process of bioremediation. The use of plants in conjunction with plant-associated bacteria offers thus much potential for bioremediation as plant and soil microbes develop a rhizospheric zone with highly complex symbiotic and synergistic relationships, which also can be used as a tool for accelerating the rate of degradation or to remove contaminants (Shukla *et al.*, 2010; McGuinness *et* Dowling, 2009).

### 1.3.2 Microbe-induced bioremediation

Microbe-induced bioremediation exploits the genetic and biochemical capacities of bacteria for the remediation of organic compounds and heavy metals.

Therefore due to the ability to tolerate metal toxicity, adsorb and accumulate heavy metals ions or degrade organic pollutants, specific microorganisms can be studied and used in bioremediation of polluted environments (Zhuang *et al.*, 2007).

It is first important to consider that every remediation approach is site-specific and has to take into account the peculiar characteristics of the contamination and contaminated area. Moreover no organisms or groups of organisms are universally applicable to all cases, although some can be metabolically versatile and are capable of degrading a wide spectrum of substrates, thus all procedures will be necessarily site-specific.

Depending on the detection in the contaminated matrix of metabolic activity functional to the contaminant detoxification, **Microbe induced-Bioremediation** relies on two approaches: **Biostimulation**, stimulating native microbial population, and **Bioaugmentation**, which imply an introduction of viable population to the to-be-treated area (Shukla *et al.*, 2010; Thompson *et al.*, 2005).

Actually if a functional metabolic activity is present, in a biostimulation protocol soil conditions are modified to enhance catalytic capacities of autochthonous microorganism by supplementing nutrients (nitrogen and phosphorus) and/or electron acceptors (oxygen) until a decontaminated desired threshold is reached. On the other side, in absence of a sufficient metabolic activity, functional to the contaminant remediation, it is possible to introduce a viable population with desired catalytic capabilities adopting a bioaugmentation protocol. In the latter case, a massive quantity of autochthonous microorganisms previously cultivated or allochthonous microorganisms with desired metabolic characteristics are bioaugmentated to the soil itself (Shukla *et al.*, 2010; Thompson *et al.*, 2005; Fantroussi *et* Agathos, 2005).

Besides bioaccumulation and biosorption applications are reported for the removal of different kinds of heavy metals by contaminated solutions. Biosorption and bioaccumulation involve interactions and concentration of toxic metals or organic pollutants in the biomass, either living (bioaccumulation) or non-living (biosorption), and are considered a potential instrument for the removal of metals from waste solutions and for precious metals recovery, offering an alternative to the conventional processes, such as those based on ion exchange or adsorption on activated carbon (Chojnacka, 2010).

Biosorption and bioaccumulation differ in that in the first process pollutants are bound to the surface of bacterial cell wall, while in the second they become also accumulated inside the cell. Biosorption and bioaccumulation are mainly used for the removal of metal cations from solutions, including wastewaters from metallurgical industry, rinse waters from electroplating, mining operations, leachates, surface and ground waters. Biosorption of Pb by *Rhizopus arrhizus* and Bioaccumulation from immobilized growing yeast are also reported in literature (Chojnacka, 2010).

*Cupriavidus metallidurans* CH34 is also a versatile heavy metal resistant soil bacterium with high adaptation behavior, and many application possibilities due to its strong genetic pool are started to be investigated; its behavior toward heavy metal binding and flotation was in fact combined and studied in a

lab-scale biometal sludge reactor to extract and separate heavy metals from metal contaminated soils (Diels *et al.*, 2009).

#### 1.3.2.1 Organic lead Microbe-induced Biodegradation cases

As far as bioremediation of soil contaminated by tetraethyl lead is concerned, few data are reported in literature.

Fallon (US patent, 1997) described a process for promoting the degradation of TEL and other organo-lead compounds in contaminated media via the stimulation of indigenous micro flora, i.e. in a biostimulation protocol (Fallon, 1997). The process was based on the stimulation of microbes supplying oxygen and with periodic addition of dilute solutions of complex biological extracts to the contaminated media, resulting in the enhancement of a population of microbes with the ability to transform organo-lead compounds into insoluble inorganic metals. In aerobic laboratory microcosms the percentage of total lead contributed by TEL declined more than six-fold from 46% to 7%. Polar organic lead declined as well from 43% to 15% while the percentage of total lead as inorganic lead increased seven-fold from 11% to 77%. On the other side in an anaerobic treatment, pulsed additions of a source of soluble sulfate were suggested, in order to ensure adequate concentrations for stimulation of the sulfate reducing bacteria. In this second case the percentage of total lead contributed by TEL declined more than three-fold from 57% to 18% and polar organic lead declined as well from 19% to 6%, while the percentage of total lead as inorganic lead increased more than three-fold from 24% to 76% (Fallon, 1997).

As reported in Gallert and Winter (2004), lab-scale experiments were performed in glass columns with sandy soil from a former anti-knocking agents factory adding oxygen-saturated water, and water hydrophobic tetraalkyl-lead was transformed to inorganic lead (Gallert *et* Winter, 2004). In particular, if the alkyl-lead containing water from these columns was diluted to concentrations of alkyl-lead compounds that were found in the groundwater of the contaminated site and were used as a source of alkyl-lead compounds in columns with non-contaminated sandy soil, elimination of tetra-, tri- and dialkyl-lead compounds followed first-order kinetics. It was also detected that in the soil 85.8-93.6% of the alkyl-lead disappeared in only 170 days with 51% being converted to inorganic lead, pointing out an in situ reasonable remediation approach (Gallert *et* Winter, 2004).

Besides soil remediation via air-injection-well (AIW) is an innovative technique combining air-lifting and stripping. Through this technology groundwater circulation is induced and used for remediation, for stripping volatile components, as well as for supporting biodegradation. It consists of a combination of two common remedial techniques: an airlift pump and a strip reactor. With an air lift pump water can be pumped in a vertical tube using pressurised air injected at the basis of the tube in order to create a groundwater circulation cell in the aquifer. Organic compounds are then removed from the water via air-stripping volatilization. A Field AIW application has been reported for a TEL contaminated site, aiming at distributing oxygen in the aquifer in order to enhance microbial degradation to mineral lead in the subsoil. The increase of the soluble Triethyl- and diethyl-lead compounds pointed out that degradation started shortly after the beginning of AIW operation. However TEL showed more or less constant level in water after 100 days operation of AIW, probably because of the release of stored organic lead from the soil. Thus the proposal of a combined nutrient enrichment was suggested, underling that AIW application for organic lead is still in the developmental stage (Luber *et al.*, 2000).

### 1.3.3 Phytoremediation

Phytoremediation, defined as the use of green plants to remove pollutants from the environment or to render them harmless, can be applied to both organic and inorganic pollutants and may involve a number of processes including: **phytoextraction** (also defined as phytoaccumulation) which involves the uptake

and concentration of pollutants into harvestable tissues, **phytodegradation** which is the use of plants and associated microorganisms to degrade organic pollutants, **rhizofiltration** that uses plant roots to absorb and adsorb pollutants, mainly metals, from water and aqueous waste streams, **phytostabilization** which is the use of plants to reduce the bioavailability of pollutants in soils and **phytovolatilization** that involves the removal of pollutants from soil and their release through leaves via evapo-transpiration processes (McGuinness *et* Dowling, 2009; Salt *et al.*, 1998).

The low cost of phytoremediation and its *in-situ* applicability are among the main advantages it offers in comparison to conventional techniques. Phytoremediation has in fact been reported to be approximately 10-fold less expensive than traditional remediation technologies and up to 1000 times cheaper than excavation and reburial (Bidar *et al.*, 2009; McGuinness *et* Dowling, 2009; Jing *et al.*, 2007).

In the last decade phytoremediation has received greater attention and its main application has so far been to remove toxic heavy metals from soil. Although these technologies are still in developmental stages, they appear to have great potential for the clean-up of soils contaminated with Pb and other heavy metals (Sharma *et* Dubey, 2005; Chaudhry *et al.*, 2005).

In particular phytoextraction - more often applied for extracting heavy metals than for organics - not only has no disruptive effect on soil quality, but through the revegetation of polluted areas it also allows establishing a plant cover that will limit the further dispersion of metal-contained soil particles through water and/or wind erosions. Besides it also helps preventing landscape destruction and enhances activity and diversity of soil microorganisms, maintaining healthy ecosystems. It is consequently considered to be a more attractive alternative than traditional methods (Bidar *et al.*, 2009; Shukla *et al.*, 2010; Jing *et al.*, 2007).

Another promising clean-up technology appears to be rhizofiltration, which involves use of plant roots to remove contaminants such as heavy metals from contaminated waters. In this approach plants with a more branched root system seem to take up more Pb and other heavy metals compared to plants with longer and less branched root systems (Sharma *et* Dubey, 2005).

#### 1.3.3.1 Plants and organic compounds

Although the main application for phytoremediation has so far been related to heavy metals removal from soil, it is well known that the presence of plants can enhance degradation of certain recalcitrant organic chemicals in soil. The phytodegradation of organic compounds can indeed take place inside the plant or within the rhizosphere of the plant. Actually *ex-planta* metabolic processes lead to the hydrolysis of organic compounds into smaller units that can be absorbed by the plant. Besides some contaminants can be absorbed by the plant and are then broken down by plant enzymes and the smaller pollutant molecules may then be used as metabolites by the plant as it grows, thus becoming incorporated into the plant tissues (Shukla *et al.*, 2010). In particular plant enzymes have been identified that breakdown ammunition wastes, chlorinated solvents such as TCE (Trichloroethane), and others that degrade organic herbicides (Singh *et* Jain, 2003; Shukla *et al.*, 2010).

In case of complex and recalcitrant compounds, such as pesticides, explosives and solvents which cannot be broken down to basic molecules (water, carbon dioxide etc) by plants, certain plants are however able to render these substances non-toxic by their metabolism. Hence the term phytotransformation represents a change in chemical structure without complete breakdown of the compound. In other cases, microorganisms living in association with plant roots are responsible for metabolizing these substances in soil or water (Shukla *et al.*, 2010; Yoon *et al.*, 2008).

Many different classes of compounds can be removed from the environment by this method, including solvents in groundwater, petroleum and aromatic compounds in soils (Chaudhry *et al.*, 2005; Newman *et* Reynolds, 2004).

However, as far as we know, in this context no data for organic Pb are reported in literature. Actually this is still a relatively new area of research and many laboratories are studying the underlying science necessary for a wide range of applications for plant-based remediation of organic contaminants (Newman *et* Reynolds, 2004; Shukla *et al.*, 2010).

### 1.3.3.2 Pb Phytoextraction

Phytoextraction (or phytoaccumulation) involves the use of plants to remove contaminants from the soil into their above-ground biomass - which can then be harvested using conventional agricultural techniques - and it has been proposed as a cost-effective, environment-friendly alternative restoration strategy for the cleanup of heavy metal contaminated soils (Manousaki *et* Nicolas, 2009; Memon *et* Schröder, 2009).
Actually at the time of plant disposal - which can be composted or incinerated - contaminants are typically concentrated in the much smaller volume of the plant matter than in the initially contaminated soil or sediment. The plants in fact absorb contaminants through the root system and store them in the root biomass and/or transport them up into the stems and/or leaves, and a living plant may continue to absorb them until it is harvested. 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 *et* Shamsuddin, 2010).
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 *et* Nicolas, 2009; Karami *et* Shamsuddin, 2010).
Metal phytoremediation research focused on hyperaccumulating plants, however, usually hyperaccumulators are slow-growing plants and low-biomass producers; in addition, they generally accumulate only one specific element and are low-depth rooted, making them impractical for application in sites with deep contamination (Doumett *et al.*, 2008; Manousaki *et* Nicolas, 2009). In contrast, plants with good growth usually exhibit a low tolerance to heavy metals, low metal accumulation or a preferential root accumulation. Therefore, if such a combination is not possible, a compromise between hyperaccumulation and biomass production must be made (Manousaki *et* Nicolas, 2009; Karami *et* Shamsuddin, 2010).
For practical reasons the shoot Pb concentration is a major physiological parameter for evaluating Pb-phytoextraction potential of plants (Xiong, 1998). Despite this, the hyperaccumulator *Thlaspi rotundifolium*, having a slow growth rate and small biomass is not suited for phytoextraction of Pb from contaminated soils (Huang *et al.*, 1997).
On the other side, for some cultivars of *Brassica juncea* - although not a Pb hyperaccumulator - is reported both a strong ability to accumulate Pb in roots and to transport Pb to the shoots (Sharma *et* Dubey, 2005; Kumar *et al.*, 1995). Indeed in literature *Brassica juncea* (Indian mustard) is reported to be able to accumulate various metals and metalloids, moreover as a crop plant is characterized by a fast and great biomass production, thus resulting interesting in phytoremediation research (Liu *et al.*, 2000; Kapourchal *et al.*, 2009; Sheng *et al.*, 2008a).
Ragweed (*Ambrosia artemisiifolia*), Indian Hemp (*Apocynum cannabinum*), *Mimosa pudica* and Poplar trees, have also been reported in literature in relation to Pb accumulation (Shukla *et al.*, 2010; Lasat, 2000). In particular, Cunningam (U.S.Patent, 1994) claimed that lead can be economically recovered from soil containing organic or inorganic lead by harvesting *Ambrosia* sp. or *Apocynum* sp., which accumulate lead in concentrations of from about 100 mg/kg dry weight of the plant in the above-ground tissues

(Cunningham, 1994). As he claimed, it is expected that one acre of these plants will produce between 15 and 30 tons of dry weight bio-mass per year. It is also expected that as much as 0.2% to 2% of that dry weight will constitute accumulated lead, resulting in the remediation of about 60 lb to 1200 lb of lead per acre per year from contaminated soil (Cunningham, 1994).

### *Chelating strategy*

One of the most critical points of phytoremediation is the phytoavailability of heavy metals in soil, as the effectiveness of phytoextraction can be limited by the sorption of metals to soil particle surfaces and their low solubility. Actually a large proportion of metal contaminants are unavailable for the root uptake by plants, because heavy metals such as Pb in soils are generally bound to organic and inorganic soil constituents, or alternatively, present as insoluble precipitates (Lu *et al.*, 2005).

However, metals can be solubilized by the addition of complexing agents promoting an increasing uptake by plants over time and chelate-enhanced phytoremediation has been proposed as an effective tool for the extraction of heavy metals from soils by plants (Doumett *et al.*, 2008; Karami *et* Shamsuddin, 2010).

To artificially enhance metal solubility in phytoremediation studies synthetic chelates have been used, which resulted in a marked increase in metal uptake by plants (Romkens *et al.*, 2002). The most widely investigated synthetic chelate ethylenediaminetetraacetic acid (EDTA) forms a soluble complex with many metals, including Pb and can solubilize Pb from soil particles. Moreover, EDTA enhances the translocation of Pb from roots to shoots and leaves and produces a decrease in specificity of transmembrane solute transport into root cells (Di Gregorio *et al.*, 2006). Actually large Pb particles cannot easily cross the casparian strip due to their size and charge characteristics but once they form a complex with chelators such as EDTA, their solubility increases, avoiding the processes that would normally prevent their unrestricted movement such as precipitation with phosphates and carbonates, or binding to the cell wall through mechanisms such as cation exchange (Sharma *et* Dubey, 2005).

However it is important to point out that the addition of persistent non-biodegradable or the least biodegradable chelating agents to the soil has to be done in a carefully controlled manner, so as not to mobilize metals into ground water or otherwise promote their off-site migration.

Actually the addition of persistent chelators with high complexant capabilities to the soil can increase the heavy metal concentration in soil solution to well over the translocation potential of plants. There is, therefore, the possibility that heavy metals mobilized in the soil leach into the subsoil or into ground- or surface water and besides create a new source of pollution by the residual chelating reagent (Romkens *et al.*, 2002; Doumett *et al.*, 2008).

Though synthetic chelators are more effective in accelerating heavy metals availability in the soil, a number of studies have therefore been conducted using the natural, low molecular weight organic acids, such as citric, oxalic and tartaric acid, which are characterized by lower toxicity and higher biodegradability (Doumett *et al.*, 2008; Liu *et al.*, 2008).

## 1.3.4 Phytoremediation assisted by soil rhizobacteria

Phytoremediation assisted by soil rhizobacteria (also called Rhizodegradation, Rhizoremediation, Enhanced rhizosphere biodegradation, Microbe-assisted phytoremediation) involves the removal or breakdown of contaminants by mutual interaction of plant roots and microbial flora in the rhizosphere (Shukla *et al.*, 2010). Rhizoremediation can either occur naturally or can be facilitated by inoculating soil with microorganisms capable of degrading environmental contaminants (McGuinness *et* Dowling, 2009).

Plant root exudates act as carbohydrate sources for the soil microflora and enhance microbial growth and activity, whereas some of these compounds may also act as chemotactic signals for microbes. The plant roots also loosen the soil and transport water to the rhizosphere thus additionally enhancing microbial

activity (Shukla *et al.*, 2010; Dzantor, 2007). Moreover the plant root system aerates the soil, distributes bacteria through soil and penetrates otherwise-impermeable soil layers while drawing soluble forms of the pollutants in the soil water towards the plant and the microbes (Belimov *et al.*, 2001).

To date, a number of toxic organic compounds in soil have been successfully remediated using rhizospheric bacteria. Researchers have in fact exploited this symbiotic relationship for rhizoremediation and have documented attenuation of compounds such as volatile organic carbon contaminants, trichloroethylene, naphthalene and polychlorinated biphenyls (Shukla *et al.*, 2010; Sipilä *et al.*, 2008).

Besides, recently microbe-assisted phytoremediation has appeared as a more successful approach for the remediation of soils and many investigations have also focused on the closer relationship between plants and plant growth-promoting rhizobacteria (PGPR), to promote the uptake efficiency of heavy metals by plants (Koo *et* Kyung-Suk, 2009).

As previously reported (par.1.2.2.4), PGPR have a capacity to enhance the growth of the host plant by various mechanisms involving production of specific compounds and increasing nutrient uptake. Moreover several established studies also indicated that PGPR can reduce the toxicity of heavy metals and promote the growth of plants under the toxicity of Ni, Pb or Zn (Koo *et* Kyung-Suk, 2009; Dary *et al.*, 2010; Zhuang *et al.*, 2007; Jing *et al.*, 2007).

Furthermore, some rhizobacteria can excrete organic acids to enhance the bioavailability of heavy metals and a variety of bacteria (mainly PGPR) have been reported as phytoextraction assistants, such as *Pseudomonas* spp., *Bacillus* spp., *Mesorhizobium* sp., *Microbacterium* spp., *Rhizobium* spp., *Variovorax* sp., *Rhodococcus* sp., *Psychrobacter* spp., *Flabobacterium* sp., *Sinorhizobium* sp. and A*chromobacter* sp. (Koo *et* Kyung-Suk, 2009). Mechanisms involved in transfer and mobilization of heavy metals by the rhizosphere microbes include acid and siderophore production, and phosphate solubilization (Jing *et al.*, 2007; Zhuang *et al.*, 2007; Khan *et al.*, 2009).

Also plant roots can increase metal bioavailability by exuding low molecular weight organic acids and protons that acidify the soil and mobilize the metals. The lowering of soil pH decreases the adsorption of heavy metals and increases their concentrations in the soil solution, moreover the formation of soluble complex of heavy metals reacting with exuded organic acids also increase bioavailability of heavy metals to plants. Soil microbes associated with plant roots are also helpful in the phytoextraction of the heavy metals in soils through the degradation of organic pollutants (Liao *et al.*, 2006; Lasat, 2000).

The recent researches of PGPR on the remediation of contaminated soils show a brilliant prospect for the successive studies. For example, rhizobacteria has proved to increase the uptake of Cd in *Brassica napus* (Sheng *et* Xia, 2006), of Ni in *Alyssum murale* (Abou-Shanab *et al.*, 2007), and significantly improved Cu uptake by *B. junce*a (Ma *et al.*, 2009). Besides a symbiotic combination of an indigenous metal-resistant rhizobial strain, *Cupriavidus taiwanensis* TJ208, and its host plant *Mimosa pudica* has been suggested for the removal of heavy-metal pollutants. In fact after nodulation via inoculation, the metal uptake ability of *M. pudica* resulted 86% higher than that of nodule-free plants, also displaying a 71% enhancement in metal adsorption efficiency (Chen *et al.*, 2008).

However, although the extensive use of PGPR for the environmental remediation with plants emerged as a promising field, there have been only very few field studies while most are controlled studies conducted in greenhouses and/or growth chambers (Zhuang *et al.*, 2007; Karami *et* Shamsuddin, 2010).

### 1.3.5 Combined approach of conventional and biological remediation

Whereas phytoextraction can be applied to clean up the soil without causing any kind of harm to the soil quality, being controlled by plant this process takes more time than traditional soil cleanup processes. As a plant-based system, Phytoremediation is not applicable at the highest level of contamination as plant survival is affected by the toxicity and general condition of the soil. Phytoremediation is also limited by

factors such as roots depth, biomass production and plant growth rate, moreover it does not completely prevent the leaching of contaminants into the groundwater. Therefore interest has been drown by the concept of a combined approach of conventional treatments and bioremediation (Shukla *et al.*, 2010; Karami *et* Shamsuddin, 2010; Romkens *et al.*, 2002).

Taking into account that every remediation process is site-specific, in presence of contamination levels too high for a direct bioremediation application, a chemical-physical approach can be applied in a first step. A following biological application can therefore be applied as a lowered contamination level is present, and at the same time it contributes to further decrease the contamination level and favors the soil restoration, reestablishing its structure, biological activity and fertility which have been affected by the contamination and by the previous disruptive approach.

Actually in a study on oil and shale oil contaminated soil, Goi *et al.* (2006) reported that a chemical processes allowed improving the subsequent biodegradability of oil and that the integrated chemical and biological processes resulted more effective than either one alone, proposing this combination as a successful treatment technology for the remediation of oil contaminated soils (Goi *et al.*, 2006). Positive evidence for a combined chemical-biological approach was moreover provided for on-site, field-scale treatment of pesticide-contaminated soil (Waria *et al.*, 2009).

# 1.4 The SLOI Srl (Trento-Nord site) case of study

## 1.4.1 The history

Located in a former industrialised pole in Trento-Nord area - which extends currently for 104.000 $m^2$ and coincides with the northern periphery of Trento - the factory SLOI Srl (*Società Lavorazioni Organiche ed Inorganiche*) produced and added TEL to petrol for almost 40 years. Actually SLOI Srl started its activities in Trento in 1939 and was closed down by the City of Trento in 1978 when a major accident happened (Fig 1.5).

The area formerly occupied by SLOI is currently vacant and it is mostly polluted with both inorganic and organic lead. Although the functioning infrastructures were decommissioned when the industrial plan closed down and the equipment such as chemical reactors and tanks were recycled or reused in other plants, the abandoned building are still visible (Fig 1.6).

The SLOI occupied an area of 48.000 $m^2$ and it was assigned to the processing of ethyl chloride and lead/tetraethyl lead and to the storage of hydrochloric acid, hypochlorite, tetraethyl lead and liquid chlorine. Tel synthesis was performed in the Reactor section, while the end product was added to petrol in the Mixer section (Fig.1.6). Fuel oil and settling tanks as well as depots for goods were also situated within this area together with offices, staff rooms, workshops, laboratories and car parks.

The maximum quantity of tetraethyl lead produced in a year was estimated to be about 13.000 tones with a resulting waste production of about 300 tons per year.

In order to obtain one molecule of TEL - $(C_2H_5)_4Pb$ - four molecules of an alloy of lead and sodium and four molecules of ethyl chloride were needed in the process:

$$4NaPb + 4C_2H_5Cl \rightarrow (C_2H_5)_4Pb + 4NaCl + 3Pb$$

In order to extract and recycle lead from the mixture so produced, the product was separated by distillation through an aerosol flow.

Ethyl chloride was produced *in-situ* - in the $C_2H_5Cl$ production section shown in Fig. 1.6D - as a result of chlorination of ethyl alcohol using hydrochloric acid from the sodium-chlorine plant. The production of sodium hypochlorite needed the reagents chlorine, sodium and mercury. Dichloroethane and dibromoethane were added to tetraethyl lead to obtain a solution more suitable for the function of the petrol antiknock additive. The finished product was a liquid ternary mixture (TEL, dibromoethane, dichloroethane) containing 60% lead.

Towards the end of 1978, during a violent storm, rainfall entered the sodium depot. A ferocious fire started and a toxic cloud rich in vapours of sodium hydroxide reached the city centre almost immediately (Fig.1.5). The flames were put down by pouring concrete onto the area affected by the fire and the dome of concrete is still visible on site. The Mayor of Trento ordered the immediate closure of the factory and after the forced closure, the local authority also requested the complete dismantling of all infrastructures. However after an investigation the degree of contamination by organic compounds of lead was found to be very high and so the buildings were left *in-situ* and have remained to this day (Fig1.6.) (Collins *et al.*, 2004).

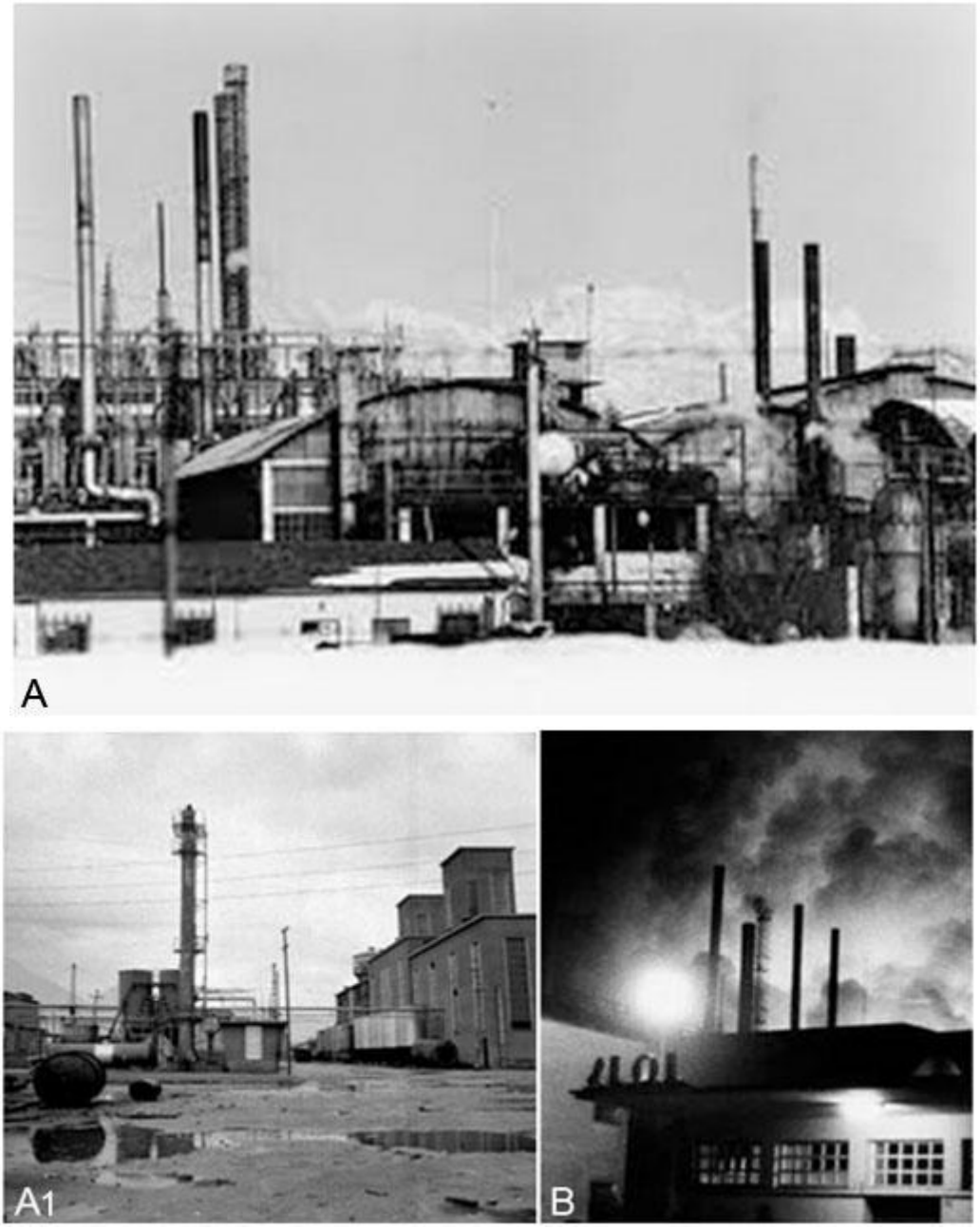



**Fig 1.5** SLOI factory during the production activity (A, A1) and during the fire (1978) (B)


**Fig 1.6** Nowadays SLOI factory's dismissed area: entrance (A), Reactor section (B), Mixer section (C) $C_2H_5Cl$ production section (D)

## 1.4.2 Remediation priority

SLOI was located in the former industrialised pole in Trento-Nord area, which is characterized by high levels of pollution and, as a result of different industrial activities, the contamination varies between areas (Fig 1.7). In fact the two included sites Ex-CarboChimica and Fransy are contaminated mainly by solvents, phenols and poly-aromatic hydrocarbons (PAHs) whereas Ex-SLOI and Nilupa-BI.MA. sites, covering together 61,300 $m^2$, are mainly contaminated by organic compounds of lead and mercury.

In the 2001 the Italian Government approved the 'National Program of remediation and environmental recovery' and in the norms of the Ministerial Decree n. 468/01 it identified highly contaminated sites - contaminated sites of national interest - as a result of risk-based analysis procedures. About 50 areas in Italy have been identified as high-risk environmental areas in need of urgent clean up and Trento-Nord, including Ex-SLOI area, is one of these sites of national concern, i.e. associated to high health risk and for which remediation is a priority.

As far as Pb is concerned, the limits laid down by Italian regulations (Legislative Decree 152/2006) for green use of soil is 100 mg $kg^{-1}$ d.w and for commercial/industrial use is 1000 mg $kg^{-1}$ d.w.

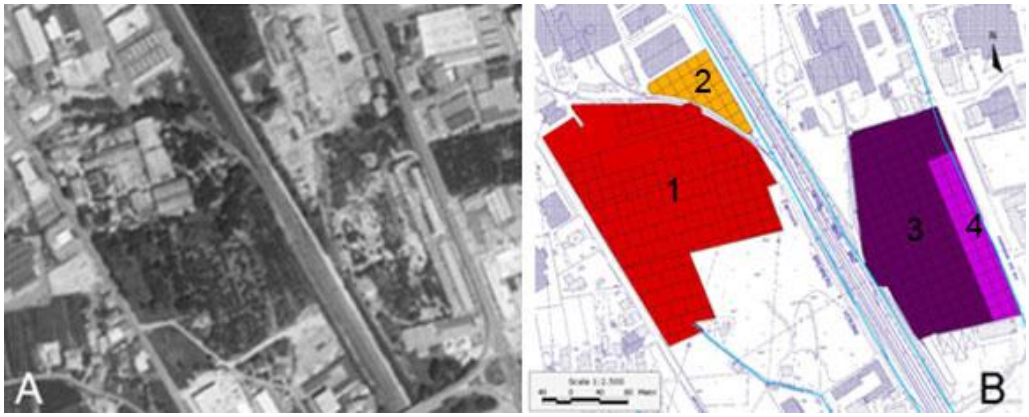



**Fig 1.7** Aerial view and geographical position of Ex-SLOI (1), Nilupa-BI.MA (2), Ex-CarboChimica (3) and Fransy (4) in the industrial pole in Trento-Nord site (B)

## 1.5 Aims of the study

Lead has been recognized as one of the most hazardous heavy metals within a list of priority environmental pollutants. Actually this metal has been widely used since ancient time and it plays a central role in the industrial economy. Besides, its spreading in the environment is also connected to both agricultural and urban activities such as land application of sewage sludge, smelting operations and use of leaded petrol (Thornton *et al.*, 2001; Hill, 2004; International Lead association, 2009).

Indeed Tetraethyl-lead (TEL) was used since the beginning of the last century as a petrol additive because of its anti-knocking properties, which increased the fuel octane rating and efficiency. Despite the use of organic Pb in petrol has been banned in most countries due to its high toxicity, its use for almost a century in automotive petrol has lead to an ubiquitous pollution with both organic and inorganic Pb and more severe soil-water contamination at oil refineries and petrol stations (Gallert *et* Winter, 2002; Lazzaro *et al.*, 2006). While inorganic Pb is an aspecific toxin interacting with metalloenzymes, TEL is essentially a severe neurotoxin affecting mainly the nervous system (World Health Organization, 2007; Godwin, 2001; Environmental and Occupational Medicine, 2007). Remediation of contaminated areas is therefore required and imposed by law.

Although the mechanism is still not clear, in soil TEL is known to be degraded both biologically and chemically in sequential de-alchylation and a reduction of environmental risk is achieved by the complete mineralization to the much less toxic and mobile inorganic Pb (Teeling *et* Cypionka, 1997; Gallert *et* Winter, 2002; Ou *et al.*, 1995). However, as far as organic lead and its degradation are concerned, only few studies are available in literature.

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 Phytoremediation 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 *et* Nicolas, 2009; McGuinness *et* Dowling, 2009).

In this context the present PhD study deals with a real case of a soil contaminated by both organic and inorganic lead in the former industrial area SLOI (*Società Lavorazioni Organiche Inorganiche*) in Trento. SLOI was one of the few leader companies in Europe, manufacturing anti-knocking additives for automotive petrol for almost 40 years till the end of the '70s. Nowadays a high contamination is present in the entire former industrial area reaching values up to 23.000 mg/kg of inorganic Pb and reporting an esteem of approximately 168 tones of total lead, of which at least 62 tones of alkyl-lead. This area was in fact declared site of national concern by the Environmental Department (DM 468/01) – i.e. implying high environmental risk and priority remediation.

This PhD study examined the soil autochthonous bacterial community selected by and acclimated to the contamination of both inorganic and organic Pb present in this ex-industrial area for over half century. At the same time this study also focused on the interaction of the indigenous micro flora and plants in relation to Pb contamination, in a Phytoremediation approach. In particular two plants were included in the study: *Brassica juncea* and *Apocynum cannabinum*. While the first one is a crop plant known to accumulate various metals including inorganic Pb (Liu *et al.*, 2000; Kapourchal *et al.*, 2009; Sheng *et al.*, 2008a), the second was suggested for both organic and inorganic Pb phytoextraction, although only few data are reported in literature (Cunningham, 1994).

In this context the present work included the following main objectives:

- to study the biodiversity and composition of the soil autochthonous bacterial community at different contamination levels within the area, to evaluate the impact of a long-term exposure to organic and inorganic Pb, and the resistance and bioremediation potential of the selected micro flora;
- to isolate and characterize members of the soil microbial community in relation to their resistance and plant growth promoting potential, even in the perspective of a Phytoremediation application in a biaugmentation protocol;
- to evaluate the interaction of the stress adapted autochthonous micro flora established within the examined area and its possible synergistic role in a Plant-Rhizobacteria system in relation to both inorganic and organic Pb;
- to study the tolerance and basic phytoremediation potential of *Brassica juncea* and *Apocynym cannabinum* in relation to the examined combined contamination of inorganic and organic Pb.

# 2. Material and Methods

# 2.1 Chemicals, culture media and solutions

Chemicals purchased from Sigma-Aldrich (Milan, Italy) were all analytical grade. Nutrient Broth, Yeast Extract and Bacteriological Agar were furnished by Oxoid Italia S.p.a (Garbagnate Milanese, Italy). TEL was furnished by Sigma-Aldrich as solution 50% wt in xylene.
When necessary Inorganic lead ions were aseptically added as $Pb(NO_3)_2$. $Pb(NO_3)_2$ was prepared as a 100 mM stock solution in deionised water and sterilized by filtration.

## 2.1.1 Growth media

**Nutrient Broth** was furnished by Oxoid Italia S.p.a. 14 g $l^{-1}$ of Bacteriological agar was added in Nutrient Agar.
**Minimal defined Medium (DM)** was prepared as previously described in (Frassinetti *et al.*, 1998). 14 g $l^{-1}$ of Bacteriological agar was added in DM Agar.
**Minimal defined Medium plus Yeast Extract (DMY)** was prepared as previously described in Minimal defined Medium (DM) and supplemented with 0.1% of Yeast extract. If higher % of Yeast extract were supplemented is specified.
**Reasonar's 2 agar (R2a):** Yeast Extract 0,5g $l^{-1}$; Peptone pancreatic digest of casein 0,5g $l^{-1}$; Casein Hydrolysate (acid) 0,5 g $l^{-1}$; D-Glucose anhydrous 0,5 g $l^{-1}$; Starch 0.5 g $l^{-1}$; Sodium pyruvate 0,3 g $l^{-1}$; $K_2HPO_4$ 0,3 g $l^{-1}$; $MgSO_4$ (anhydrous) 0,05 g $l^{-1}$; 10 g $l^{-1}$ of Bacteriological agar were added in R2a Agar.
**Malt Medium:** Malt Broth 20,0 g $l^{-1}$; Yeast Extract 5,0 g $l^{-1}$ . 10 g $l^{-1}$ of Bacteriological agar were added in Malt Agar. After sterilization, Malt medium was supplemented with 15 mg $l^{-1}$ of rifampicin (broad-spectrum antibacterial).
**Waksman Medium (pH 7):** Glucose 10,0 g $l^{-1}$ ; Sodium Chloride 5,0 g $l^{-1}$; Bacteriological peptone 5,0 g $l^{-1}$ ; Lab Lemco Powder 3,0 g $l^{-1}$. pH was adjusted to 7 prior to sterilization, afterwards Waksman medium was supplemented with a mix of antibiotics, namely 50 mg $l^{-1}$ of nistatin (with fungistatic and fungicidal action against a wide variety of yeasts and yeast-like fungi), 5 mg $l^{-1}$ of polymixin and 4 mg $l^{-1}$ of anfotericin B (antifungal agents).
**Luria Bertani (LB):** 10 g $l^{-1}$ tryptone; 5 g $l^{-1}$ yeast extract; 10 g $l^{-1}$ NaCl. 14 g $l^{-1}$ of Bacteriological agar was added in LB Agar.
**SOC broth:** 10 g $l^{-1}$ tryptone; 5 g $l^{-1}$ yeast extract; 0.5 g $l^{-1}$ NaCl; 10 ml $l^{-1}$ KCl [250 mM]; 5 ml $l^{-1}$ $MgCl_2$ [2M]; 5 ml $l^{-1}$ glucose [1M].
**Dworkin-Foster (DF)** salts minimal medium was prepared as previously described in (Penrose *et* Glick, 2003).

## 2.1.2 Solutions

**Salkowski's reagent**: 150ml concentrated $H_2SO_4$, 250 ml deionised $H_2O$, 7.5 ml 0.5 M $FeCl_3 \cdot 6H_2O$.
**Half-strength Hoagland solution** was prepared as described in (Zayed *et al.*, 1998), and sterilized by filtration.
**Tris-Acetate EDTA (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 (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 $H_2O$.

If not otherwise specified, all media were sterilized by heating at 121°C under a pressure of 105 kPa for 15 minutes.

## 2.2 The Ex- SLOI sampling area

Nowadays the whole Ex-SLOI area of 48.000 $m^2$ results to be mostly polluted with inorganic and organic lead, with an estimated contamination of about 168 metric tons of total lead of which 62 metric tons are represented by alkyl lead.

Actually after the closure of SLOI, serial surveys have been performed by the local authorities (*Provincia Autonoma di Trento*) on the entire area since 1982, with numerous probing and positioning of various piezometers, the latter to allow the monitoring of ground waters. Pb has been detected in 11 prospectings on 32, with the highest detected concentration of 23.0000 mg/kg soil and 1050 mg/kg soil for respectively inorganic and organic Pb detected in the probing K2 (shown in Fig 2.1) and hereafter named Hot spot.


**Fig 2.1** Hot Spot in the map of surveys performed on Ex-SLOI area (nov.1997-febr.1998)

### 2.2.1 Trento-Nord area's geological characteristics

The Trento-Nord site is located in the northern part of the city of Trento - in the *Trentino Alto Adige* region of Italy - in an alluvial plain between the two rivers *Avisio* and *Fersina*, both tributaries of the river *Adige*, the main river in the valley. In the last part of the XVIII century, the course of the river *Adige* was subjected to a major realignment towards the western part of the valley, in order to protect Trento town centre from flooding events and at the same time a complex network of ditches and streams was created.

Geological investigations carried out by the geological department of the local authorities identified the presence of a succession of alluvial deposits in this area, as result of the inundation of the river Adige and the deposition of the secondary streams flowing from the valley sides. The granulometry of these deposits ranges from fine to medium-fine and medium-large. The local soil type ranges from clayey silt and sandy silt, silty sand and sand with gravel, in a mainly alkaline soil.

These alluvial deposits can reach a depth of several dozens of meters and generally the upper stratum appears with finer sediments. The soil profile of Trento-Nord can be described by a thin layer of filling material (about 1m), a horizon of silty soil (about 5m), the aquifer characterized by gravel and sand

(about 7m), a layer of clay and sandy lime with organic matter (about 2m), another stratum of sands and gravel (about 5m), a stratum of weakly clayey silt with sand layers and finally some alternations of fine sand, silt and clay (about 15m), to the depth of 40 m below ground level (Fig. 2.2) (Collins *et al.*, 2004).

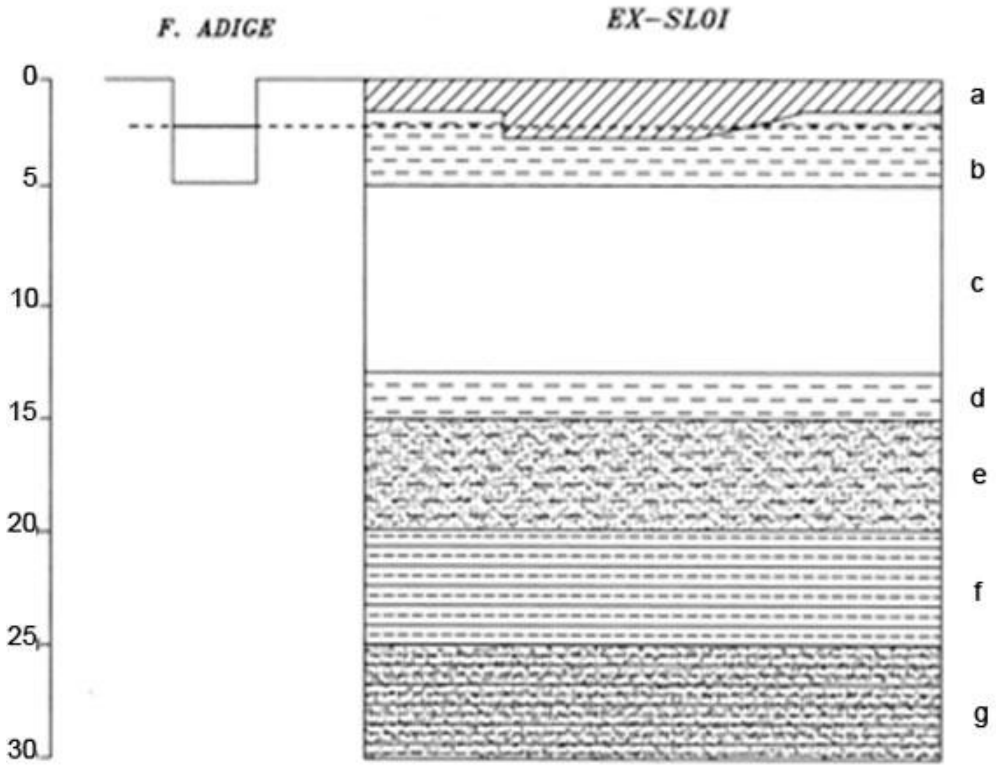



**Fig 2.2** Lithologic structure of Trento Nord and Ex-SLOI area: a) filling material, b) silty soil c) gravelly sand to sandy gravel, d) clay and sandy limestone with organic matter, e) sands and gravel, f) weakly clayey silt with sand layers, g) alternations of fine sand, silt and clay

## 2.2.2 Sampling locations within Ex-SLOI area

Within the Ex-SLOI area three different reference locations - named Point I, Point II and Point III (shown in Fig 2.3, 2.4) - were selected as sampling points to be studied.

These 3 locations have been chosen on the basis of their contamination level, for which the following concentration ranges were reported by the local authorities (*Provincia Autonoma di Trento)* in charge of the area.

**Point I** presents a contamination >2000 mg/kg of inorganic Lead and <50 mg/kg of organic Pb. Besides it also contaminated by about 20-30 mg/kg hydrocarbons. This sampling point was in fact located between the Reactor for TEL synthesis and the Mixer, where the antiknock mixtures were added to petrol.

**Point II** was located just behind the reactor section and it is in fact the most polluted of the 3 locations, with a reported contamination >4000 mg/kg for inorganic Pb and >100 mg/kg of organic Pb.

**Point III** is characterized by a lower content of both organic and inorganic Pb, but at the same time it is also contaminated by Hg, with concentration in the order of tens of mg/kg. Actually this location was next to $C_2H_5Cl$ production section, which implied mercury in its processes.

Together with these 3 sampling points, the above mentioned Hot spot (par.2.2) - with the highest contamination detected within the Ex-SLOI area - was included as reference sampling point under study.

The **Hot spot** was reported to be characterized by the maximum concentration of 1050 mg/kg for organic Pb, including about 450 mg/kg TEL, and reaching the maximum concentration of 23.000 mg/kg for inorganic Pb.

Samples at the three sampling points (Point I,II,III) were collected directly at the Ex-SLOI area at 0.10-0.20 m depth and stocked in hermetic polyethylene barrels, at room-temperature.
For the Hot spot two sampling from a probing were included: Hot spot A collected from 0 to 1m depth and Hot Spot B collected at a higher depth of 3 meters.
The collected samples were air-dried under chemical hood and sieved through a 2mm sieve for the subsequent study.
Within this vast contaminated area a real control was not available. However as approximate mean of comparison a soil sample was collected in the Ex-SLOI parching area, the location within the Ex-SLOI area most far from the TEL synthesis reactor and reporting a much lower contamination in the order of hundreds mg Pb/kg (fig 2.3,2.4).
In this PhD study the three sampling points and the hot spot have been analyzed through two main approaches: culture dependent (described in par. 2.3) and culture independent (described in par. 2.6), both focused on the study and characterization of the autochthonous bacterial community selected by and acclimated to the Pb contamination present in the area.
As far as the Hot spot is concerned, both samplings Hot spot A and B were object of the molecular analysis, while the culture study was exclusively performed on the sampling Hot spot A - simply named Hot spot - collected from 0 to 1m depth.
The soil of the three reference locations Point I, II and III was also used in the Phytoremediation study, described in par. 2.7.

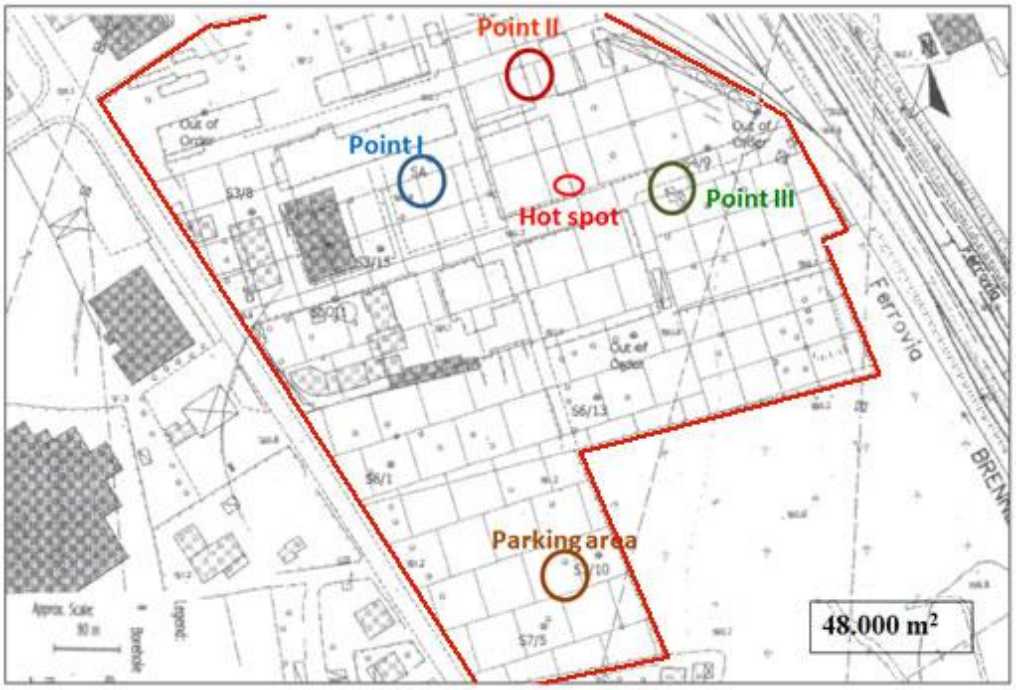



**Fig 2.3** Position of reference points in the planimetric Map of the Ex-SLOI area in Trento-Nord.


**Fig 2.4** Indicative position of the reference points (Point I: P.I, Point II: P.II, Point III:P.III and Hot Spot:H.S), the parking area (P.a.) and of the Reacor section (A), $C_2H_5Cl$ production section (B) and Mixer section (C) within the Ex-SLOI area

# 2.3 Culture-dependent study

For each sampling point (I,II,III) and the Hot Spot the culture-dependent approach involved:

- Enumeration of total cultivable microflora: heterotrophic aerobic eubacteria enumerated using rich-medium Nutrient and low-nutrient medium Reasonar's2 agar (R2a), Actinobacteria counted with Waksman medium and Fungi with Malt medium.
- Direct isolation and identification of heterotrophic aerobic eubacterial strains from Nutrient and R2A enumeration's plates
- Isolation and characterization of bacterial strains by enrichment culture performed on minimal medium DM containing $Pb(C_2H_5)_4$ respectively as sole carbon source and in presence of Yeast exctract 0,1%.

As next described, the Operational Taxonomic Units (OTUs) thus obtained (par. 2.5.1.4) were studied in relation to their resistance and degrading potential towards the contaminant, and screened for Plant growth promoting (PGP)traits.

## 2.3.1 Enumeration of cultivable microflora

In 250-ml Erlenmeyer flasks 10 g of sieved soil - from each of the sampling point Point I,II,III and Hot spot - were suspended in 100 ml of physiologic solution (0.9% wt/vol NaCl) and magnetically stirred for 1hour, to separate microbial cells from the soil. After decanting for 15 minutes, 10-fold serial dilutions were spread on solid agar plates. The rich-medium Nutrient and low-nutrient medium Reasonar's 2 agar (R2A) allowed enumeration of heterotrophic aerobic eubacteria, Waksman medium was used for Actinobacteria and Malt medium for Fungi. For all media, agarized plates were inoculated in duplicate at each dilution level 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 Waksmann medium.

## 2.3.2 Isolation of soil bacterial strains

### 2.3.2.1 Direct isolation

After 5 days of incubation and enumeration of the total cultivable microflora on Nutrient and R2a agar plates, for each culture medium colonies of different morphological characteristics - such as colony pigmentation, shape, size, edge - were isolated and repeatedly streaked on fresh growth medium plates and incubated at 27°C, until axenic culture were obtained. This was performed for all the sampling points under study (I,II,III) and the Hot spot. Pure cultures of the isolated strains were then stocked at -80°C in glycerol 60%.

### 2.3.2.1 Enrichment Cultures

Enrichment cultures for bacterial isolation were inoculated with soil samples from each sampling point (Point I,II,III) in 100 ml minimal medium DM amended with 125 mg/L TEL, respectively as sole carbon source (named DM), and with addiction of 0,1% yeast extract (named DMY).

For the Hot Spot it was performed only the enrichment culture DM, namely with TEL as sole carbon source.

Both enrichment cultures (DM and DMY) were carried out in 250-ml Erlenmeyer flasks and incubated at 27°C on an orbital shaker (250rev/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 125 mg/l TEL.

After 8 weeks the isolation of the selected resistant microorganisms was made plating 100µl 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 6 days and, as in the direct isolation, single colonies were then isolated and axenic cultures of morphologically different bacteria were obtained.

### 2.3.3 MIC (Minimum inhibitory concentration) determination for organic and inorganic Pb

The minimum inhibitory concentration (MIC) of Pb was determined for all OTUs obtained by enrichment culture and Hot Spot. MIC was moreover determined for the OTUs most represented between the isolates obtained by direct isolation from the 3 sampling points (I,II,III).

Minimum inhibitory concentration (MIC) for TEL and $Pb(NO_3)_2$ was determined on DMY0,2% agarized plates and performed in duplicate. One representative strain for each tested OTU was scrapped on plates containing different concentrations of TEL - 200-500-700-1000 mg/kg TEL - and $Pb(NO_3)_2$ - 1mM, 2mM, 3mM, 5mM, 7mM, 8mM, 10mM $Pb(NO_3)_2$. Plates were checked for microbial growth after 5 days of incubation. The lowest concentration of Pb that prevented growth was the MIC.

### 2.3.4 Growth Test with Tel as sole carbon source

Growth test were performed in 100 ml flask in liquid DM medium supplemented with TEL as sole carbon source. Briefly, a pre-inoculum of the selected OTU was grown in 4 ml of DMY medium for 48 h. The cells were collected by centrifuge (5000 rpm for 5 min at 4°C) and washed twice with physiologic solution (0.9% wt/vol NaCl). The bacterial inoculum was supplied to 50 ml of DM supplemented with TEL (125, 250 and 500 mg/L TEL) to obtain an initial OD(600nm)=0.1. Flasks - hermetically closed with a metal ring - were incubated at 27°C on an orbital shaker (250rev/min) in the dark for 10 days. A sample of this suspension was collected periodically throughout the incubation period. The bacterial growth was determined evaluating the absorbance of the suspension at 600 nm by spectrophotometer. In parallel for each tested strain a positive control - to check for cells viability - was performed with no TEL and the addiction of yeast extract. One trial with no bacterial inoculum was carried out as negative control. This test was performed for all OTUs selected by Enrichment culture and from Hot Spot.

# 2.4 Plant growth promoting (PGP) traits analysis

## 2.4.1 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 *et* 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 $l_{-1}$). 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-Ammonium Sulfate and DF-ACC, the latter containing 3.0mM 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 (250rev/min) in the dark for 7 days. An aliquot was collected throughout the incubation period and the bacterial growth was determined evaluating the absorbance of the suspension at 600 nm by spectrophotometer.

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.

## 2.4.2 Assay for indoleacetic acid (IAA) production

The bacteria were cultured for 4 days in flasks containing 20 mL DF medium supplemented with N as $(NH_4)_2SO_4$ (2 g $l^{-1}$) and with 0.5 mg $ml^{-1}$ tryptophan, precursor of IAA. After incubation, 1ml of the cell suspension was transferred into a tube and then mixed vigorously with 2mL *Salkowski*'s reagent and allowed to stand at room temperature for 20 min, afterwards the cell suspensions color was checked (Cavalca *et al.*, 2010). The development of pink color indicates IAA production.

# 2.5 Phylogenetic analysis through molecular taxonomic techniques

The molecular methods 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 by both direct and enrichment cultures.

## 2.5.1 ARDRA (Amplified Ribosomal DNA Restriction Analysis)

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

### 2.5.1.1 Genomic DNA extraction with the bead-beater method

Microbial genomic DNA of the selected 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, 0.2g/ml Yeast Extract, 0.02g/ml Triton X-100, 0.1M NaCl, 5mM EDTA), 0.3g of glass beads (diameter 0.4-0.5 mm sterilized in absolute ethanol) and 0.2ml of Phenol:Chloroform:Isoamyl Alcohol 24:25:1 (saturate with 10mM Tris, 1mM EDTA, pH 8.0) were added and the microtubes were vortexed for 2min. Next, 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 incubation was performed for 5 min at room temperature. Next the samples were centrifuged at 10.000 rpm for 5 min and the supernatant was removed. The pellet was washed with ethanol 70%, centrifuged at 10.000 rpm for 5 min and the supernatant was removed. Finally, the pellet was air-dry and resuspended in 50µl 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 0.5µg/ml of ethidium bromide, and run in TAE 1X buffer at 100V for 20 min.

### 2.5.1.2 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 0.4 µM of each primers, 0.4mM of dNTPs, 1 U of GoTaqTM DNA polymerase and 5 µl of 5x PCR-buffer (Promega).

| Primer | sequence 5'-3' | Reference |
|---|---|---|
| R11 | ACGGCTACCTTGTTACGACT | (Weisburg *et al.*, 1991). |
| F8 | GAGTTTGATCCTGGCTCAG | (Weisburg *et al.*, 1991). |

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

Approximately 5-20ng of target DNA was added to each reaction, and the following cycle conditions were used: 95°C for 2 min; followed by 30 cycles of 95°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.

### 2.5.1.3 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 50µl. Afterwards, the digested products were

loaded in 1.5% (wt/vol) agarose gel with 0.5µg/ml of ethidium bromide and run in TBE 1Xbuffer at 100V for 60 minutes. The gel was visualized with the UV transilluminator (Eppendorf).

#### 2.5.1.4 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.

### 2.5.2 Sequence analysis and phylogenetic tree construction

For each OTU total DNA of one representative strain was extracted, the gene encoding for 16S rRNA (1500-pb) was amplified (as described in par. 2.5.1.2) 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 the Basic local alignment search tool (BlastN) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997).

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.

# 2.6 Primer-specific PCR for heavy metal resistances and degrading capabilities towards hydrocarbons

As heavy metal resistances are often associated, this molecular study targeted both resistances to Pb and other heavy metals; it also targeted the degrading capabilities towards hydrocarbons: polycyclic aromatic hydrocarbons (PAH), associated to petrol, and aliphatic hydrocarbons.
This study was performed by Primer-specific PCR amplifications targeting total genomic DNA extracted from soil of the three sampling points (I,II,III) and of the Hot Spot, and from all OTUs obtained from enrichment cultures, from Hot Spot and the most represented OTUs directly isolated from soil.

## 2.6.1 DNA extraction

Total DNA from strains was obteined as reported in par 2.5.1.1.
Total soil DNA from soil samples was extracted with Fast DNA® SPIN for Soil Kit in conjunction with FastPrep®Instrument (MP Biomedicals), following the manufacturer's instructions. To determine the quality and quantity of the DNA extracted, 5 µl of genomic DNA were loaded in 1% (wt/vol) agarose gel with 0.5µg/ml of ethidium bromide in TAE 1X buffer and run at 100V for 20 minutes.

## 2.6.2 PCR conditions

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 GoTaqTM 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 gel with 0.5µg/ml of ethidium bromide in Joule Box™ Mini Gel Electrophoresis System (Stratagene), and visualized with ultraviolet illumination. All reagents were from Promega.

### 2.6.2.1 Purification of PCR product and sequencing

PCR products were loaded and run in 1-2% (wt/vol) agarose gels containing 0.5µg/ml ethidium bromide in TAE 1X buffer for 40 minutes at 60V, the fragment of the correct size was cut and purified by gel elution using QIAEX® II Gel Extraction Kit (500) (Quiagen) following the manual instructions.
Sequencing was performed as reported in par 2.5.2.

## 2.6.3 PCR analysis targeting Heavy metals resistance genes

The heavy metal determinants in study were *pbr, czc, chr, ncc* and *mer* genes, respectively responsible for resistance to Pb, Cd-Zn-Co, Cr, Ni and Hg (table 2.2) (Abou-Shanab *et al.*, 2007; Borremans *et al.*, 2001).
In this analysis *Cupriavidus metallidurans* CH34 (Genbank Accession CP000352-CP000355), in which all these resistance systems have been detected and studied, was included as positive control.
In particular *pbr* resistance determinant – as described in par.1.2.1.1 - encodes for the active resistance efflux system mediated by the P-type Pb(II) ATPase, the *czc* determinant mediates resistance to cobalt, zinc and cadmium through the resistance nodulation cell division (RND)-driven trans-envelope exporter CzcCBA and a cation diffusion facilitator czcD, while *mer* resistance includes the uptake of the toxic Hg(II) before reductive enzymatic detoxification of Hg(II) to Hg(0) (Von Rozycki *et* Nies, 2009; Borremans *et al.*, 2001). The *chr* resistance determinant is also based on efflux catalyzed by ChrA chemiosmotic pump, while *ncc* encodes RND-driven transenvelope efflux systems (Von Rozycki *et* Nies, 2009; Legatzki *et al.*, 2003).
The oligonucleotide sequences used as primers for the partial amplification of the *pbrA, czcD, chrB, merA* and *nccA* loci are reported in Table 2.3 (Abou-Shanab *et al.*, 2007; Borremans *et al.*, 2001). In particular

*pbrA* encodes for a P-type Pb(II) ATPase; *czcD* encode for a cation diffusion facilitator (CDF) involved in detoxification of Cd, Zn and Co metals and in regulation of expression of the czcCBA exporter, and *chrB* encodes for a regulator of the ChrA efflux pump. Besides the *ncc* operon was amplified as a 1141-bp fragment that spanned the *nccA* and *nccN* genes; and *merA* encodes for a mercuric reductase enzyme (Von Rozycki *et* Nies, 2009; Abou-Shanab *et al.*, 2007; Legatzki *et al.*, 2003).

| | ***pbrA*** | ***merA*** | ***chrB*** | ***nccA*** | ***czcD*** |
|---|---|---|---|---|---|
| **Resistence encoded** | Pb | Hg | Cr | Ni | Zn, Cd,Co |
| **Lenght bp** | 2300 | 1011 | 450 | 1141 | 1000 |

**Tab. 2.2** Heavy metal determinants under study

### *PCR cycle conditions*

***pbrA:*** Specific PCR-amplification of pbrA was performed in the following condition: 95°C for 5 min; followed by 40 cycles of 95°C for 45 s, 58°C for 1 min, and 72°C for 3 min; and final extension at 72°C for 5 min. PCR products were run on a 1% (wt/vol) agarose TAE gel.

***czcD*, *merA* and *nccA*:** The PCR-amplification of czcD and merA determinants were carried out as follow: 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min 30 s; with a final extension at 72°C for 5 min. PCR products were run on a 1% (wt/vol) agarose TAE gel. The same conditions were applied to primer pair nccAfor/nccARrev for nccA amplification, except that primer annealing was carried out at 58°C.

***chrB*:** The PCR-Amplification of chrB determinant was performed in the following conditions: 95°C for 5 min; followed by 40 cycles of 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min. Product formation was confirmed by 2 % (w/v) agarose gel electrophoresis.

| **Genetic determinant** | **Primer** | **Sequence 5'-3'** | **Reference** |
|---|---|---|---|
| *pbrA* | pbrAF | ATGAGCGAATGTGGCTCGAAG | (Borremans *et al.*, 2001) |
| | pbrARev | CGACGCAACAGCCTCAA | |
| *merA* | merAF | GAGATCTAAAGCACGCTAAGGC | (Abou-Shanab *et al.*, 2007) |
| | merAREV | GGAATCTTGACTGTGATCGGG | |
| *nccA* | nccAFor | ACGCCGGACATCACGAACAAG | Abou-Shanab et al, 2007 |
| | nccARev: | CCAGCGCACCGAGACTCATCA | |
| *chrB* | chrBFor | GTCGTTAGCTTGCCAACATC | (Abou-Shanab *et al.*, 2007) |
| | chrBRe | CGGAAAGCAAGATGTCGATCG | |
| *czcD* | czcD for | TTTAGATCTTTTACCACCATGGGCGCAGGTCACTCACACGACC | (Abou-Shanab *et al.*, 2007) |
| | czcD rev | TTTCAGCTGAACATCATACCCTAGTTTCCTCTGCAGCAAGCGACTTC | |

**Tab. 2.3** Primers used for the partial amplification of the Heavy metal determinants under study

## 2.6.4 PCR primers-specific for degrading capabilities towards hydrocarbons

Concerning the study on hydrocarbon degradation, highly specific primers were chosen to target genes *phnAc* and *nahAc*, which encode the iron sulfur protein large (α) subunits of PAH (Polyciclic aromatic hydrocarbons) dioxygenases in *nah*-like and *phn* catabolic operons for the degradation of PAH (Tab. 2.4) (Smits *et al.*, 1999; Laurie *et* LLoyd-Jones, 2000).

Oligonucleotide sequences used as primers for the partial amplification of these operons are given in Table 2.5. *Pseudomonas putida* G7 *nahAc* (GenBank accession no.M83949), and *Burkholderia* sp. strain RP007 *phnAc* (GenBank accession no.AF061751), on whose sequences the chosen primers were designed, were respectively used as positive control for *nahAc* and *phnAc* PCR-amplifications (Smits *et al.*, 1999; Laurie *et* LLoyd-Jones, 2000).

| | ***nahAc*** | ***phnAc*** | ***alkB*** |
|---|---|---|---|
| **Degradation capability encoded** | PAH | PAH | n-alkane |
| **Lenght bp** | 992 | 993 | 557 |

**Tab.2.4** Hydrocarbons degradation determinants under study

| **Genetic determinant** | **Primer** | **Sequence 5'-3'** | **reference** |
|---|---|---|---|
| *nahAc* | nahAcfor | TGGCGATGAAGAACTTTTCC | (Laurie *et* LLoyd-Jones, 2000) |
| | nahAcrev | AACGTACGCTGAACCGAGTC | |
| *phnAc* | P8073 | TTCGAGCTGGAATGTGAGC | (Laurie *et* LLoyd-Jones, 2000) |
| | P9047 | AATAACCGGCGATTCCAAA | |
| *alkB* | Ts2sF | AAYAGAGCTCAYGARYTRGGTCAYAAG<br>key to symbols Y = C+T, R = A+G | (Smits *et al.*, 1999) |
| | deg1R | CACCTTAAGCGNACNACNAGICTNAC<br>key to symbols N = A+C+G+T, I=INOSINE | |

**Tab.2.5** Primers used for the partial amplification of the hydrocarbon degradation determinants under study

In both cases the following PCR cycling conditions were used: 94°C for 5 min; 35 cycles consisting of 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s; final extension of 72°C for 10 min.

In relation to aliphatic hydrocarbons, highly degenerate oligonucleotides were chosen for the amplification of genes related to the *Pseudomonas oleovorans GPo1* and *Acinetobacter* sp. ADP1 alkane hydroxylases, enzyme which oxidize n-alkanes (Tab. 2.4, 2.5) (Smits *et al.*, 1999).

In this case a strain belonging to the species *Acinetobacter calcoaceticus* - isolated from a soil contaminated by petrol in Pistoia (Italy) (Albertarelli *et al.*, 2006) - was used as positive control.

The following amplification conditions were used: 95°C for 5 min; followed by 25 cycles of 95°C for 45 s, 40°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min. After a first round of PCR, 2 µl (out of 25 µl) of product were used in a second round of PCR using the same primers. Product formation was confirmed by 2%(w/v) agarose gel electrophoresis.

## 2.6.5 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 soil samples from each sampling point and Hot Spot.

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.

PCR-DGGE analysis was performed as followed:

### 2.6.5.1 Total DNA extraction and amplification of 16S rDNA reaction

Total soil DNA from soil samples was extracted with Fast DNA® SPIN for Soil Kit (MP Biomedicals) as described in section 2.6.1, and it was used as template for PCR amplification of the gene encoding for the 16S rRNA (see par. 2.5.1.2.)

### 2.6.5.2 Nested-PCR of V3 hypervariable region

The universal bacterial primers targeting 16SrDNA V3 hypervariable region, p3 and p2, were used to amplify fragments sized 233 bp (tab.2.7) (Muyzer *et al.*, 1993). The reactions were set up using as template a suitable dilution of the PCR products obtained from the amplification of the entire 16S rDNA. 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'** | **reference** |
|---|---|---|
| P3 | (40bp-GCclamp)CCTACGGGAGGCAGCAG | (Muyzer *et al.*, 1993) |
| P2 | ATTACCGCGGCTGCTGG | (Muyzer *et al.*, 1993) |

**Tab. 2.6**: Primers used for amplification of V3 region

### 2.6.5.3 Electrophoretic run in denaturing gel

The PCR products 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 Dcode™ Universal Mutation Detection System (Bio-Rad) and gels were run for 20 hrs at 50 V in TAE 1Xbuffer at 65°C. Afterwards the polyacrylamide gel was stained for 30 min in ethidium bromide (1 mg $l^{-1}$) in TAE 1Xbuffer and were visualized by UV illumination. The image was detected by the software AB.EL CAT with CAMERA CONTROL, version 2.0.0.

### 2.6.5.4 Bands' sequencing

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). The PCR products obtained were transformed in *E. coli* XL1Blu CaCl-competent cells using the pGEM®-T Easy vector system, following the manufacturer's instructions (Promega), sequenced and subsequently searched for homology using the BLASTN database (see par. 2.5.2).

### 2.6.5.5 DGGE Marker

A marker was made to allow the comparison of band profiles between distinct DGGE gels. Therefore strains of different genera – isolated and identified as described in par. 2.3 - with presumably different GC content, were used as template for PCR-DGGE analysis. Afterwards a combination of 6 isolates - with bands at different positions along the DGGE gel - was chosen to cover the DGGE run lenght. The Marker obtained was used in all DGGE analysis performed.

# 2.7 Phytoremediation assay

## 2.7.1 Plants species examined in Phytoremediation trials

A lab-scale and a field scale trials were performed with two plant species: *Brassica juncea* (Indian mustard) and *Apocynym cannabinum* (Indian Hemp).

Seeds of two cultivar of *Brassica juncea* - PI173874 and PI426308 - were provided by the *USDA North Central Regional Plant Introduction Station (NCRPIS), Iowa State University* (Ames, Iowa, USA). *Apocynum cannabinum* seeds were obtained from Bonn University - Botanical Gardens, Germany.

Besides in a lab-scale mesocosm trial in a biaugmentation protocol, a third plant has been studied: the arboreal plant Poplar, *Populus sp.*. For this study, Hybrid Poplar AL35 selected for Cd and Zn resistences were provided from the University of Salerno.

## 2.7.2 Lab-scale Phytoremediation trial

A lab-scale mesocosm trial was set up with *Brassica juncea* cultivar 173874 and *Apocynum cannabinum*, with the soil of each sampling point I, II and III. In order to evaluate the role of the soil autochthonous community on the phytoremediation processes, for each sampling point mesocosms were set up in parallel respectively with the soil untreated and sterilized.

### 2.7.2.1 Experimental design

Cultivation experiments were carried out in a glasshouse equipped with supplementary lighting, at temperature $\geq$ 24°C and with a photoperiod of 12 hours.

Mesocosms of 1 kg were set up using the soil collected from each sampling point and previously sieved through a 2mm sieve (named untreated mesocosms), and in parallel with the same soil treated by heat sterilization (named sterilized mesocosms). Heat sterilization was performed in autoclave by 1h at 121 °C for three times, at intervals of 24 hours. The sterilization was performed in pyrex glass jars with a correct headspace in order to avoid overpressure and explosion upon heating.

For both untreated and sterilized mesocosms, three replicates for each sampling point were set up. Moreover for each plant, controls were set-up in triplicate using uncontaminated agricultural soil - collected from agricultural field with maize cultivation near Verona. With both untreated and sterilized soils, control mesocosms with no plant were also set-up in duplicate for each sampling point (I,II,III).

Seeds of *Brassica juncea* (cultivar 173874) and *Apocynum cannabinum* were sown directly on pots in number of respectively 5 and 20 seedlings per pot. A higher number of *A.cannabinum* seeds were sawn because a low germination % was detected in previous germination tests. Sowing was considered the T0 of the experiment.

Every other day pots were watered alternatively with a proper quantitative of half-strength Hoagland's solution and deionised water, excess soil moisture was trapped in plastic saucers placed below each pot to prevent leaching from pots.

**Fig. 2.5** Lab-scale mesocosms with *Brassica juncea* and *Apocynum cannabinum*

In the case of *B.juncea* mesocosms for untreated, sterilized and control mesocosms - set up with the agricultural soil - plants were collected at three different times:

- T1: after 2 months from sowing
- T2: after 3 months from sowing
- T3: after 4 months from sowing

For *Apocynum cannabinum* mesocosms, a unique sampling was carried out:

- Tf: after 6 months from sawing.

The sampling of one pot was made at each sampling time, collecting all plants grown in the pot and the rhizosphere soil. Soil samples were stocked at -20 °C in case of following molecular analysis, and at 4°C for chemical analyses and microbial enumerations.
For the unplanted mesocosms, the sampling of soil was performed at T0 (corresponding to the sowing time) and in parallel with the final sampling of *B.junca* (T3) and of *A.cannabinum* trial (Tf).

### 2.7.2.2 Determination of biomass production and lead content

Plants collected at the different sampling times 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.
Soil and plants samples (if there was enough biomass production as minimal amount needed is 0.5 g) were analysed for total Pb content. Moreover for *Brassica juncea* mesoscosms, the alkyl-lead content in soil explored by their roots was analysed.

#### *Lead content determination*

Total Pb and organic Pb analysis were performed by the laboratory *Trentino Servizi Spa* (Trento, Italy). To measure Total Pb, samples were digested using the microwave digestion system in the Method EPA 3052

2006. After the digestion was completed, the mineralized samples were conveniently filtered and diluted to be analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) via EPA Method 6010B 1996. A Method MI-144 rev 1 2007: SPME + GC/ MS, based on solid-phase microextraction (SPME) and gas chromatography coupled with mass spectrometry (GC–MS), was used to measure organic Pb.

#### 2.7.2.3 Microbial enumeration

Microbial total counts were evaluated for rhizosphere soil samples on Nutrient and R2A media, as described in section 2.3.1. In mesocosm control (CTR) set-up with the agricultural soil and in unplanted mesocosms values were registered at T0 and in parallel with the final sampling of *B.junca* (T3) and of *A. cannabinum* trial (Tf).

#### 2.7.2.4 Molecular analyses

PCR-DGGE analyses was performed on *Brassica juncea* rhizosphere soil samples collected at the each sampling times (T0, T1, T2, T3), in order to characterize the composition of the bacterial community during the phytoremediation process.
DNA extraction from soil and PCR-DGGE analyses were carried out as described in par. 2.6.4..

### 2.7.3 Field-scale Phytoremediation trial

As in lab-scale mesocosms, the two plant species *B. juncea* and *A. cannabinum* were tested on a field trial. In this case for *Brassica juncea* in addition to the cultivar PI173874 (named A) - used in lab-scale - a second cultivar PI426308 (named B) was included in field trial.

#### 2.7.3.1 Experimental design

Within the contaminated Ex-SLOI area at the location of the three sampling points (I, II and III), parcels of respectively 60, 23 e 45 square meters were set up, depending on the size of accessible and available area.
In particular, Point I parcel was 12 m long and 5 m large, while Point III parcel was 5 m long and 9 m large (fig 2.6). These two parcels were subdivided in three smaller area 1,5 m wide and 0,25 m apart. The middle area - in the center of each parcel - was left unplanted, while the outer ones were planted in 2 rows 50 cm apart, respectively with *B. juncea.* and *A. cannabinum*. Point II parcel - which was the smallest 4,5 m long and 5 m large - was subdivided in three area 1,35 m wide and plants were sawn in rows 45 cm apart.
For *Brassica juncea*, one row was used for cultivar PI173874 (A), one for cultivar PI426308 (B) and one seed was sawn every 15 cm along the raw.
For *Apocynum cannabinum* 5 seeds were sawn every 15 cm because of its lower germination percentage.
Seeds were watered just once at the sowing time, with water pumped from piezometers near the parcel. No water or fertilizers were supplied during the trial.
In the case of *Brassica juncea*, at least 4 plants for each cultivar were collected at each sampling times:

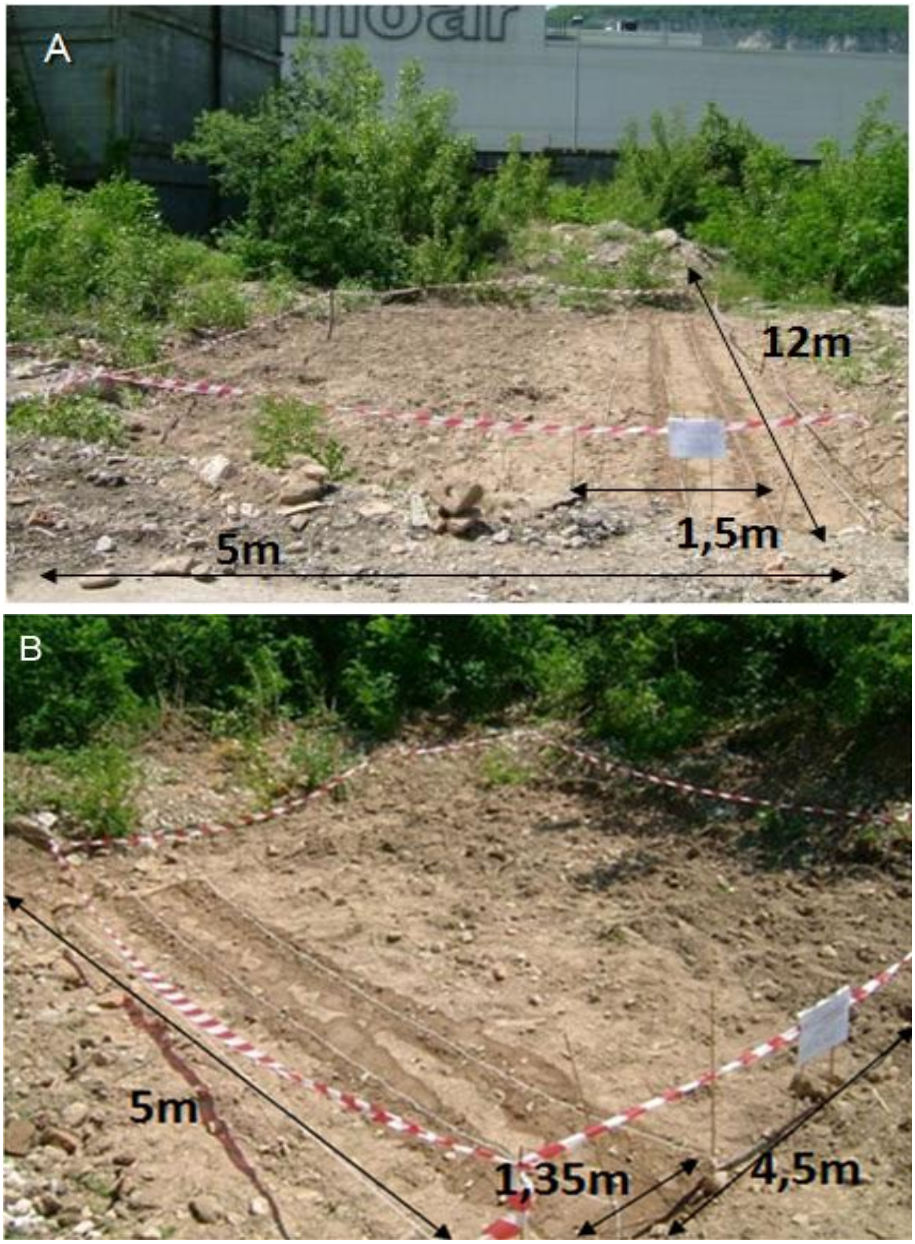
- T1: after 6 weeks from the sowing
- T2: after 14 weeks from the sowing
- T3: after 18 weeks from the sowing

At the end of the experiment, i. e. at T3, all plants of both *B.juncea* cultivars were collected and analysed.
For *A. cannabinum,* as in lab-scale mesocosms, due to the slow growth rate a unique sampling of all plants was carried out:

- T1: after 16 months from sowing.

### 2.7.3.2 Analyses performed on plant and soil samples

For each sampling time, dried weight of roots and shoots and total Pb content in plant tissues were determined as described for lab-scale trial (section 2.7.2.2.). Rhizosphere microbial enumeration was also performed on Nutrient and R2A media as for lab-scale (par 2.7.2.3).



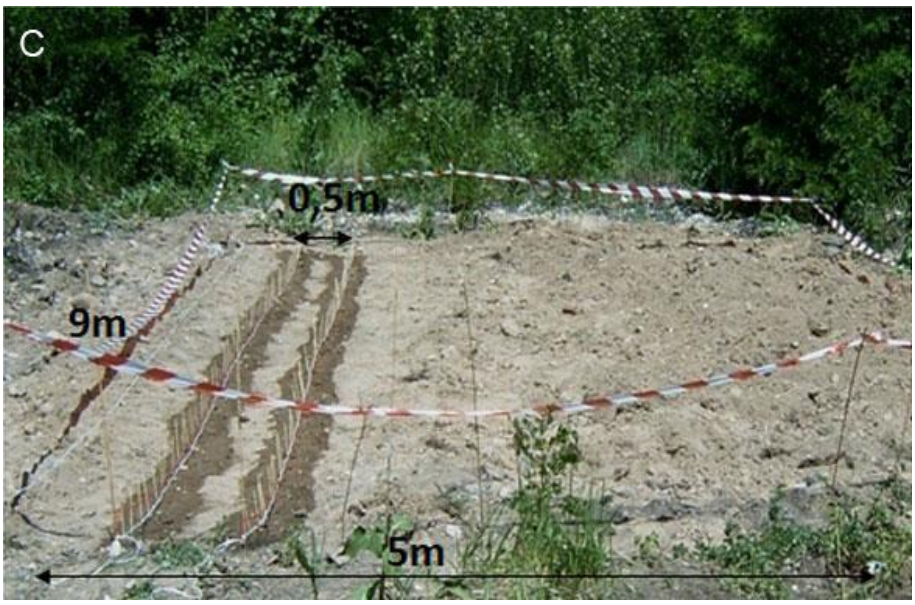



**Fig 2.6** Field trial parcels at the three sampling points, A) Point I: 60m$^2$, B) Point II: 23m$^2$, C) Point III: 45m$^2$

## 2.7.4 Lab-scale Phytoremediation trial with biaugmentation protocol

A lab-scale trial has been set up with the arboreal plant Hybrid Poplar using a bioaugmentation protocol. In particular Hybrid Poplar AL35 selected for Cd and Zn resistences were provided from the University of Salerno.

### 2.7.4.1 Experimental design

Shoot cuttings were left in water for 20 days to allow roots development (Fig 2.7A); next rooted cuttings were transplanted in 5 kg pots with a mixture of 27% (wt/wt) sand and sieved soil from the sampling Point III. The soil of this sampling point was chosen because of its lower level of contamination, a better soil structure and higher accessibility.

In particular 16 pots were set up with Point III soil, while 16 pots were set up as control with an agricultural uncontaminated soil collected near Verona from a maize field.

In parallel for both contaminated and uncontaminated soils, unplanted control mesocosms were set-up in triplicate. All pots were incubated in glasshouse at temperature $\geq$ 24°C and with a photoperiod of 12 hours and the experiment was carried out for 8 months.

After 5 month from planting - for both planted and unvegetated controls set-up with contaminated and uncontaminated soil - mesocoms were inoculated (Tab. 2.7) with a consortium of bacteria, isolated from the contaminated soil under study and chosen on the basis of the study previously described in relation to heavy metal resistances (par 2.6) and PGPR traits (par 2.4).

The bacterial inoculum was bioaugmented at an initial concentration of $10^7$ CFU/g of soil.

Therefore previously, for each strain, a curve growth was evaluated, growing the strain in 250-ml Erlenmeyer flasks containing 100 ml Nutrient Broth medium – with an initial OD(600nm)=0.1 - and incubating on an orbital shaker at 200 rpm at 27°C for 3 days; periodically aliquots of bacterial cultures were seeded on agarised Nutrient plates to count the colony forming units (CFU). Hence the inoculum of each axenic strain was obtained by growing the culture in the same conditions of the growth curve. After 24h a proper aliquot of the cell cultures were centrifuged at 5000 rpm for 10 min, at 4°C. Subsequently, cells were resospended in physiologic solution (0.9% wt/vol Na Cl) and distributed uniformly to the surface of the soil.

Two days later mesocosms in triplicate - for both inoculated and not inoculated pots - were amended with the chelating agent ethylenediaminetetraacetic acid (EDTA) with 5 mmol EDTA/kg soil, adding in two times 25 ml per pot of a EDTA stock 0,5 M pH8.
The experimental trial is summarized as follow (Table 2.7).

| **Mesocosm set up in triplicate** | **Inoculum** | **EDTA** |
|---|---|---|
| **Hybrid Poplar** | + | - |
| **Hybrid Poplar** | + | + |
| **Hybrid Poplar** | - | + |
| **Unplanted** | + | - |
| **Unplanted** | + | + |
| **Unplanted** | - | + |

**Tab 2.7** Test set up in lab-scale mesocosms – with both contaminated and uncontaminated soil – in a bioaugmentation protocol

A unique sampling of the plants for all mesocosms was carried:

- Tf: after 8 months from planting

At the final sampling all plants and soil from each pot were collected to be analysed as performed in the lab-scale trial described in par 2.7.2.
Along the trial soil samples were also collected before planting, before and after inoculum biaugmentation, before and after EDTA amendments, to carry out total microbial enumeration and the PCR-DGGE analysis to monitor the bacteria bioaugmented.

### 2.7.4.2 Analyses in progress on plant and soil samples

Plant tissues are being analysed for both biomass production and Pb content, whereas soil aliquots from the plant rhizosphere are being investigated through microbial total counts on Nutrient media and DGGE analysis, as described for lab-scale mesocosms (par. 2.7.2).
These analyses are now in progress, thus no date regarding this lab-scale trial is reported in this document.


**Fig 2.7** Hybrid Poplar cuttings left in water (A) and lab-scale mesocosms set up in glasshouse (B)

# Results and discussion

## Subdivision of the thesis study

This PhD study can be divided in two main sections. The first one - presented in the following chapter 3. - is focused on the study and characterization of the soil autochthonous bacterial community selected by and acclimated to the contamination of both organic and inorganic Pb, present in the Ex-SLOI area for over half century, to assess its composition, biodiversity richness, resistance and degrading potential.
Indeed, the study of the microbial community response in a contaminated site as the one here examined can allow evaluating the bioremediation potential of the soil, and at the same time it give information on the response of ecosystems to increasing environmental change and disturbance.
The second section of this PhD Thesis - presented in chapter 4. - studies the interaction of autochthonous microorganisms and plants in relation to Pb contamination, in a Phytoremediation approach. In particular two plants have been included in the study: *Brassica juncea* and *Apocynum cannabinum*.
*Brassica juncea* is a crop plant reported to tolerate salinity and other toxic conditions, and it is well known to accumulate various metals. Although it is not a hyperaccumulator, it has drawn much attention due to its rapid growth and high biomass production, factors which positively affect phytoextraction efficiency (Liu *et al.*, 2000; Sheng *et al.*, 2008a).
*Apocynym cannabinum* is an interesting quite unexplored subject of study, it is in fact reported to accumulate Pb and its use is suggested in Pb-phytoremediation (Cunningham, 1994), however at the best of our knowledge few literature data are available.
The study on a third plant - Hybrid poplar - has been started. This arboreal plant is in fact characterized by a huge biomass production and an extensive root system, while at the same time it is reported to accumulate metals (Di Lonardo *et al.*, 2010). The study in a biaugmentation protocol with selected components of the autochthonous community is still in progress, thus no data on this experiment are reported in this document.

# 3. Results and discussion Chapter I: Characterization of the soil autochthonous micro flora

The study of the autochthonous micro flora has been performed by complementing two different approaches: culture-dependent and culture-independent.

The first approach relies on cultivation techniques of classical microbiology and it allows the isolation, subsequent characterization and use of strains obtained in pure culture from the matrix under study.

On the other side a culture-independent approach, based on metagenomic DNA extraction and on molecular techniques, it is independent from strains' cultivability and allow a study comprehensive of the unculturable fraction of a microbial community. It has in fact been estimated that only 0.1-10% of the total soil population can be cultured by using standard cultivation techniques, because of the ignorance of the culture conditions under which microorganisms thrive in their natural environment (Daniel, 2004; Marschner *et al.*, 2004).

In the meantime due to this selective discrimination, the culture-dependent approach can provide a useful and rapid assessment of biological responses to heavy metal pollution, as readily culturable bacteria are probably the largest, most active prokaryotes in a given sample (Ellis *et al.*, 2003; Margesin *et al.*, 2010). Wherefore a combination of both approaches can lead to a more complete overall study.

The primary objective of this first part was to assess the structure and biodiversity of the autochthonous bacterial community which inhabits a soil with a long history of Pb pollution and to study the impact of both inorganic and organic Pb compounds – on the latter in particular only few data are available in literature. Further attention goes towards isolating and characterizing components of the indigenous microbial community, to identify microorganisms of high resistence and bioremediation potential and interesting even in the perspective of a phytoremediation approach enhanced by microorganisms.

While only few studies have examined the effects of long-term, chronic exposure to particular contaminants, typically metal is added to an uncontaminated soil and the resulting changes in the community are monitored (Becker *et al.*, 2006). The soil object of this study has been contaminated for over half century and the bacterial communities have already undergone selection for a metal-tolerant community, thus it allows ascertaining the effect of prolonged exposure to organic and inorganic Pb on the microbial community.

On the other side a soil with all the same characteristics except for Pb contamination as control is consequently not available. Although influence on the phylogenetic composition of a microbial community is exerted from environmental chemical-physical characteristics (Cavalca *et al.*, 2010) and soils' heterogeneity is present in a vast area as Ex-SLOI, a soil sample was collected in the Ex-SLOI parking area – presenting the lowest level of contamination within the entire area - as approximate mean of comparison.

# 3.1 Culture-dependent study of the soil autochthonous micro flora

## 3.1.1 Enumeration of the cultivable bacterial community at the 3 sampling points within the Ex-SLOI area

Three sampling points have been chosen within the Ex-SLOI area - identified as Point I, Point II and Point III - on the basis of their contamination levels (par 2.2), in order to study the established autochthonous microbial community selected by and adapted to the Pb contamination for over half century.

Point I presents a contamination >2000 mg/kg of inorganic Lead and <50 mg/kg of organic Pb, and it is also contaminated by about 20-30 mg/kg hydrocarbons; Point II has been reported to be the most contaminated of the three, with levels >4000 mg/kg for inorganic Pb and >100 mg/kg of organic Pb, it was in fact located just behind the reactor for TEL synthesis. Point III has the lowest content of both organic and inorganic Pb among the three, but at the same time it is also contaminated by Hg in the order of tens of mg/kg, due to the proximity to $C_2H_5Cl$ production system which implied mercury in its processes.

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 Eubacteria was performed by using rich-medium Nutrient and low-nutrient medium Reasonar's 2 agar (R2a), as the use of oligotrophic media prevents the overgrowth of fast-growing microbial species so thus unique populations of microorganisms can be cultured (Daniel, 2004). At the same time Actinobacteria were enumerated with Waksman medium and Fungi with Malt medium.

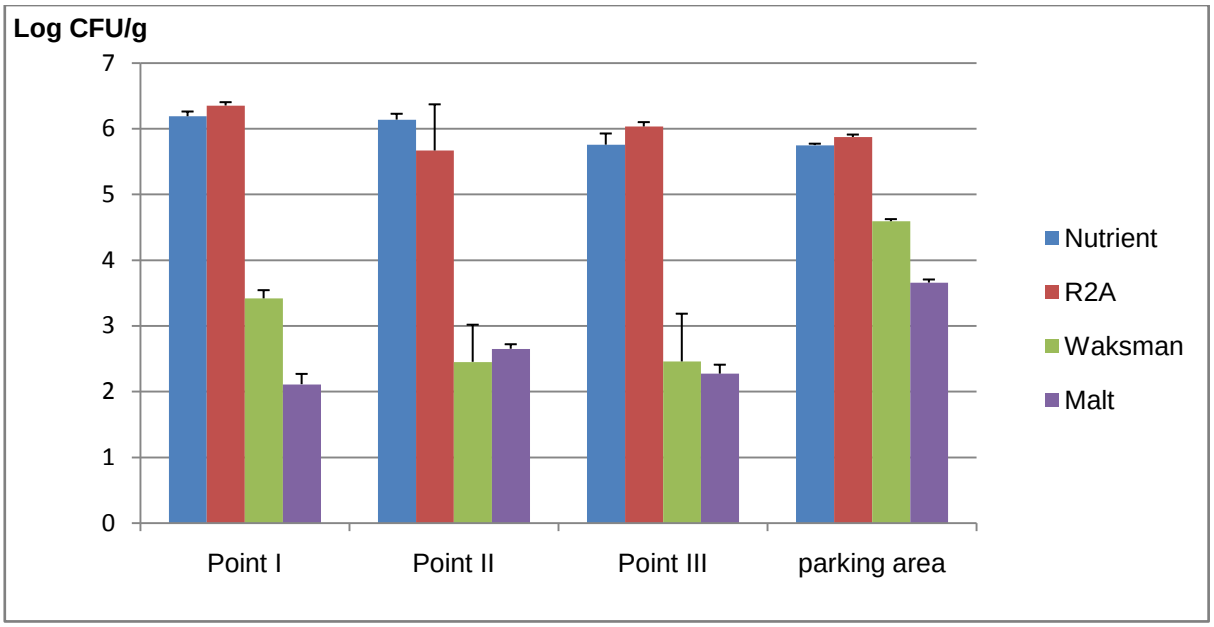



**Fig. 3.1**: Culturable counts at the 3 examined sampling points (I,II,III) and control parking area

As reported in Fig. 3.1, data obtained showed a $\log_{10}$ value of about 6 for the heterotrophic aerobic Eubacteria in both media used, and no great differences can be detected among the three sampling points in analysis.

Although the microbial diversity in soils exceeds that of other environments and one gram of soil can contain approximately $10^7$ up to $10^{10}$ microorganisms of possibly thousands of different species (Daniel,

2004), data obtained report quite a high microbial charge for a perturbed soil at all the three sampling points under study (Nakatsu *et al.*, 2005). This first datum seems to suggest a good adaptation and resistance to the contamination by the established heterotrophic eubacterial population.
While the immediate reported effect of metal stress is a decrease in microbial biomass as metal-sensitive microbes are inhibited, over a longer period there can be a gradual change in the microbial composition in which natural selection, gene exchange, and immigration can all contribute to a microbial community that is adapted to the perturbed conditions. If such a tolerant community develops in soils - as this seems to be the case - then the decrease in biomass and activity may be temporary (Joynt *et al.*, 2006).
Contrasting examples are reported in literature on phylogenetic distribution at metals contaminated sites. While some studies have shown an increase in the fungal population proportion compared to bacteria in heavy metal amended soil (Akmal *et* Jianming, 2009) and a predominance of gram-positive organisms - in particular of Actinobacteria (Becker et al., 2006) - in others Actinobacteria were reported to be the microbial group mostly affected by Cd, Cu and Zn contamination (Lorenz *et al.*, 2006).
As in the latter case, in this study gram-positive Actinobacteria and Fungi showed a lower detection with counts of 3 orders of magnitude lower, they seem therefore to be the most affected by the Pb contamination present in the area. Moreover an antagonistic action exerted by the fast and high growth of a metal-tolerant eubacterial population could also be connected and partly explain the lower detection of gram-positive bacteria.
A slight lower abundance in the eubateria fraction with a concomitant increase of more than 1 order of magnitude in gram-positive Actinobacteria and Fungi was in fact observed in the control sample, collected in the ex-parking area within the Ex-SLOI area – i.e. the location most far from the Tel synthesis reactor and reporting a much lower contamination in the order of hundreds mg Pb/kg. Hence this indicates a change under Pb contamination in favor of the Eubacteria fraction, which has been further analyzed as hereafter reported.

### 3.1.2 Direct Isolation of soil bacterial community members at the 3 sampling points within the Ex-SLOI area

After incubation and enumeration of total cultivable Eubacteria on Nutrient and R2a agarized media, colonies with different morphologies were selected from the plates to obtain axenic cultures. By direct isolation from Nutrient and R2a respectively 84 and 93 isolates were thus obtained.
They were therefore screened by the molecular technique ARDRA - some of the main profiles obtained are reported in fig 3.2 and 3.3 - and grouped into different Operational Taxonomic Units (OTUs). Through sequencing of about 500 bp of the 16S rRNA gene and alignment to databases all OTUs were taxonomically allocated, as reported in tab 3.1 and 3.2, where percentage homology of one representative strain for each OTU to GenBank relatives for 16S rDNA sequences are reported.
Therefore 37 and 52 OTUs were identified in Nutrient and R2a respectively, and as only 9 OTUs coincided between the two media, 80 OTUs where altogether obtained at the three sampling points; their distribution into different and heterogeneous taxa is also detectable in the phylogenetic trees (fig 3.4, 3.5). This first datum attests therefore a high biodiversity within the eubacterial population established within the Ex-SLOI area under study.
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* of Gamma-proteobacteria (Pages *et al.*, 2008), genera reported for high resistances to heavy metals such as *Cupriavidus* of Beta-proteobacteria (Von Rozycki *et* Nies, 2009) and reporting degrading capabilities towards organic compounds and hydrocarbons such as *Sphingomonas* of Alfa-

proteobacteria (Kertesz *et* Kawasaki, 2010), thus suggesting a high resistance and degrading potential within the indigenous bacterial community.

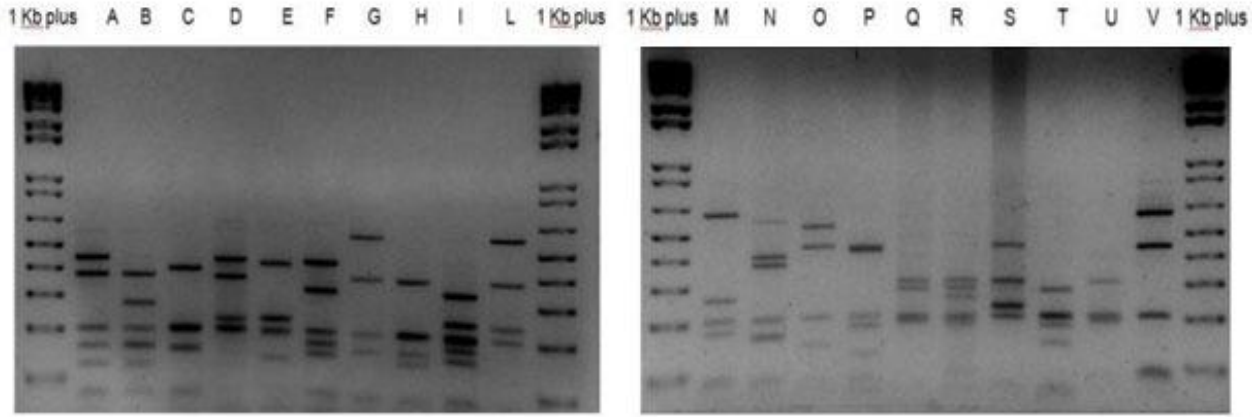



**Fig. 3.2:** Main restriction profiles obtained with AluI enzyme

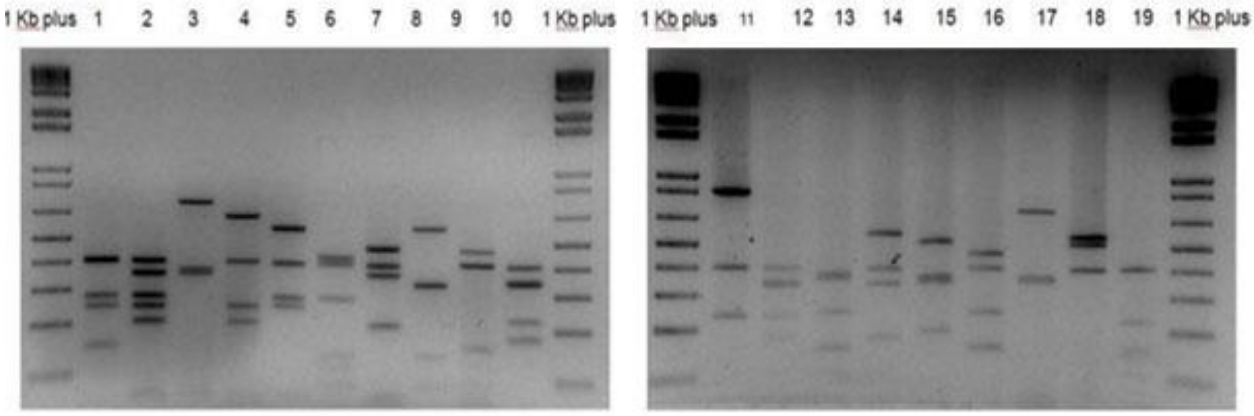



**Fig. 3.3**: Main restriction profiles obtained with HhaI enzyme

| Nutrient OTU | Taxonomic Reference ID | Phylogentic group | Homology % |
|---|---|---|---|
| **A** | *Stenotrophomonas humi* AM403587 | γ-proteobacteria | 99% |
| **B** | *Pseudomonas putida* DQ833753 | γ-proteobacteria | 100% |
| **C** | *Stenotrophomonas sp.* DQ984206 | γ-proteobacteria | 100% |
| **D** | *Micrococcus* sp. EF 491956 | Actinobacteria | 100% |
| **E** | *Pseudomonas grimontii* EU169177 | γ-proteobacteria | 100% |
| **F** | *Sinorhizobium morelense* AM922194 | α-proteobacteria | 100% |
| **G** | *Alcaligenes sp.* EU37500 | β-proteobacteria | 100% |
| **H** | *Pseudomonas sp. DQ377767* | γ-proteobacteria | 100% |
| **L** | *Cupriavidus sp.* EF435017 | β-proteobacteria | 99% |

| | | | |
|---|---|---|---|
| **N** | *Enterobacter sp.* DQ481480 | γ-proteobacteria | 99% |
| **O** | *Chryseobacterium sp.* DQ530108 | Flavobacteria | 99% |
| **P** | *Bacillus firmus* AJ491843 | Firmicutes | 99% |
| **Q** | *Cryseobacterium sp.* AY514020 | Flavobacteria | 97% |
| **R** | *Bacillus firmus* EU418717 | Firmicutes | 99% |
| **S** | *Sinorhizobium morelense* AM285019 | α-proteobacteria | 100% |
| **T** | *Brachybacterium sp.* DQ643203 | Actinobacteria | 100% |
| **U** | *Bacillus pumilus* EU333893 | Firmicutes | 100% |
| **Z** | *Enterobacter amnigenus* DQ481471 | γ-proteobacteria | 99% |
| **AA** | *Stenotrophomonas sp.* EU102280 | γ-proteobacteria | 100% |
| **AC** | *Bacillus cereus* EU368180 | Firmicutes | 99% |
| **AD** | *Bacillus sp.* DQ358682 | Firmicutes | 99% |
| **AE** | *Bacillus sp.* EU652859 | Firmicutes | 100% |
| **AG** | *Pseudomonas sp*. AY0303289 | γ-proteobacteria | 100% |
| **AH** | *Microbacterium hydrocarbonoxydans* AJ698726 | Actinobacteria | 99% |
| **AI** | *Bacillus sp.* DQ14538 | Firmicutes | 100% |
| **AM** | *Pseudomonas sp.* EF491969 | γ-proteobacteria | 100% |
| **AN** | *Pseudomonas chlororaphis* EF620458 | γ-proteobacteria | 100% |
| **AO** | *Sinorhizobium sp.* EU571251 | α-proteobacteria | 98% |
| **AP** | *Pseudomonas sp.* DQ984204 | γ-proteobacteria | 100% |
| **AQ** | *Bacillus sp.* EU182839 | Firmicutes | 100% |
| **AR** | *Pseudoxanthomonas sp.* EF540482 | γ-proteobacteria | 99% |
| **AS** | *Pseudomonas sp*. AY032726 | γ-proteobacteria | 99% |
| **AT** | *Enterobacter hormaechei* AM943033 | γ-proteobacteria | 99% |
| **AU** | *Pseudomonas sp*. EU195324 | γ-proteobacteria | 100% |
| **AZ** | *Arthrobacter sp.* DQ208673 | Actinobacteria | 100% |

| | | | |
|---|---|---|---|
| **BB** | *Pseudomonas sp.* AY690672 | γ-proteobacteria | 100% |
| **BC** | *Agrobacterium sp.* EF189105 | α-proteobacteria | 100% |

**Tab. 3.1**: Taxonomic collocation of OTUs isolated from Nutrient medium

| OTUs R2A | Taxonomic Reference ID | Phylogentic group | Homology % |
|---|---|---|---|
| **RA** | *Stenotrophomonas maltophilia* EU622536 | γ-proteobacteria | 100% |
| **RB** | *Bacillus sp.* AM950301 | Firmicutes | 100% |
| **RC** | *Bacillus sp.* EU124558 | Firmicutes | 100% |
| **RD** | *Sphingomonas sp.* EU580708 | α-proteobacteria | 100% |
| **RE** | *Uncultured Cupriavidus* EU268605 | β-proteobacteria | 99% |
| **RF** | *Lysobacter sp*. EF601813 | γ-proteobacteria | 100% |
| **RH** | *Bacillus sp.* EU182839 | Firmicutes | 100% |
| **RI** | *Caulobacter sp.* EU723141 | α-proteobacteria | 99% |
| **RL** | *Sinorhizobium sp.* AF35722 | α-proteobacteria | 99% |
| **RM** | *Sphingopyxis panaciterrae* AB245354 | α-proteobacteria | 100% |
| **RN** | *Xanthomonas sp*. AY599706 | γ-proteobacteria | 99% |
| **RO** | *Streptomyces sp.* EU098026 | actinobacteria | 100% |
| **RP** | *Stenotrophomonas sp.* AY689084 | γ-proteobacteria | 100% |
| **RQ** | *Stenotrophomonas maltophilia* EF620448 | γ-proteobacteria | 100% |
| **RR** | *Pseudomonas aeruginosa* EF620448 | γ-proteobacteria | 100% |
| **RS** | *Pseudomonas fluorescens* DQ095891 | γ-proteobacteria | 100% |
| **RT** | *Pseudomonas chlororaphis* EF6204581 | γ-proteobacteria | 100% |
| **RU** | *Acinetobacter junii* EU418715 | γ-proteobacteria | 99% |
| **RV** | *Pseudomonas chlororaphis* DQ525599 | γ-proteobacteria | 100% |
| **RZ** | *Delftia tsuruhatensis* EU019989 | β-proteobacteria | 100% |
| **RAA** | *Delftia sp.* EU310441 | β-proteobacteria | 99% |
| **RAB** | *Uncultured Xanthomonas sp.* AY834297 | γ-proteobacteria | 99% |

| | | | |
|---|---|---|---|
| **RAC** | *Uncultured Alcaligenes sp.* EF11196 | β-proteobacteria | 100% |
| **RAD** | *Rhizobium giardinii* RGU86344 | α-proteobacteria | 100% |
| **RAE** | *Uncultured Lysobacter sp.* EU300545 | γ-proteobacteria | 98% |
| **RAF** | *Pseudomonas sp.* AY690672 | γ-proteobacteria | 99% |
| **RAG** | *Lysobacter sp* D249997 | γ-proteobacteria | 99% |
| **RAH** | *Pseudomonas chlororaphis* EF620458 | γ-proteobacteria | 100% |
| **RAI** | *Bacillus sp.* AM934688 | Firmicutes | 100% |
| **RAL** | *Nocardia* sp. AF277212 | Actinobacteria | 99% |
| **RAM** | *Rhizobiumsp. FM173709* | α-proteobacteria | 100% |
| **RAO** | *Ochrobactrum sp.* EU529846 | α-proteobacteria | 100% |
| **RAP** | *Streptomyces sp.* DQ846817 | Actinobacteria | 100% |
| **RAQ** | *Streptomyces variabilis* EU593632 | Actinobacteria | 99% |
| **RAR** | *Comamonas sp.* AM412126 | β-proteobacteria | 100% |
| **RAS** | *Bacillus simplex* EU427320 | Firmicutes | 100% |
| **RAT** | *Bacillus sp.* EU563373 | Firmicutes | 100% |
| **RAU** | *Streptomyces sp.* AY465256 | Actinobacteria | 99% |
| **RAV** | *Streptomyces sp. EF012098* | Actinobacteria | 99% |
| **RAZ** | *Bacillus sp.* AM950301 | Firmicutes | 100% |
| **RBA** | *Bacillus sp. EU734600* | Firmicutes | 100% |
| **RBC** | *Delftia tsuruhatensis* DQ864991 | β-proteobacteria | 100% |
| **RBD** | *Cupriavidus campinensis* AF312020 | β-proteobacteria | 100% |

**Tab. 3.2**: Taxonomic collocation of OTUs isolated from R2a medium

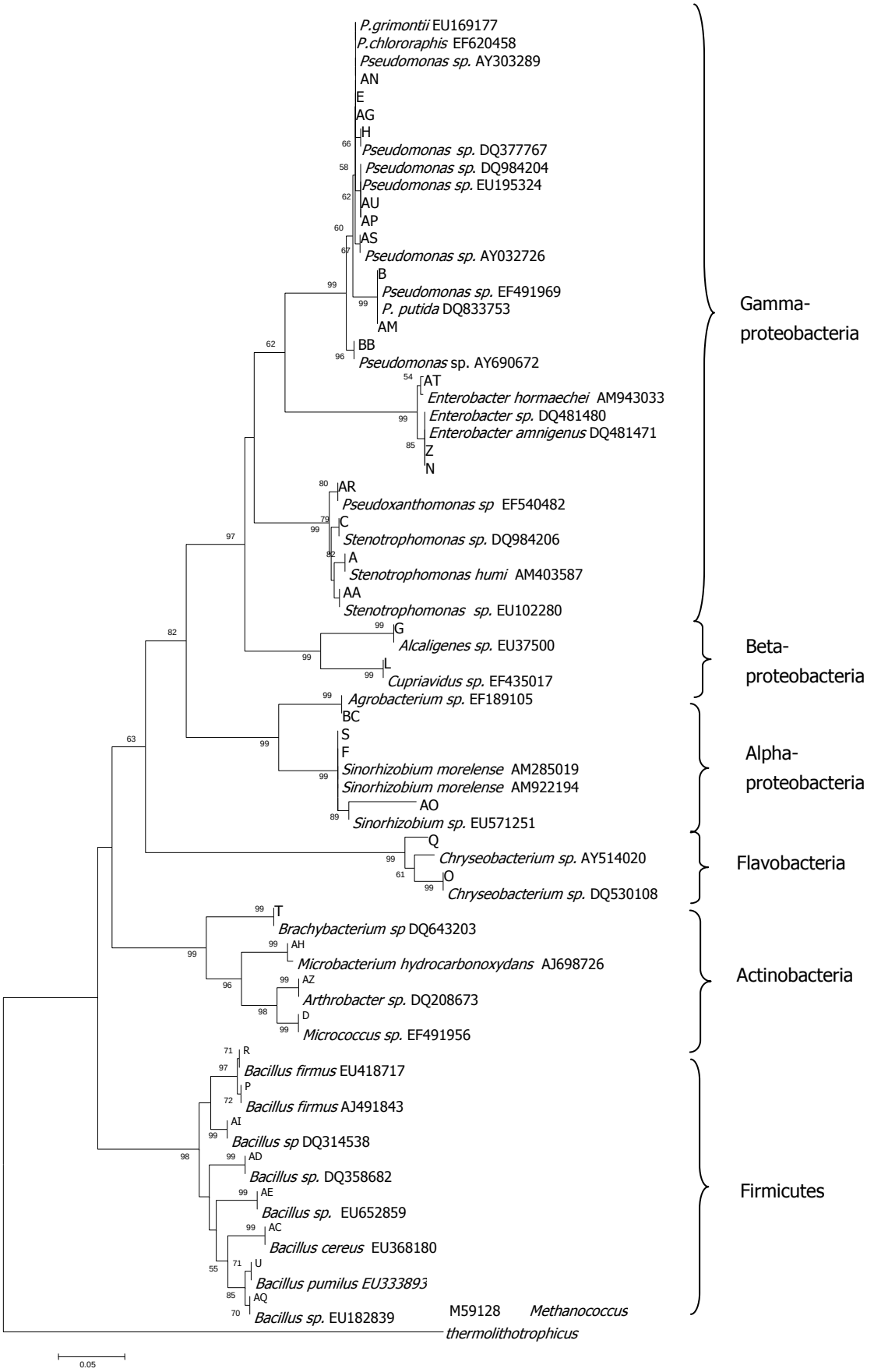



**Fig.3.4:**Phylogenetic neighbour-joining tree of the 16S rRNA gene sequences of Nutrient OTUs(A,B,C,D,E,F,G,H,L,N,O,P,Q,R,S,T,U,Z,AA,AC,AD,AE,AG,AH,AI,AM,AN,AO,AP,AQ,AR,AS,AT,AU,AZ,BB, BC) and their closest database relatives with Species Name and GenBank accession numbers. Bootstrap values(*n*_1,000) >50% are indicated at the nodes. The scale bar represents genetic distance (nucleotide substitutions per site).

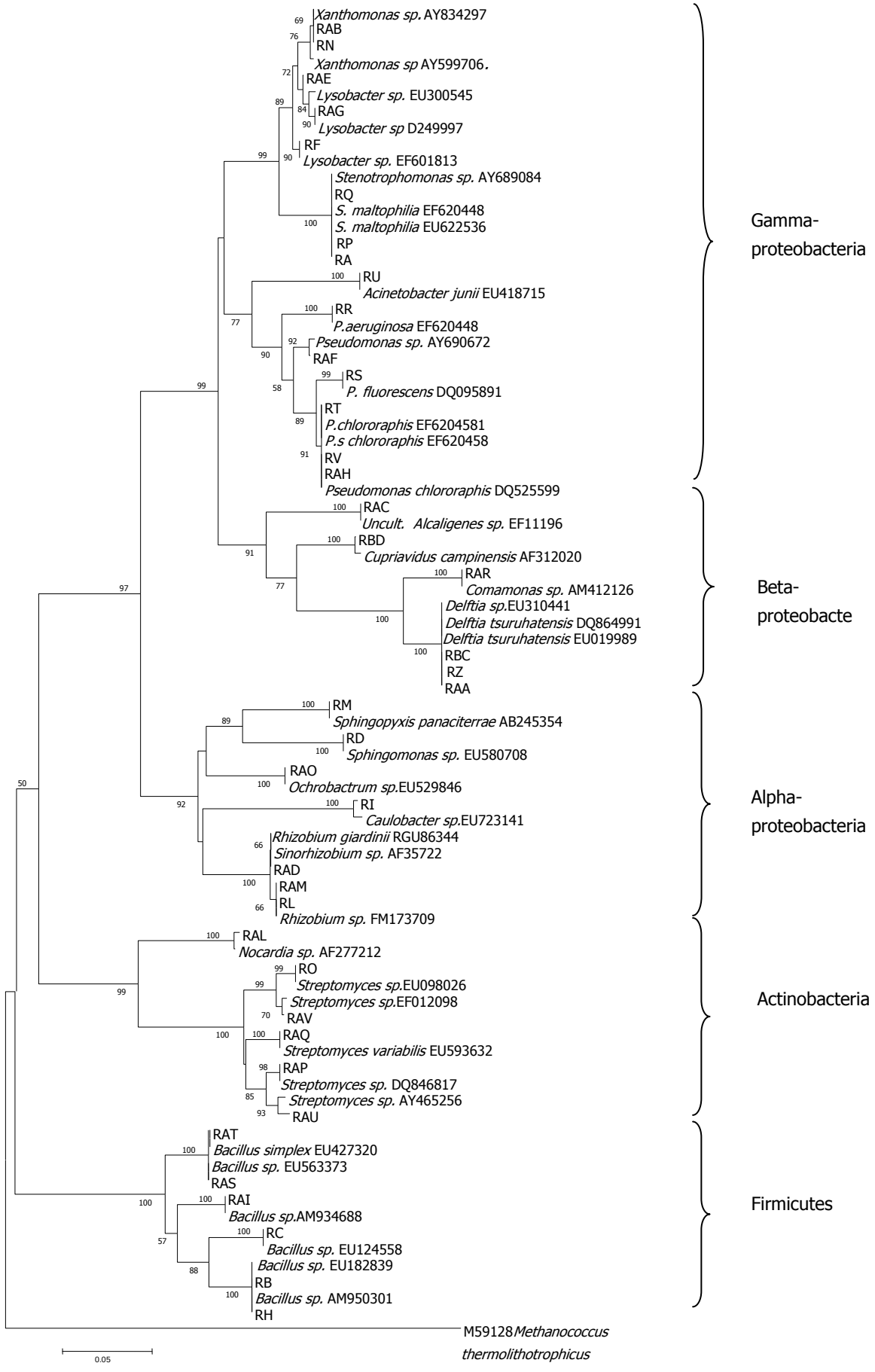

Xanthomonas sp. AY834297
RAB
RN
Xanthomonas sp AY599706.
RAE
Lysobacter sp. EU300545
RAG
Lysobacter sp D249997
RF
Lysobacter sp. EF601813
Stenotrophomonas sp. AY689084
RQ
S. maltophilia EF620448
S. maltophilia EU622536
RP
RA
RU
Acinetobacter junii EU418715
RR
P.aeruginosa EF620448
Pseudomonas sp. AY690672
RAF
RS
P. fluorescens DQ095891
RT
P.chlororaphis EF6204581
P.s chlororaphis EF620458
RV
RAH
Pseudomonas chlororaphis DQ525599
RAC
Uncult. Alcaligenes sp. EF11196
RBD
Cupriavidus campinensis AF312020
RAR
Comamonas sp. AM412126
Delftia sp.EU310441
Delftia tsuruhatensis DQ864991
Delftia tsuruhatensis EU019989
RBC
RZ
RAA
RM
Sphingopyxis panaciterrae AB245354
RD
Sphingomonas sp. EU580708
RAO
Ochrobactrum sp.EU529846
RI
Caulobacter sp.EU723141
Rhizobium giardinii RGU86344
Sinorhizobium sp. AF35722
RAD
RAM
RL
Rhizobium sp. FM173709
RAL
Nocardia sp. AF277212
RO
Streptomyces sp.EU098026
Streptomyces sp.EF012098
RAV
RAQ
Streptomyces variabilis EU593632
RAP
Streptomyces sp. DQ846817
Streptomyces sp. AY465256
RAU
RAT
Bacillus simplex EU427320
Bacillus sp. EU563373
RAS
RAI
Bacillus sp.AM934688
RC
Bacillus sp. EU124558
Bacillus sp. EU182839
RB
Bacillus sp. AM950301
RH
M59128 Methanococcus thermolithotrophicus
0.05
Gamma-proteobacteria
Beta-proteobacte
Alpha-proteobacteria
Actinobacteria
Firmicutes

**Fig.3.5**: Phylogenetic neighbour-joining tree of the 16S rRNA gene sequences obtained from R2A OTUs (RA,RB,RC,RD,RE,RF,RH,RI,RL,RM,RN,RO,RP,RQ,RR,RS,RT,RU,RV,RZ,RAA,RAB,RAC,RAD,RAE,RAF,RAG,RAH,RAI,RAL,RAM,RAN,RAO,RAP,RAQ,RAR,RAS,RAT,RAU,RAV,RAZ,RBA,RBC,RBD) and their closest database relatives with Species Name/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).

### 3.1.2.1 Taxonomic distribution of all heterotrophic aerobic eubacteria isolated in Nutrient and R2a media

Considering the distribution into different taxa - shown in fig 3.6 - OTUs are divided respectively for Nutrient and R2a between three classes of gram-negative Proteobacteria, namely Gamma-proteobacteria (49%-39%), Alpha-proteobacteria (8%-17%) and Beta-proteobacteria (5%-15%); and the gram-positive Firmicutes (22%-17%) and Actinobacteria (11%-12%). Moreover 5% of OTUs from Nutrient medium belong to the Flavobacteria class of Bacteroidetes *phylum*.

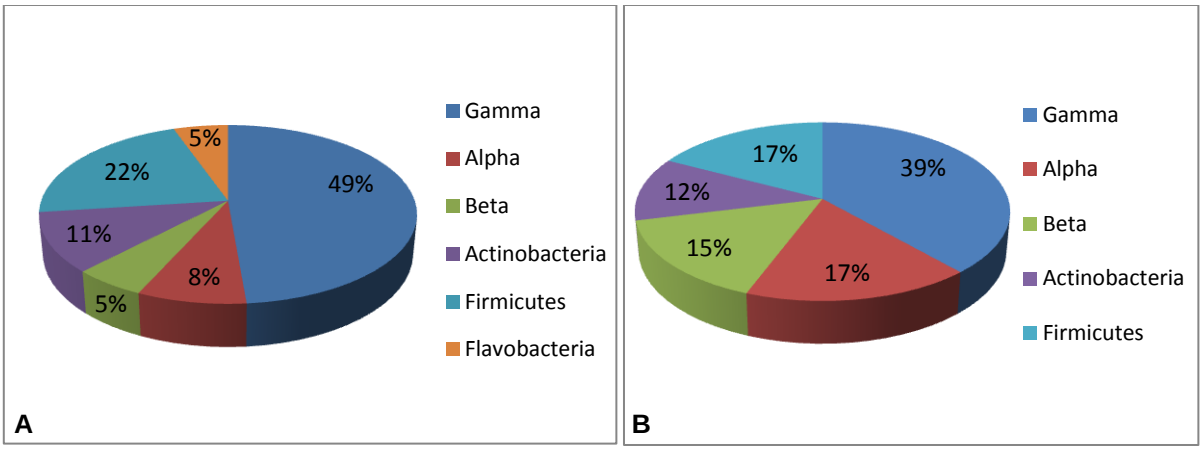



**Fig. 3.6**: Taxa distribution of Nutrient (A) and R2a (B) OTUs

Briefly considering isolates' distribution, few OTUs showed a percentage higher than 5%, in particular OTU C *Stenotrophomonas* sp. - the only OTU from Nutrient detected in all the 3 sampling points - which includes 16% of Nutrient isolates and is by far the most represented in the whole culturable community, followed by OTU AN *Pseudomonas chlororaphis* (7%) and OTU AM *Pseudomonas* sp. (6%). In fact the 28% of OTUs from Nutrient and 34% from R2a included just one isolate. Even in R2a only 3 OTUs had a percentage of isolates higher than 5%, namely RA *Stenotrophomonas maltophilia* (8%) and two OTUs identified in both culturing media: AN (9%) e OTU AQ (8%). This points out a high biodiversity within the microbial community with few dominant members and at the same time the presence of a variety of strains low represented.

Considering OTUs from both media it is worth noticing the prevalence of the *phylum* of Proteobacteria, and of the class Gamma in particular, which in fact includes by itself respectively the 49% and 39% of OTUs identified in Nutrient and R2a (Fig. 3.6). Moreover the three classes of Proteobacteria altogether respectively comprehend the 66 % and 73 % of Nutrient and R2a OTUs, pointing out an overall prevalence of gram-negative.

This gram-negative preponderance within the eubacterial culturable population - mainly of the class Gamma-proteobacteria - can be further observed in the distribution of genera within Nutrient and R2a phylogenetic trees (Fig 3.4 and 3.5). In connection to this high representation, the easy cultivability of Proteobacteria and of the Gamma class in particular has to be mentioned.

As shown in Fig 3.7, most of the isolated strains affiliated to the Gamma-proteobacteria class are associated with members of the genus *Pseudomonas* (59% in Nutrient and 50% in R2a), whose members are able to grow well on minimal medium and under laboratory culture conditions (Kim *et al.*, 2008).

*Pseudomonadaceae* are also reported to have a remarkable nutritional versatility with the ability of decomposing a large variety of organic molecules, including many that are often toxic to microorganisms of other groups (Palleroni, 2010), such as aromatic and aliphatic compounds (Ben Said *et al.*, 2008).

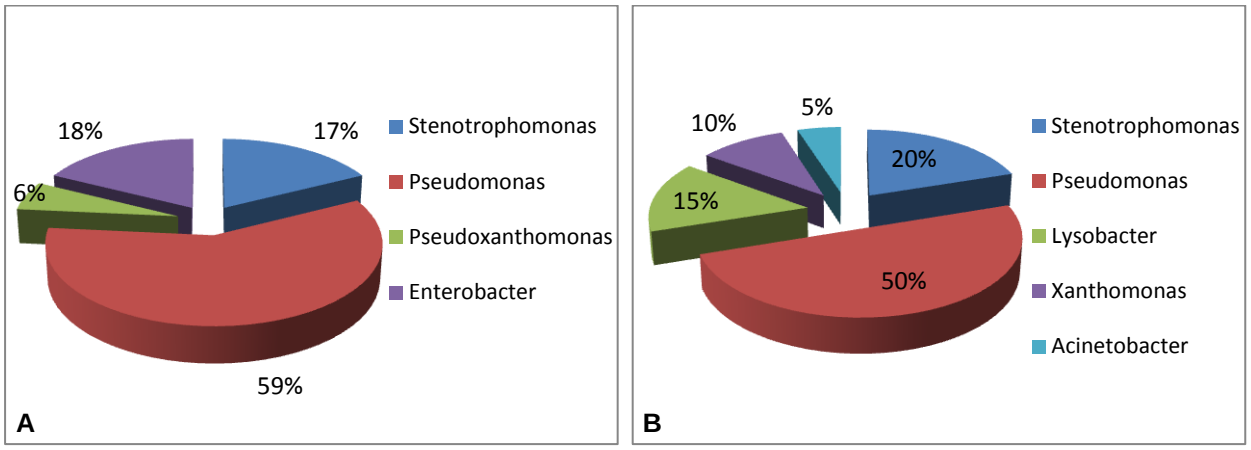



**Fig. 3.7**: Taxa distribution of Nutrient (A) and R2a (B) OTUs within Gamma-proteobacteria class

Studies have moreover reported the predominance of both Gamma and Beta classes of Proteobacteria in oil-contaminated areas (Ben Said *et al.*, 2008). To the Beta-proteobacteria also correlates *Cupriavidus* genus, identified in both culture media (Fig.3.8), and which includes the strain *Cupriavidus metallidurans* CH34, the most deeply investigated metal resistant bacterium (Von Rozycki *et* Nies, 2009; Janssen *et al.*, 2010). This suggests, as expected under a constant long-term Pb stress in soil, the selection towards a more resistant population.

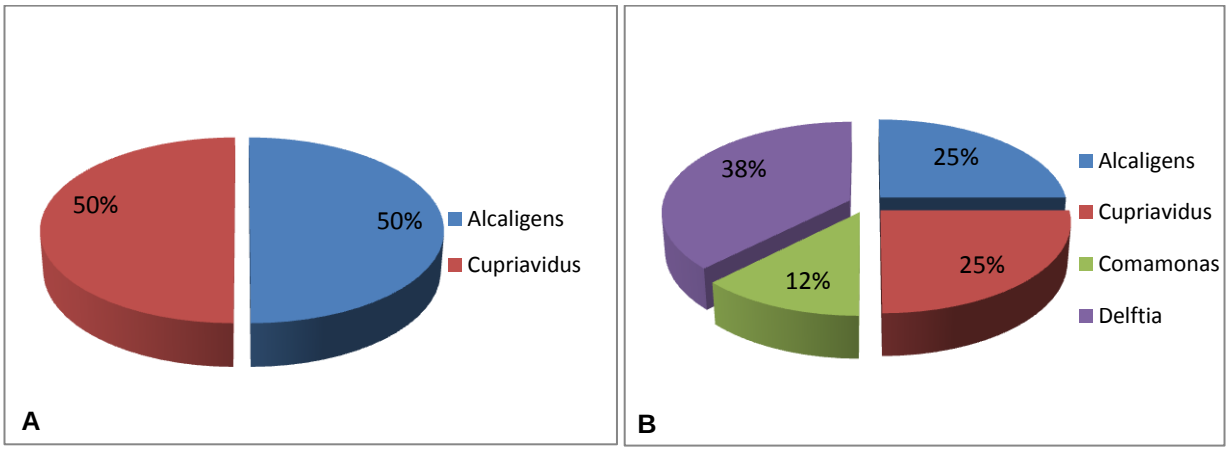



**Fig. 3.8:** Taxa distribution of Nutrient (A) and R2a (B) OTUs within Beta-proteobacteria class

The prevalence of gram-negative could partly be connected to a easier nutrient uptake from the environment in comparison to gram-positive bacteria - due to binding proteins in the periplasmic space - and a subsequent higher gram-negative population density in more oligotrophic environments (Saida *et al.*, 1998) such as perturbated soils. In fact, disturbances caused by heavy metals to microbial biomass and activity are known to be reflected in decreased litter decomposition and subsequently less-efficient soil nutrient cycling (Pennanen *et al.*, 1996; Hu *et al.*, 2005).

Although mixed results are reported in literature, the prevalence of gram-negative bacteria in heavy metal and metalloids contamination has been pointed out. This prevalence is in fact reported in the rhizobacterial community of an arsenic polluted soil (Cavalca *et al.*, 2010) where Proteobacteria seemed more tolerant towards As than Actinobacteria and Firmicutes, whose numbers were in fact greatly reduced. Other studies have shown that Proteobacteria were not affected by either As or Cd and

appeared to be more tolerant to both of them than several other groups of bacteria (Lorenz *et al.*, 2006). Besides in soils rich in heavy metals a predominance of the bacterial communities was observed compared to fungi in the rhizosphere of hyperaccumulators soils, with a high percentage of bacteria belonging to the Alpha-proteobacteria (Kamaludeen *et* Ramasamy, 2008; Idris *et al.*, 2004).

In particular as far as Gamma-proteobacteria are concerned, 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.*, 2007b), and the dominance of this class - mainly *Stenotrophomonas*, *Pseudomonas* and *Enterobacter* genera - was detected by clone library screening in sub-superficial soil levels and connected to the heterotrophic feature of these genera (Zhang *et al.*, 2007b).

Considering the Alpha-proteobacteria class, it has to mentioned that members of this group may have specific physiological requirements and therefore may have been lost during subculture. Being generally slow-growing, this class results in fact more highly detected in oligotrophic R2a medium (Fig.3.9), showing a much higher biodiversity with 9 OTUs belonging to 7 different genera, whereas only 4 OTUs of 2 genera were isolated in Nutrient (Ellis *et al.*, 2003). In particular, the detection of members closely related to well-known nitrogen fixers such as *Rhizobium* and *Agrobacterium* is interesting in the context of a Phytoremediation approach.

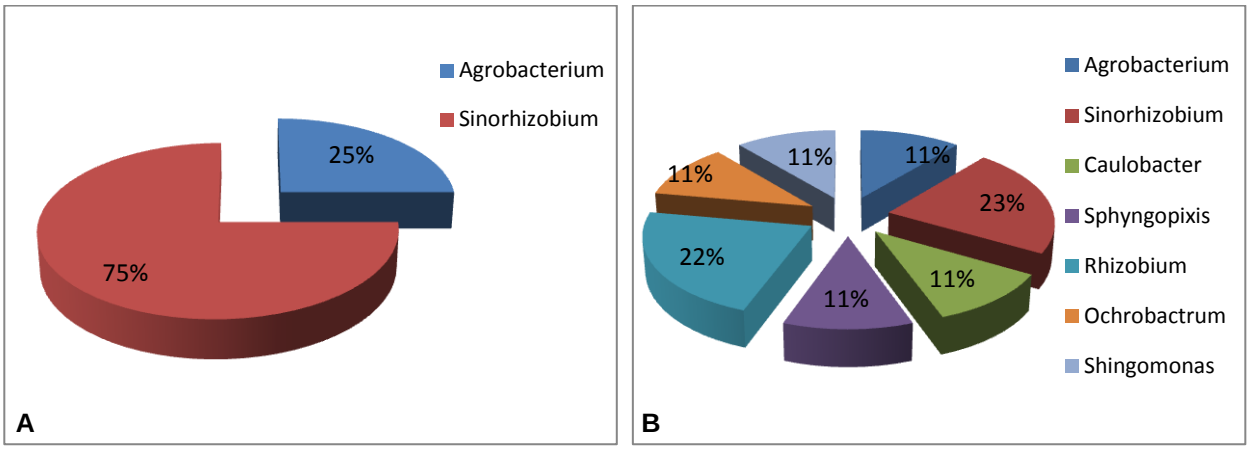



**Fig. 3.9**: Taxa distribution of Nutrient (A) and R2a (B) OTUs within Alpha-proteobacteria class

Even if less represented, an interesting potential can relays in the gram-positive Actinobacteria community members, with OTUs belonging to genera such as *Arthrobacter* (Fig. 3.10), for which tolerance and resistance to various heavy metals such as Pb is reported (Margesin *et* Schinner, 1996; Benyehuda *et al.*, 2003). Moreover the detected *Nocardia* genus includes hydrocarbon degrading members even proposed for the bioremediation of petroleum pollutants (Van Hamme *et al.*, 2003; Periello, 2000). Other isolated strains of this *phylum* most closely related to *Micrococcus, Brachybacterium, Streptomyces* and *Microbacterium* genera. To the latter in particular is reported a strain with different multiple heavy metal resistance characteristics, able to increase water-soluble Pb in solution and in Pb-added soil and reporting Plant growth promoting (PGP) activities (Sheng *et al.*, 2008a), consequently particularly interesting in a phytoremediation approach.

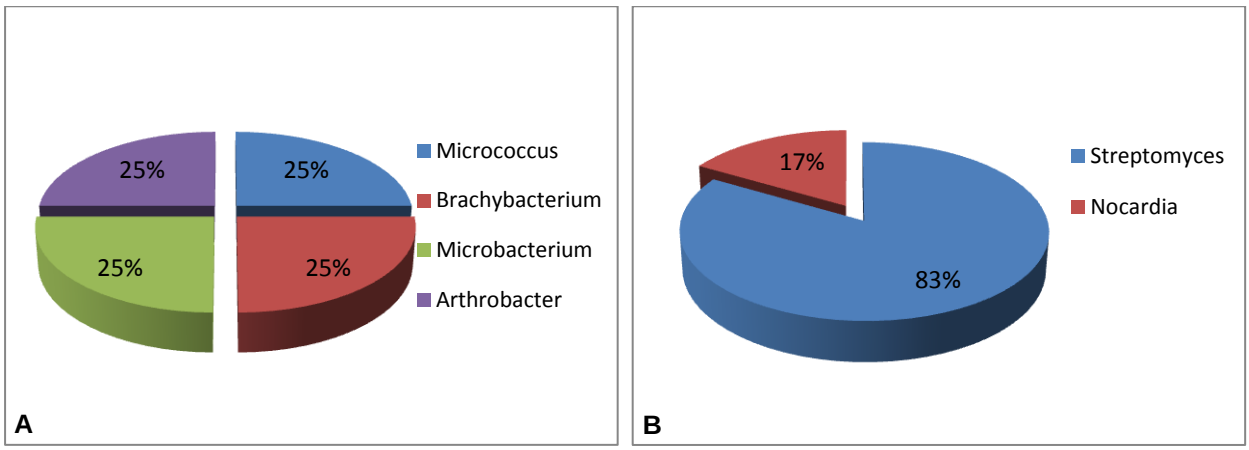



**Fig 3.10** Taxa distribution of Nutrient (A) and R2a (B) OTUs within Actinobacteria *phylum*

As far as the Firmicutes *phylum* is concerned (cake graph not presented), all isolated OTUs belong to *Bacillus* genus as *B. firmus*, *B. pumilus* and *B.cereus*, whose PGP activities, abundance in heavy metals polluted soil and resistance to conditions of stress including heavy metal are reported in literature (Kusum *et* Kumar, 1998; Ellis *et al.*, 2003; Belimov *et al.*, 2001).

### 3.1.2.2 Taxonomic distribution at the 3 sampling points

Considering the taxonomic distribution of all OTUs directly isolated at each sampling point, it is possible to notice the predominance of proteobacteria gram-negative in point I and II, which are the most polluted ones, including respectively the 97% and 85% of all OTUs directly isolated (Fig 3.11). On the other side in point III, characterized by a lower Pb contamination level, gram-positive bacteria predominate including 59% of all OTUs, in particular 38% belong to Firmicutes *phylum* and 22% to Actinobacteria. This datum indicates therefore not only a numerical predominance of gram-negative within the culturable bacterial community of this area, but also suggests a higher adaptation and resistance acquired and spread within this group under the selective pressure imposed by decades of Pb contamination.

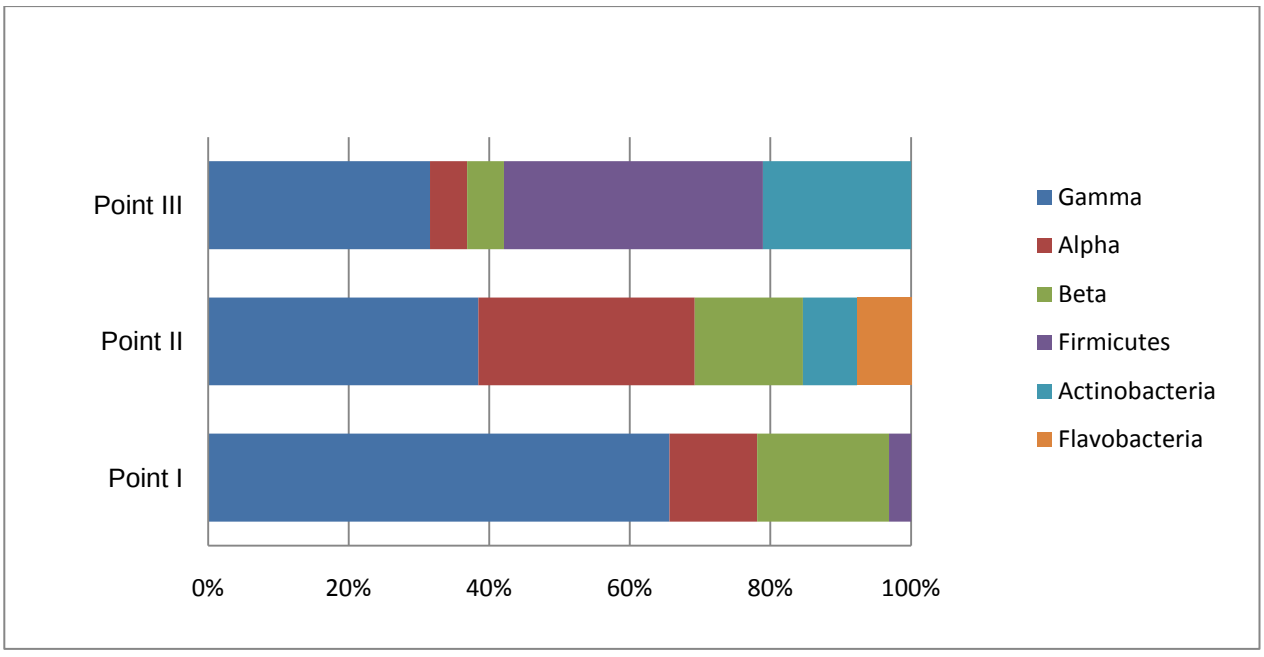



**Fig 3.11** Taxonomic distribution of all OTUs from direct isolation at each sampling point

Considering the most represented class of Gamma-proteobacteria - which includes respectively 66%, 38% and 30% of all OTUs at the 3 sampling points I, II and III - in particular to the genus *Stenotrophomonas* correlate the only 2 OTUs detected at all the 3 sampling points: OTU C *Stenotrophomonas* sp.*,* previously reported (par 3.1.2.1) as the most represented in the whole cultivable microbial community, and OTU RA *Stenotrophomonas maltophilia*.

*Stenotrophomonas maltophilia* is an aerobic, non-fermentative gram-negative bacterium widespread in the environment - it can be found in aquatic environments, in soils, on vegetation and even on some animals – and reported to tolerate very high concentrations of various toxic metals (Pages *et al.*, 2008; Ryan *et al.*, 2009). Moreover, this species constitutes one of the dominant rhizosphere inhabitant showing plant growth-promoting activity as well as antagonistic properties against plant pathogens (Ryan *et al.*, 2009). *S. maltophilia* is also able to degrade xenobiotic compounds, to detoxify high molecular weight polycyclic aromatic hydrocarbon, possessing therefore a potential for soil decontamination even in a Phytoremediation perspective (Seo *et al.*, 2007; Ben Said *et al.*, 2008).

Other 3 OTUs (OTU A, L, BC) of the genera *Stenotrophomonas, Cupriavidus* and *Agrobacterium* - respectively of Gamma-, Beta- and Alpha-proteobacteria classes - have been detected in both points I and II. At the same time 3 OTUs of Gamma-proteobacteria, belonging to *Stenotrophomonas* and *Pseudomonas* genera, and 1 *Alcaligenes* species of Beta class have been isolated in both point I and III (OTUs AA *Stenotrophomonas* sp., OTU AN *Pseudomonas chlororaphis*, OTU AM *Pseudomonas* sp., OTU G *Alcaligenes* sp.). It is worth noting that there is no coincidence of OTUs between point II and III, respectively the most and the less contaminated sampling points, suggesting the establishment of a peculiar type of microbial community related to the different contaminant level.

In fact the low recurrence of OTUs among the different soil samples indicates a rich biodiversity within the indigenous micro flora and a heterogeneity in the community structure among the 3 sampling points, which reflects the different contamination level and heterogeneity implied in a complex matrix such as soil.

Moreover as far as gram-positive members are concerned, 15 of the 16 OTUs of Firmicutes - all belonging to *Bacillus* genus - have been identified exclusively in the less contaminated point III, although to these microorganisms is reported a high stress resistance (Kusum *et* Kumar, 1998; Ellis *et al.*, 2003). Just one OTU clustering in the *Bacilli* class has been identified in point I and no one in the most polluted Point II.

Considering the Actinobacteria respectively 2 and 8 OTUs were isolated from the most and less contaminated points II and III. Even if low represented, this can suggest a higher resistance potential of the gram-positive bacteria within this *phylum.*

Considering the Flavobacteria Class, its really low representation can be partly connected with its difficulty of cultivation. The only two OTUs of this class, belonging to *Chryseobacterium* genus, have been identified in the most polluted Point II. Species of this genus have been detected in the rhizosphere of plants colonizing on mine tailings, and include alkane-degrading strains, suitable to degrade pollutants comprising petroleum compounds (Periello, 2000; Zhang *et al.*, 2007b).

It has to be considered that along with metal 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 the addition of other contaminants such as hydrocarbons detected at point I and Hg in trace at point III. 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.

### 3.1.2.3 Taxonomic distribution of the most represented Proteobacteria classes

Considering the above-mentioned high representation of Proteobacteria, it is therefore interesting to focus on the genera identified at the 3 sampling points within the gram-negative Proteobacteria *phylum*.
As far as the Gamma class is concerned, the dominance of *Pseudomonas* and *Stenotrophomonas* genera is reflected at each sampling point (Fig 3.12).

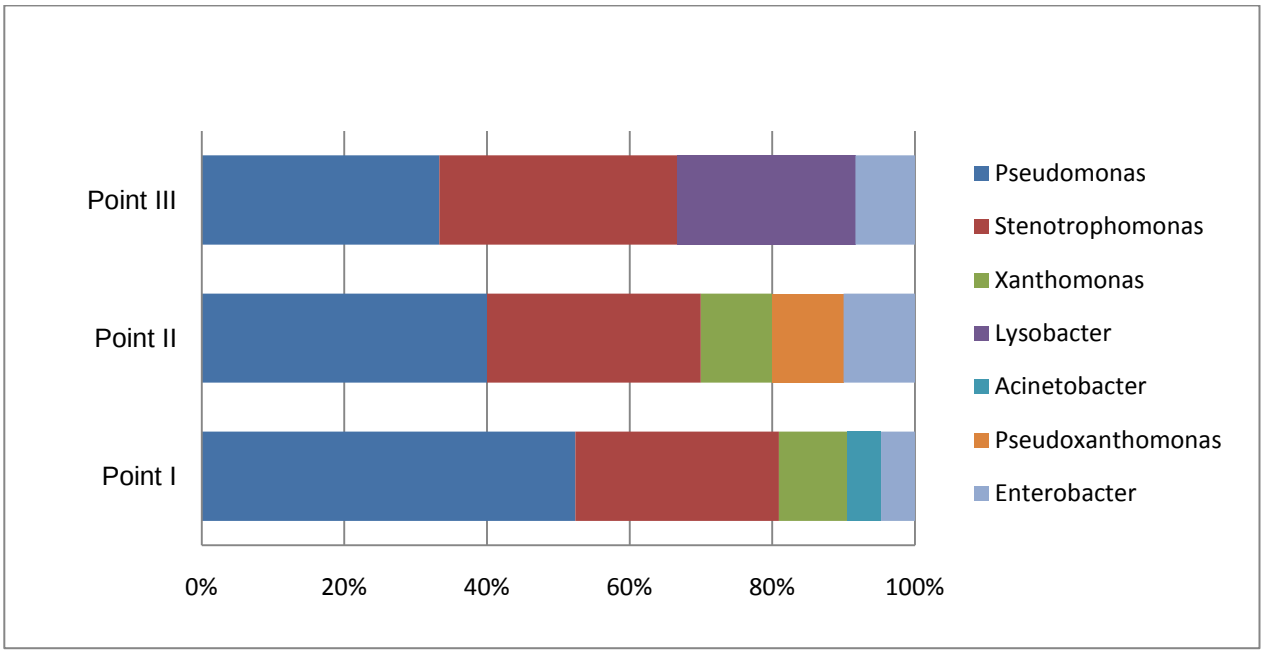



**Fig 3.12** Taxonomic distribution of all Gamma-proteobacteria OTUs at each sampling point

These genera are reported to be involved in the mineralization of polycyclic aromatic hydrocarbons (PAH) and to the *Pseudomonas* genus belong species such as *P. putida* and *P.chlororaphis* reported in literature for degrading capabilities towards organic compounds, resistances to metals and metalloids (Seo *et al.*, 2007; Cánovas *et al.*, 2003). In particular the species *Pseudomonas chlororaphis,* members of which have been isolated in distinct OTUs at all the 3 sampling points, includes a strain able to mineralize tetrachlorobenzene, a highly recalcitrant pollutant, pointing out the high degrading potential within this genus (Potrawfke *et al.*, 1998).
One OTU isolated in Point I, characterized also by a hydrocarbon contamination, relates to the *Pseudomonas aeruginosa* group, members of which have been described as hydrocarbonoclastic and are also well known as resistant to metals (Ben Said *et al.*, 2008).
For *Pseudomonas putida,* species identified in Point II, is also reported an unexpected capacity to tolerate heavy metals and metalloids (Cánovas *et al.*, 2003), and a strain with the ability to degrade hydrocarbon has been successfully applied for the bioremediation of crude oil in open environment (Raghavan *et* Vivekanandan, 1999).
*Pseudomonas* members show even PGP activities: a strain of *Pseudomonas putida* is in fact reported in literature as strong candidate for development as a soil inoculant to enhance crop yields and resulting in substantial promotion of seedling root growth (Patten *et* Glick, 2002).
Moreover the improvement in growth of *Triticum aestivum* (wheat) seedling, in presence of different Pb concentrations, and of both growth and Pb accumulation in rape seedlings have been detected when inoculated with *Pseudomonas* strains (Karami *et* Shamsuddin, 2010; Hasnain *et* Sabri, 1997; Sheng *et al.*, 2008b). This genus seems therefore interesting also in a Phytoremediation perspective.

Considering the *Stenotrophomonas* genus, as above reported the species *Stenotrophomonas malthophilia* includes strains with degrading capabilities of PAH, PGP activity and resistances to metals (Seo *et al.*, 2007).

Within this class the genera *Xanthomonas, Pseudoxanthomonas* and *Lysobacter* of the *Xanthomonadacea* family have also been identified (Fig.3.12). Six out of 23 genera of the family *Xanthomonadaceae* are involved directly or indirectly in oil or petroleum hydrocarbon degradation including *Pseudoxanthomonas,* the *Stenotrophomonas* genus, above described, and *Xanthomonas* (Chang *et* Zylstra, 2010). Considering *Pseudoxanthomonas* genus – detected in the most contaminated Point II - strains have indeed been isolated from soil contaminated with PAH (Harada *et al.*, 2006) and a species able to utilize pyrene as sole source of carbon and energy was isolated from soil (Klankeo *et al.*, 2009). Moreover *Xanthomonas* includes both PAH degraders (Viñas *et al.*, 2005) and heavy metal resistant strains isolated from highly polluted metal-containing soil (Sheng *et* Xia, 2006).

Besides, *Acinetobacter* genus has also been isolated from Point I, while 3 distinct OTUs of *Enterobacter* genus have been isolated at all the three sampling points.

The co-cultivation of rice with PAH-degrading *Acinetobacter* sp. increased biomass production and PAH removal, suggesting its great potential to accelerate the bioremediation process (Gao *et al.*, 2006). Besides surfactants produced by a thermophilic strain of *Acinetobacter calcoaceticus* isolated from a petroleum contaminated soil were demonstrated to be effective in enhancing solubility and desorption, and consequently degradation of PAHs (Wong *et al.*, 2010).

Considering the *Enterobacter* genus, it includes members capable of degrading various organophosphorus pesticides while a high heavy metal resistance has been detected in a strain isolated from an industry wastewater treatment plan, along with the biosorption capability of over 50 mg Pb per gram of dry cell (Lu *et al.*, 2006).

Moreover, from a refinery waste sludge microorganisms able to degrade petroleum hydrocarbons, including monoaromatic, PAHs and linear and branched alkanes, were identified to belong to the above-mentioned *Enterobacter* and the genus *Ochrobactrum* of Alpha class, both identified within the analyzed soil community (Katsivela *et al.*, 2003).

Literature data indicate therefore that a high degrading and resistance potential is associated to Proteobacteria members. In fact even if the two classes of Alpha and Beta proteobacteria are less represented between the isolated OTUs than the Gamma class, it is interesting to observe the variety of their detected genera, connected to high degrading capabilities and resistances to metals.

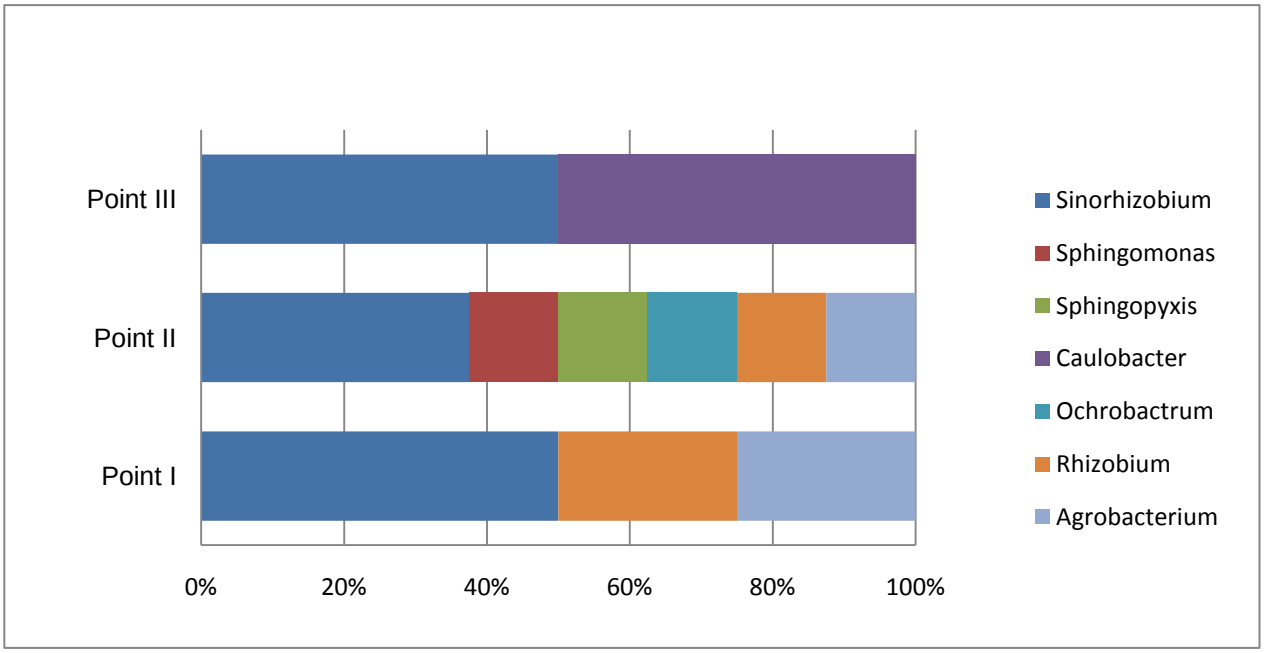



**Fig 3.13** Taxonomic distribution of all Alpha-proteobacteria OTUs at each sampling point

In particular considering the Alfa-proteobacteria, six different genera have been identified in the highest polluted Point II by itself (Fig 3.13). Among the genera identified from Point II, *Sphingomonas* genus is an organism of major interest for the degradation of organic contaminants in soils and other environments.
To *Shingomonadaceae* family - known to degrade a wide range of environmental pollutants - also belong *Sphingopyxis* genus, which includes strains able to degrade xenobiotics such as Polyethylene glycols (PEGs) and their derivatives, used as surfactants and lubricants in many fields (Tani *et al.*, 2007). Both *Sphingomonas* and *Sphingopyxis* include hydrocarbon-degrading members that have been isolated from a wide variety of environments, including temperate and polar soils, marine sediments, and plant tissues where they occur as endophytes, and they degrade a broad range of mono- and polycyclic aromatic compounds (Kertesz *et* Kawasaki, 2010).
*Sphingomonas* strains are in fact often isolated for their capability to degrade recalcitrant organic pollutants, strains are able to use PHA as only carbon and energy source, and used in bioremediation protocols (Vanbroekhoven *et al.*, 2004; Seo *et al.*, 2007).
Moreover for the genus *Ochrobactrum* - detected in the highest contaminated point as well - are reported high metal resistance, hydrocarbon degradation and the ability to use halogenated phenols as the sole carbon source (Faisal *et* Hasnain, 2006; Sultan *et* Hasnain, 2007). This genus also includes Plant growth promoting Rhizobacteria (PGPR) members (Faisal *et* Hasnain, 2006; Príncipe *et al.*, 2007).
Considering the *Caulobacter* genus – which included 50 % of OTUs isolated from the point III co-contaminated by Hg – it includes species such as *Caulobacter crescentus* and related stalk bacterial species known for their distinctive ability to live in low-nutrient environments - a characteristic of most heavy metal-contaminated sites – and heavy metal resistant members such as mercury-resistant *Caulobacter* strains, which shows an inducible mercury volatilization activity (Hu *et al.*, 2005; Chiu *et al.*, 2007).
Besides OTUs related to nitrogen fixers *Agrobacterium* and *Rhizobium* genera were isolated from both the higher contaminated points I and II. *Agrobacterium* includes also members able to completely degrade recalcitrant solvents as chlorinated aliphatic compound (Effendi *et al.*, 2000).

*Sinorhizobium* genus in particular includes 50% of Alfa bacteria isolated in point I and III, and almost the 40% in point II. This genus, as *Sphingomonas* and *Agrobacterium,* includes bacteria involved in the mineralization of PAHs and PGPR (Galleguillosa *et al.*, 2000; Seo *et al.*, 2007; Kaymak *et al.*, 2008; Kamaludeen *et* Ramasamy, 2008). A literature study on a soil contaminated by toxic spill from a pyrite mine in Southern Spain, reports of several *Sinorhizobium* and *Rhizobium* strains resistant to the toxic metals and metalloids - including Pb - which are in the meantime fully symbiotically effective, even under contamination conditions, thus confirming the potential of these $N_2$-fixating microorganisms also in a Phytoremdiation approach enhanced by microorganisms (Carrasco *et al.*, 2005).

It is also interesting to consider that to genera *Pseudomonas* and *Enterobacter* of the Gamma class and *Rhizobium* and *Agrobacterium* of the Alpha class belong phosphate-solubilizing microorganisms, which can therefore promote in plant both growth - solubilizing insoluble P forms and make them available to plants - and metal uptake, solubilizing metal from inorganic phosphate precipitates (Hamdali *et al.*, 2007; Khan *et al.*, 2009).

Besides as previously briefly mentioned, studies have reported the predominance of both Gamma and Beta classes of Proteobacteria in oil-contaminated areas (Ben Said *et al.*, 2008). Within the Beta-proteobacteria class, four genera have been detected and the prevalent are *Alcaligenes, Cupriavidus* and *Delftia* (fig 3.14).

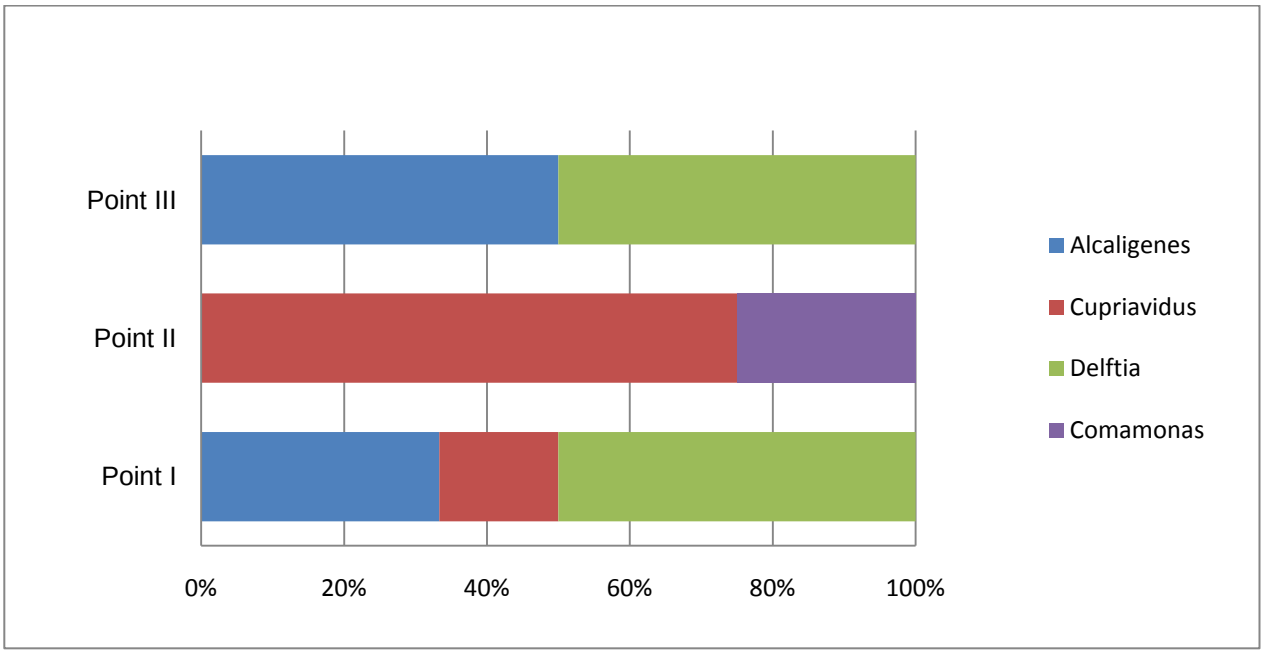



**Fig 3.14** Taxonomic distribution of all Beta-proteobacteria OTUs at each sampling point

These genera include strains reported for high degrading capabilities and for bioremediation applications, such as *Alcaligenes* genus which include both heavy metal resistant and alkane-utilizing bacteria suggested for bioremediation of petroleum pollutants (Periello, 2000; Trajanovska *et al.*, 1997), while *Cupriavidus* genus is well-known for the heavy metal resistance of its members (Chen *et al.*, 2008). OTUs of the *Comamonas* genus - which include bacteria able to degrade simple PAH such as naphthalene, anthracene, and phenanthrene - were moreover detected at the most contaminated point II (Goyal *et* Zylstra, 1996). Along with *Alcaligenes* and *Comamonas*, *Delftia* genus also includes PGPR species (Erturk *et al.*, 2010; Han *et al.*, 2005).

Therefore the genera identified within the culturable autochthonous bacterial community at the 3 examined sampling points indicate both resistance and degrading potential associated to the detected

Proteobacteria classes, even to the less represented Beta class. Moreover the detection of bacteria reporting potential growth promoting activities suggests a possible synergy of the indigenous bacterial cenosis in a Phytoremediation approach enhanced by microorganisms.

### 3.1.3 Isolation of most resistant/potential TEL-transforming bacterial strains through Enrichment cultures from the 3 sampling points within Ex-SLOI area

Direct isolation has allowed the identification of the main components of the culturable population, describing the structure of the indigenous bacterial community established at the different contamination levels of the three sampling points chosen within the Ex-SLOI area. The analysis of the soil bacterial cenoses trough direct isolation is in this context of particular interest, due to the cronic contamination of the site exposed to the selective Pb pressure by over half century. It allows therefore to infer the impact of long-term stress by inorganic and organic Pb - the latter by far less studied - and the effect on the soil microflora biodiversity.

At the same time Enrichment cultures were performed in minimal medium DM by further adding to soil samples of each sampling point (I,II,III) 125 mg TEL/L, respectively added as sole carbon source (named DM) and together with the addition of 0,1% yeast extract (named DMY).

The Enrichment was aimed at pushing the selection further on the strains within the soil community which are most resistant to organic Pb or potentially able to degrade it in cometabolism or using it as carbon source.

After 8 weeks of incubation, the isolation of the microbial species selected within each enrichment culture (DMY and DM) was made by plating serial dilutions on agar plates of respectively the same selective medium. As in the direct isolation axenic cultures of morphologically different bacteria were screened by the molecular technique ARDRA - main profiles obtained are reported in fig 3.15 and 3.16 - grouped into different Operational Taxonomic Units (OTUs) and taxonomically identified through sequencing of 16S rRNA gene. Sequencing results of obtained OTUs are reported in tab. 3.3 with percentage homology to GenBank relatives.

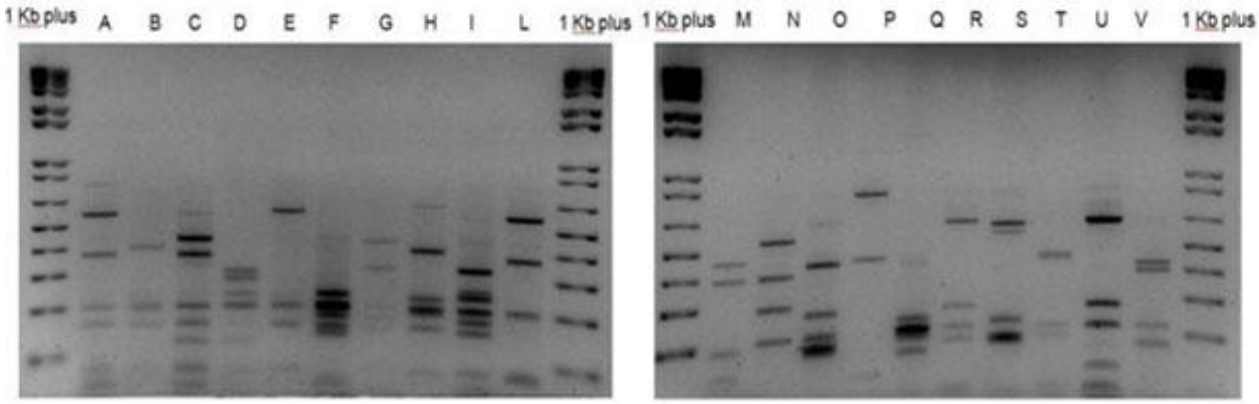



**Fig 3.15**: Main restriction profiles obtained with AluI enzyme

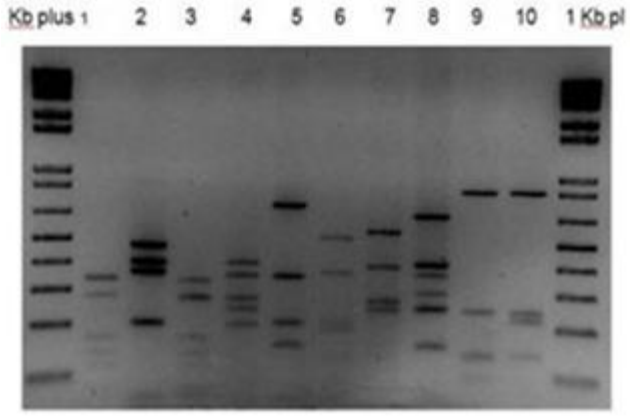



**Fig 3.16**: Main restriction profiles obtained with Hha1 enzyme

Through the Enrichment cultures 30 and 32 isolates were obtained, and 11 and 14 OTUs were identified respectively with TEL as sole carbon source (DM) and in concomitant presence of yeast extract (DMY). As 5 OTUs were detected in both enrichment conditions, 20 OTUs where altogether obtained (Tab 3.3).
It is interesting to notice that only 4 OTUs thus obtained had already been isolated also in direct isolation - i.e. A6, A9, A10 and A14 respectively corresponding to OTU AR, BC, B and G from direct isolation – and belonging to the 3 Proteobacteria classes detected in the direct isolation. Actually enrichment protocols allows the selection of resistant and metabolically interesting members of the bacterial community even from the pool of probably numerically minor components or of bacteria disadvantaged in the laboratory culture conditions, whose growth and isolation has been probably favoured in the stricter selective conditions of the Enrichment cultures.

| Enrichment OTU | Enrichment culture | Taxonomic Reference ID | Phylogentic group | Homology% |
|---|---|---|---|---|
| **A1** | DM | *Pseudomonas aeruginosa* FJ009393 | γ-proteobacteria | 100% |
| **A2** | DM | *Cupriavidus sp.* AB266611 | β-proteobacteria | 100% |
| **A3** | DM-DMY | *Pseudoxanthomonas* sp. AY635897 | γ-proteobacteria | 100% |
| **A4** | DM-DMY | *Ochrobactrum* sp. GU248309 | α-proteobacteria | 100% |
| **A5** | DM-DMY | *Delftia* sp. Fj594443 | β-proteobacteria | 100% |
| **A6** | DM-DMY | *Pseudoxanthomonas* sp. EU276093 | γ-proteobacteria | 99% |
| **A7** | DM-DMY | *Ochrobactrum* sp. FJ598332 | α-proteobacteria | 100% |
| **A8** | DM | *Variovorax* sp. EU734636 | β-proteobacteria | 100% |
| **A9** | DM | *Agrobacterium* sp. EF189105 | α-proteobacteria | 100% |
| **A10** | DMY | *Pseudomonas putida* DQ833753 | γ-proteobacteria | 100% |
| **A11** | DMY | *Pseudomonas sp.* EU375660 | γ-proteobacteria | 100% |

| | | | | |
|---|---|---|---|---|
| **A12** | DMY | *Microbacterium sp.* DQ227343 | Actinobacteria | 100% |
| **A13** | DMY | *Variovorax sp., AM285013* | β-proteobacteria | 100% |
| **A14** | DM | *Alcaligenes sp.* EU37500 | β-proteobacteria | 100% |
| **A15** | DM | *Variovorax sp. AY689053* | β-proteobacteria | 100% |
| **A16** | DMY | *Stenotrophomonas maltophilia* EF620462 | γ-proteobacteria | 100% |
| **A18** | DMY | *Microbacterium sp.* EF612319 | Actinobacteria | 100% |
| **A19** | DMY | *Pseudomonas sp.* GQ180165 | γ-proteobacteria | 100% |
| **A20** | DMY | *Arthrobacter sp. EU342346* | Actinobacteria | 100% |
| **A21** | DMY | *Microbacterium sp.* AB461019 | Actinobacteria | 99% |

**Table 3.3**: Taxonomic collocation of all OTUs isolated by Enrichment cultures with TEL as sole carbon source (DM), and with addition of yeast extract (DMY)

### 3.1.3.1 Taxonomic characterization of strains isolated through Enrichment cultures

Considering the taxonomic distribution of OTUs isolated in the stricter conditions of Enrichment cultures (Fig 3.17), it is worth noting the dominance of gram-negative Proteobacteria previously detected in the direct isolation.

In fact the three previously detected gram-negative classes – Gamma-, Alpha- and Beta-proteobacteria - together accounted for 71% of OTUs isolated in presence of Tel and yeast extract (DMY Enrichment culture), and for the totality of OTUs isolated with TEL as sole carbon source (DM Enrichment culture), thus attesting the resistance and metabolic potential within this *phylum*. This gram-negative preponderance can be observed even in the distribution of genera within the phylogenetic tree (fig 3.18).

In particular the Gamma-proteobacteria class is the most represented in DMY Enrichment cultures, accounting by itself for 43% of isolated OTUs, while in DM Enrichment cultures in presence of TEL as sole carbon source prevails the Beta-proteobacteria class, which includes 45% of all OTUs thus obtained (Fig. 3.17).

No Firmicutes was detected in Enrichment conditions. Despite the resistance to heavy metals reported in literature for the isolated *Bacilli* class (Kusum *et* Kumar, 1998; Ellis *et al.*, 2003), a low representation of this *phylum* within the indigenous community had been also observed by both microbial counts and direct isolation (par 3.1.1 and 3.1.2). On the other side exclusively in the enrichment cultures DMY - with the concomitant presence of yeast extract as carbon source - gram-positive Actinobacteria were detected and accounted for 29% of OTUs selected in those conditions (Fig 3.17).

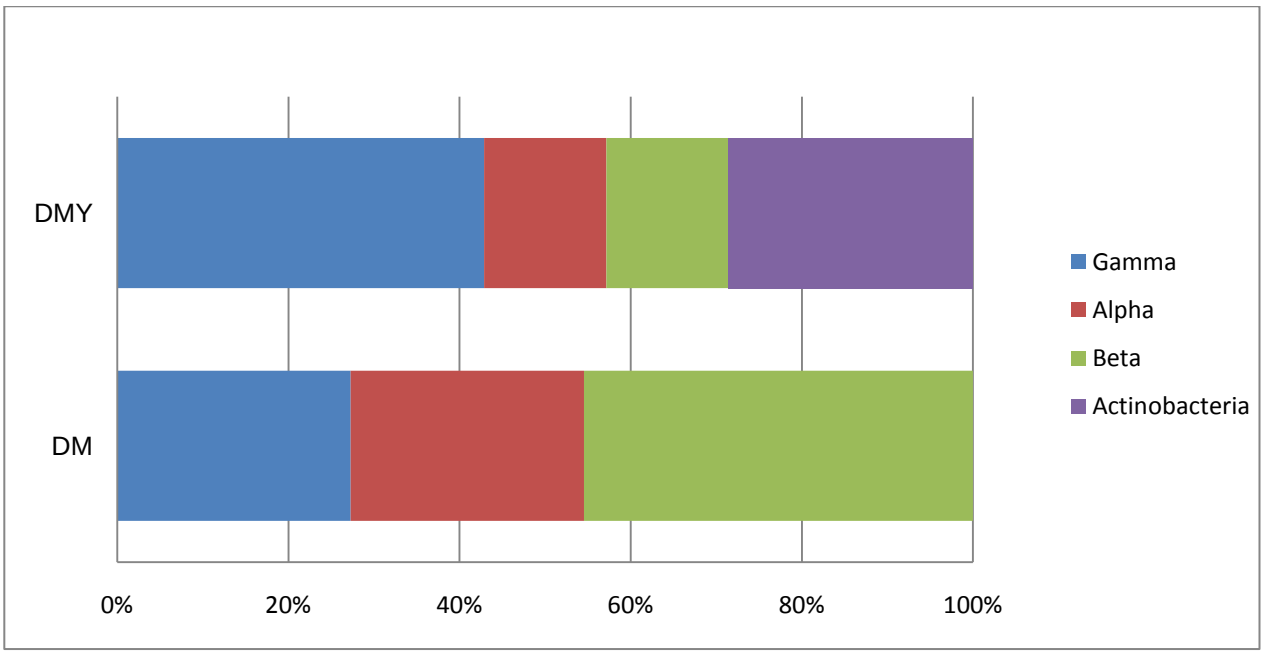



**Fig. 3.17** Taxonomic distribution of OTUs isolated by Enrichment cultures with TEL as sole carbon source (DM), and with addition of yeast extract (DMY)

Considering the taxonomic distribution within Gamma-proteobacteria (fig 3.19) - the most represented class both in direct isolation and in DMY Enrichment cultures - *Pseudoxanthomonas* genus dominates including 2 of the 6 OTUs isolated in presence of yeast extract (DMY) and 2 of the 3 OTUs isolated with TEL as only carbon source (DM), whereas in direct isolation only 1 OTUs of *Pseudoxanthomonas* had been isolated in rich medium from the most polluted sampling point (par 3.1.2.3).

For the genus *Pseudoxanthomonas* is in fact reported a strain able to degrade all six BTEX (benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylene) compounds, most common groundwater and soil contaminants associated to petrol (Kim *et al.*, 2008). Except for *Pseudomonas* or closely related species, only a few isolated microorganisms are known to degrade these compounds. To this regard it is worth remembering the use and spills of petrol within the Ex-SLOI area and in particular at the mixing level near point I. Both OTUs of this genus have in fact been isolated by Enrichment cultures from point I soil, and one even from point III.

Within the Gamma-proteobacteria class, the other OTU isolated in DM and 3 of the 6 OTUs isolated in presence of yeast extract (DMY) belong to the *Pseudomonas* genus, dominant component of the bacterial culturable community detected by direct isolation together with the *Stenotrophomonas* genus (par.3.1.2.1). A member of this latter genus, *Stenotrophomonas maltophilia*, has been moreover identified in DMY from the soil of the most contaminated point II. The genus *Stenotrophomonas* comprises at least eight species found throughout the environment, particularly in close association with plants and *Stenotrophomonas maltophilia* is the most predominant species associated to this genus (Ryan *et al.*, 2009).

Considering *Pseudomonas* genus, *P. aeruginosa* has been detected in DM enrichments from both the most and less contaminated points II and III. This species, already described as hydrocarbonoclastic and resistant to metals (par 3.1.2.3), was moreover reported to increase shoots uptake by a factor of 3.8 in Maize plants in presence of Pb contamination (Braud *et al.*, 2009). Microbial populations are in fact known to be able to affect metal mobility and availability to the plant, through release of chelators, acidification, solubilizing metal-phosphates and by redox changes. The presence of rhizosphere bacteria has in fact been reported to increase the concentrations of metals Zn, Cu, Cr and Pb in plants, thus enhancing the

efficiency of a Phytoremediation approch (Abou-Shanab *et al.*, 2008; Sheng *et* Xia, 2006; Ma *et al.*, 2009; Chen *et al.*, 2008).

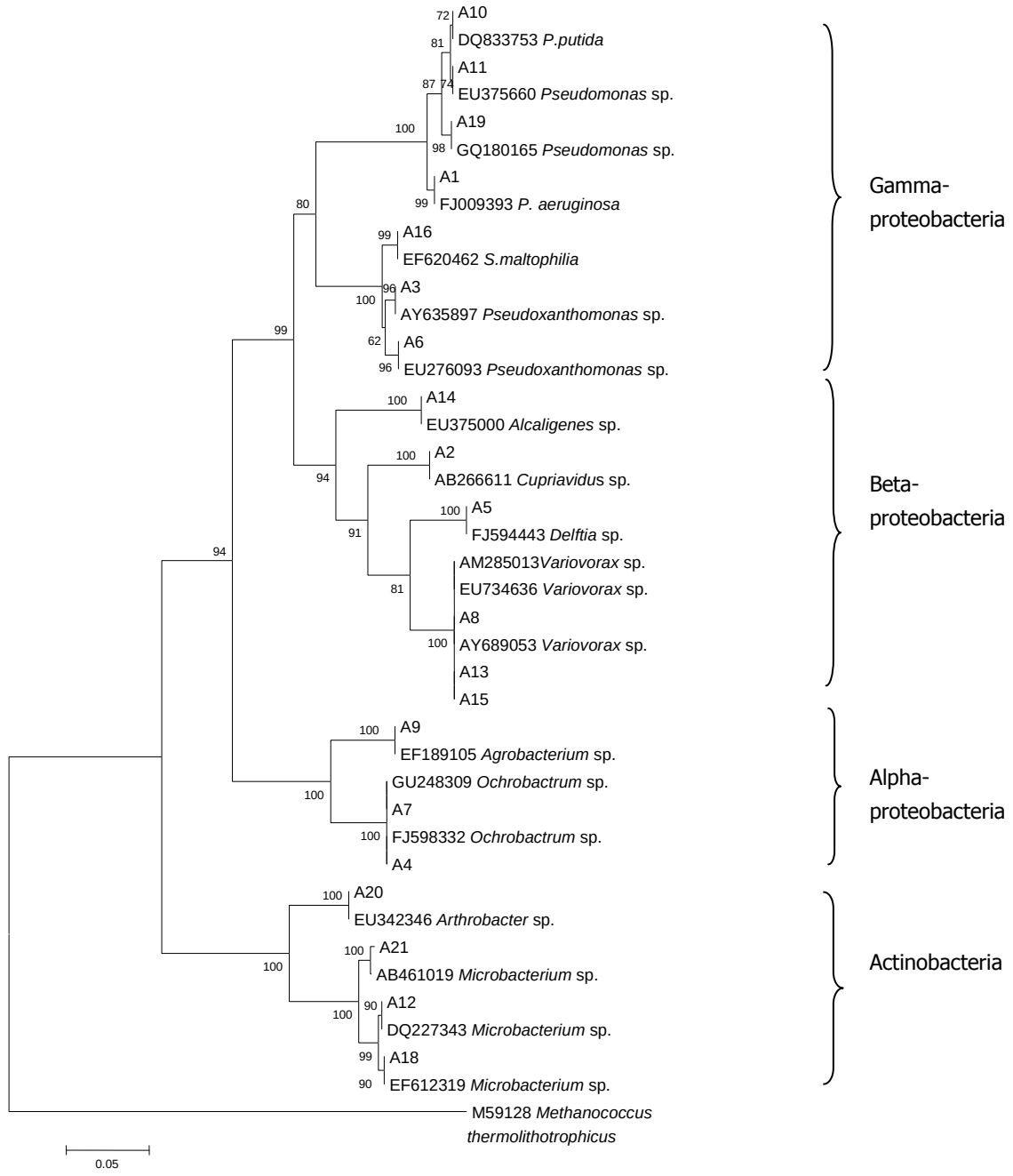



**Fig 3.18:** Phylogenetic neighbour-joining tree of the 16S rRNA gene sequences obtained from Enrichment OTUs ( A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A18, A19, A20, A21) and their closest database relatives with Species Name and GenBank accession numbers. Related species reference strains. Bootstrap values (*n*_1,000) above 50% are indicated at the nodes. The scale bar represents genetic distance (nucleotide substitutions per site).

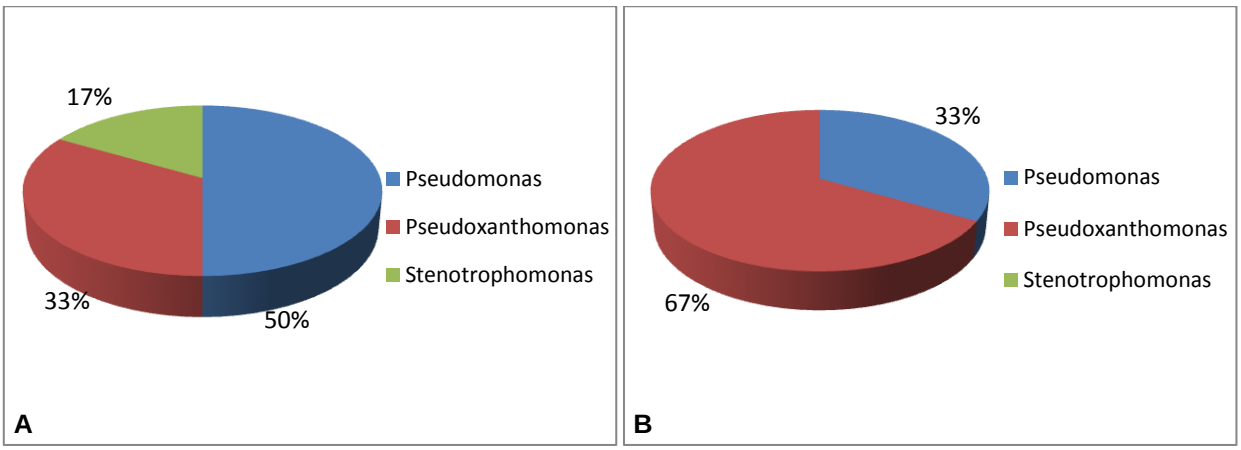



**Fig 3.19**: Taxa distribution of OTUs isolated by Enrichment culture DMY with addition of yeast extract (A) and by Enrichment culture DM with TEL as sole carbon source (B), within Gamma-proteobacteria class

Considering the OTU homologue to the species *Pseudomonas putida* identified in Point II by direct isolation (OTU B), it was also isolated from the same point in the stricter conditions of Enrichment culture DMY (OTU A10), reflecting a high capacity to tolerate heavy metals as reported in literature (Cánovas *et al.*, 2003).

Considering the Alpha-proteobacteria class, a rich biodiversity was observed by direct isolation with 7 different genera detected and 6 from the most polluted point II by itself (par 3.1.2.3), whereas only 2 genera, *Ochrobactrum* and *Agrobacterium* are present among enrichment OTUs, but both selected even in the stricter condition with TEL as sole carbon source (DM) (fig. 3.20). To both genera are in fact reported species and strains with degrading ability towards hydrocarbons or recalcitrant compounds (Effendi *et al.*, 2000; Katsivela *et al.*, 2003).

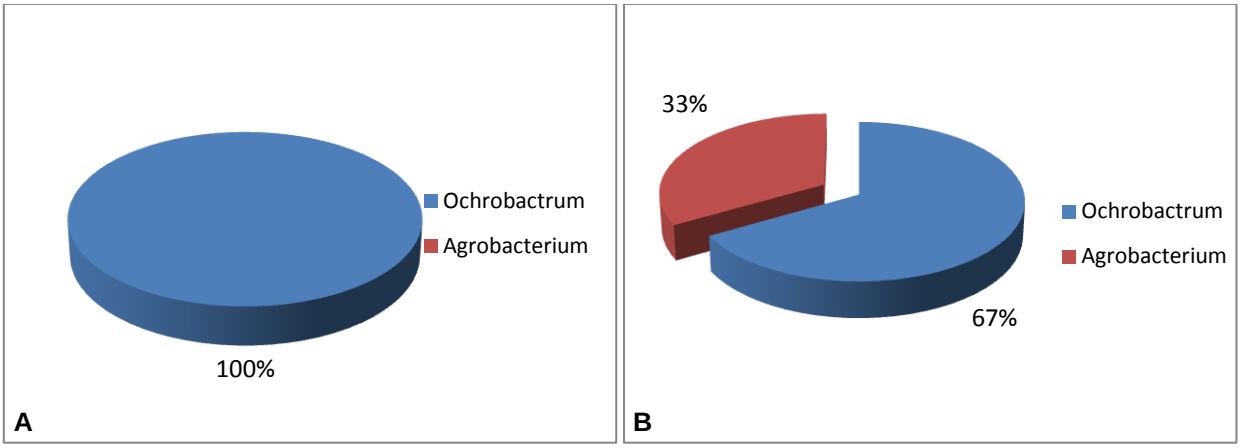



**Fig 3.20**: Taxa distribution of OTUs isolated by Enrichment culture DMY with addiction of yeast extract (A) and by Enrichment culture DM with TEL as sole carbon source (B), within Alpha-proteobacteria class

Considering the Beta-proteobacteria class – the less represented of the proteobactera classes by direct isolation – it is the predominant class within the stricter conditions of TEL as sole carbon source (DM) (Fig 3.17). Actually the Beta class includes almost half of all OTUs thus obtained within 4 different genera: *Cupriavidus, Delftia, Alcaligenes* and *Variovorax* (fig 3.21). The letter genus is the only one exclusively detected by both Enrichment cultures and not in direct isolation.

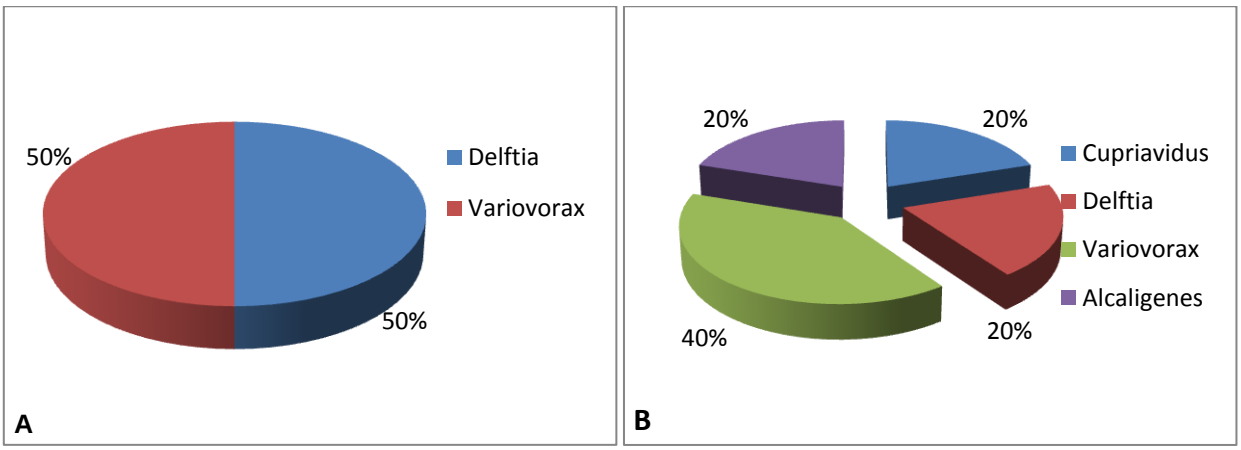



**Fig 3.21**: Taxa distribution of OTUs isolated by Enrichment culture DMY with addiction of yeast extract (A) and by Enrichment culture DM with TEL as sole carbon source (B), within Beta-proteobacteria class

*Variovorax* genus can probably be numerically a minor component of the bacterial community, whose growth and isolation has been probably favored by its resistance to the stricter enrichment selective conditions.

As previously indicated PGPR species belong to *Delftia* and *Alcaligenes* genera (Han *et al.*, 2005; Erturk *et al.*, 2010), and strains of *Alcaligenes* and *Cupriavidus* are known for high heavy metal resistance, degrading capabilities and for bioremediation applications (Periello, 2000; Chen *et al.*, 2008).

Moreover in literature to *Variovorax* genus are reported PGPR species able to promote plant growth in presence of metal contamination, and acting as phytoextraction assistants (Belimov *et al.*, 2005). This genus further includes strains able to grow on phenol as the sole carbon source (Futamata *et al.*, 2005) and characterized by multiple heavy metal resistances (Abou-Shanab *et al.*, 2007), showing therefore high resistant and bioremediation potential.

The data obtained, confirmed by literature studies, suggest therefore a particular high resistance and potential of the Beta-proteobacteria class.

As far as the gram-positive are concerned (Fig. 3.22), the only bacteria detected by enrichment culture were exclusively isolated in concomitant presence of yeast extract in DMY cultures (Fig 3.17) and belong to 2 genera of Actinobacteria: *Microbacterium* - which account for 3 of the 4 OTUs isolated - and *Arthrobacter.* Both these genera were previously detected in the direct isolation from the less contaminated point III (data not shown).

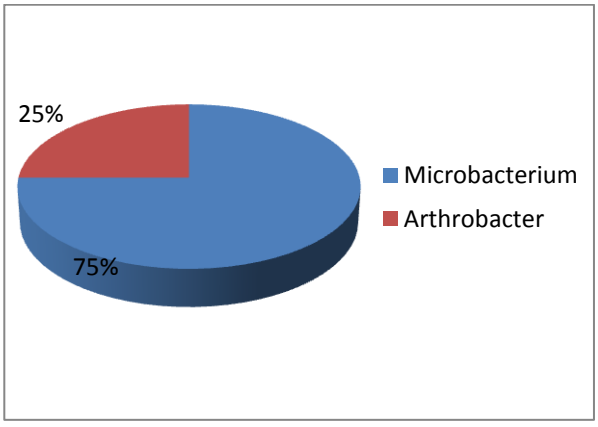



**Fig 3.22:** Taxa distribution of OTUs isolated by Enrichment culture DMY with addiction of yeast extract, within Actinobacteria *phylum*

Resistance and tolerance of *Arthrobacter* – detected by enrichment culture from point I and III - have been demonstrated to a wide variety of heavy metals, including lead (Margesin *et* Schinner, 1996; Benyehuda *et al.*, 2003) and further exploration of the *in-situ* metal reduction potential of this genus has been suggested (Horton *et al.*, 2006). To both *Arthrobacter* and *Microbacterium* strains multiple heavy metal resistances have also been reported (Abou-Shanab *et al.*, 2007).
Moreover for *Microbacteriun* genus – detected through enrichment from all the 3 sampling points – species have been reported acting as PGPR on plants in Pb contaminated soils and as phytoextraction assistants, in fact increase in root elongation of inoculated rape seedlings and in total Pb accumulation has been reported (Sheng *et al.*, 2008b). Therefore even if less represented gram-positive strains are interesting and potential component of the bacterial community even in a bioremediation perspective.

### 3.1.3.2 Taxonomic distribution of Enrichment OTUS between the 3 sampling points

Considering all enrichment OTUs isolated it is possible to notice that members of the 3 proteobacteria classes - highly represented by direct isolation - have been identified from all the 3 sampling points, even if accounting for different percentages in the two enrichment conditions (DMY and DM) performed (Fig 3.23). This suggests that various members of high resistance and degrading potential are associated to this *phylum* and are widespread within the soil under study. In fig 3.23 it is shown the taxonomic distribution between the 3 sampling points of the OTUs isolated in DMY, DM and in both Enrichment cultures.

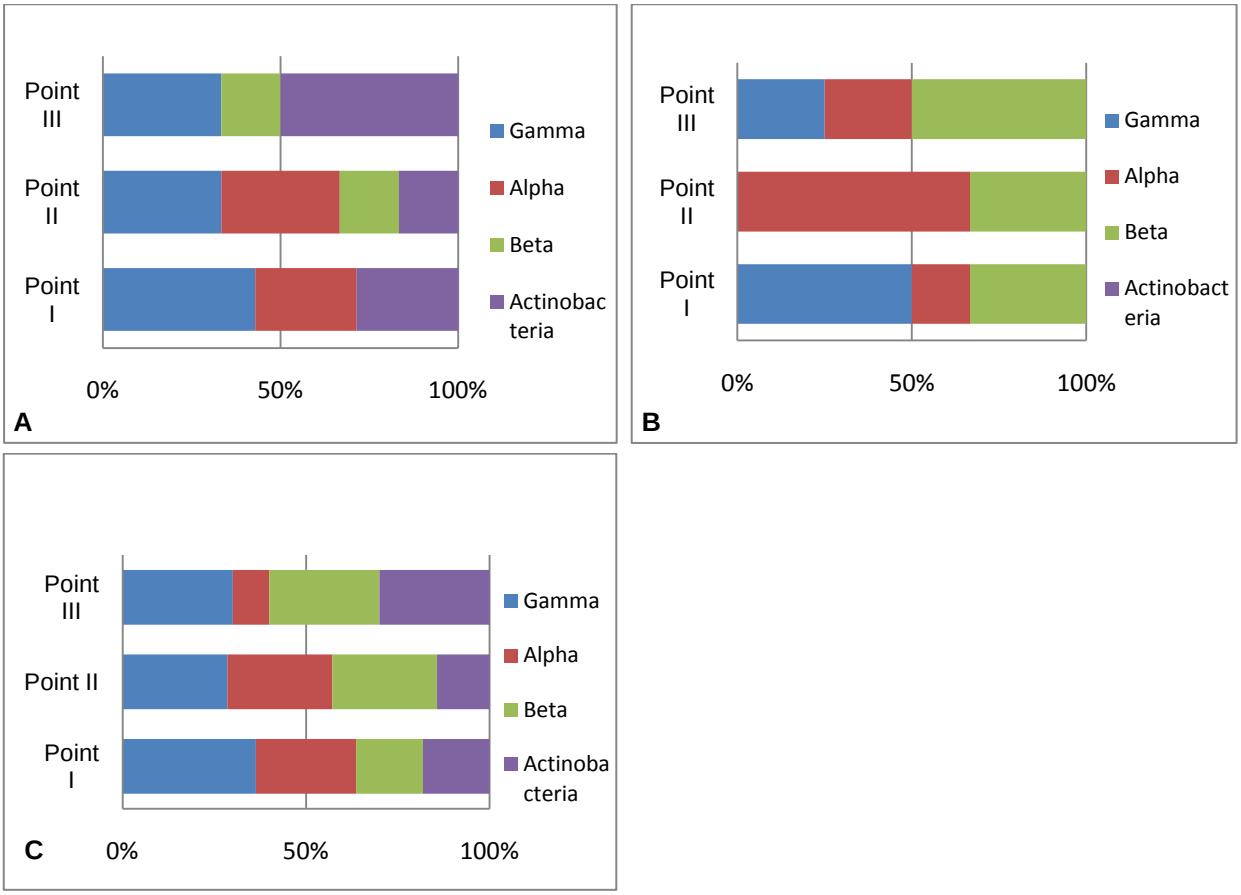



**Fig 3.23** Taxonomic distribution at each sampling point of OTUs isolated by enrichment culture DMY with the addition of yeast extract (A), by enrichment culture DM with TEL as only carbon source (B) and of all OTUs isolated from both Enrichment cultures DMY and DM (C)

Considering the distribution of gram-positive OTUs isolated by enrichment cultures with yeast extract DMY (Fig.3.23A), it is possible to notice a higher presence, accordingly to the direct isolation, of Actinobacteria

at the less contaminated point III, accounting in fact for the 50% of all OTUs isolated in DMY. On the other side Actinobacteria even accounted for the 29% and 17 % of OTUs isolated from point I and from the most contaminated point II. It is worth noting that by direct isolation no gram-positive Actinobacteria was detected at point I and only 2 OTUs - accounting for 8% of isolated OTUs - at point II (par.3.1.2.2). On the other side the low representation of Actinobacteria among the cultivated bacteria directly isolated probably stems from the slow growth of many bacteria of this *phylum* (Hui *et al.*, 2009), and it is also connected to the culturing media Nutrient and R2a for eubacteria growth from which isolation of strains was performed.

These results confirm therefore the hypothesis that to the Actinobacteria class - although less represented within the cultivable fraction of the community - are associated members of high resistance and degrading potential.

### 3.1.4 Characterization of Enrichment strains and of the most represented strains isolated directly from the 3 sampling points within Ex-SLOI area

Cultivation-based and molecular methods were used to characterize the bacteria selected from the 3 sampling points (I,II,III) through the strict conditions of Enrichment cultures and the most represented OTUs from direct isolation, i.e. including at least 4 isolates. Isolate percentages of the examined OTUs from direct isolation are reported in Tab. 3.4. Thus this study targeted both the most represented and numerically dominant OTUs, and the components selected in the strictest conditions of Enrichment. These populations of cultivable bacteria were therefore analyzed in relation to their Pb resistance, degrading capabilities toward organic Pb and PGP characteristics, in order to identify the actual potential of the autochthonous microflora and the OTUs potentially interesting in a bioremediation-phytoremediation perspective.

| **OTU** | **Taxonomic reference ID** | **Phylogenetic group** | **Nutrient %** | **R2a %** |
|---|---|---|---|---|
| **A** | *Stenotrophomonas humi* AM403587 | γ-proteobacteria | 5% | - |
| **C** | *Stenotrophomonas sp.* DQ984206 | γ-proteobacteria | 16% | - |
| **G** | *Alcaligenes sp.* EU37500 | β-proteobacteria | 5% | 2% |
| **AM** | *Pseudomonas sp* EF491969 | γ-proteobacteria | 6% | 3% |
| **AN** | *Pseudomonas chlororaphis* EF620458 | γ-proteobacteria | 7% | 9% |
| **AP** | *Pseudomonas sp.* DQ984204 | γ-proteobacteria | 4,7% | 3% |
| **AO** | *Sinorhizobium sp.* EU571251 | α-proteobacteria | 4% | 1% |
| **AQ** | *Bacillus sp.* EU182839 | Firmicutes | 5% | 8% |
| **RA** | *Stenotrophomonas maltophilia* EU622536 | γ-proteobacteria | - | 8% |

**Tab 3.4** Percentages of isolates from Nutrient and R2a for the most represented OTUs from direct isolation

### 3.1.4.1 MIC (Minimum inhibitory concentration) for organic and inorganic Pb

The minimum inhibitory concentration (MIC) of inorganic and organic Pb - namely respectively the lowest concentration of $Pb(NO_3)_2$ and TEL that prevented strain growth - was determined for all OTUs obtained by Enrichment culture and for the most represented from direct isolation (shown in Tab.3.4).

As reported in tab. 3.6, elevate MIC were obtained, with values of 7-8 mM for $Pb(NO_3)_2$ and 1000 mg/kg (3mM) TEL in almost all OTUs identified by Enrichment cultures. In fact only 2 OTUs of *Pseudomonas* genus (A1 and A10) and 1 of the gram-positive *Microbacterium* (A18) showed MIC value of 5 mM. To the letter genus of the less represented Actinobacteria also belong the OTU A21 showing the lowest MIC of 2mM. On the other side the highest MIC values of 8mM inorganic Pb and >1000mg/kg organic Pb were detected in members of all the 3 Proteobacteria classes identified, but even in one Actinobacteria OTU of the *Arthrobacter* genus (OTU A20), confirming a possible high resistance - even if less widespread - within the gram-positive Actinobacteria *phylum*.

Moreover the above-mentioned OTUs A10 and A21, together with the *Pseudoxanthomonas* OTU A3, were the only ones from Enrichment cultures not to produce a brown colour on agar plates supplemented with inorganic Pb, connected to Pb precipitation in a sequestration mechanism. In fact a variety of bacteria precipitate lead, most typically as a lead phosphate in the few cases in which the compound has been examined and, depending on the strain and the growth conditions, the precipitate can either accumulate at the cell membranes or be expelled conferring this brown colour (Mire *et al.*, 2004).

On the other hand only half of the OTUs analyzed by direct isolation showed this precipitate and this seems not to correlate with the MIC value registered, suggesting the presence of various resistance mechanisms (Tab 3.5).

Brown precipitate formation has been detected by plate cultures of several different eubacteria, suggesting that Pb precipitation is a common, albeit not universal, property of bacteria. It is worth noting that both lead-sensitive and lead-resistant bacteria were reported to precipitate lead as a brown compound and it has been raised the issue of whether lead precipitation confers lead resistance or is simply a chemical consequence of high intracellular lead concentrations (Mire *et al.*, 2004).

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).

Although as expected a general lightly lower resistance was detected in the analyzed OTUs by direct isolation (tab.3.5), their results show an overall good resistance to lead, with MIC ranging from 500 mg/kg to >1000 mg/kg TEL - the last value registered in one OTUs of *Stenotrophomonas* genus. Besides they showed MIC for $Pb(NO_3)_2$ >5 mM, higher than the values reported in literature for lead-resistant strains (Pacheco *et al.*, 1995; Konopka *et* Zakharova, 1999).

It has although to be mentioned that the chemical interaction with medium components such as hydroxyl ions and organic components in the agar and, in the case of TEL the hydrophobicity and subsequent low solubility in aqueous solutions, may overestimate the resistance level of the microbes (Konopka *et* Zakharova, 1999).

Nevertheless the results obtained point out the selection in the soil of the Ex-SLOI area towards a resistant microbial community, with members of particular high resistance belonging to the most represented gram-negative Proteobacteria classes but even to the gram-positive Actinobacteria *taxa* identified.

| OTU | Taxonomic reference ID | Phylogenetic group | MIC TEL mg/kg | MIC Inorg.Pb mM | Precipitate formation |
|---|---|---|---|---|---|
| **A** | *Stenotrophomonas humi* AM403587 | γ-proteobacteria | 700 | 5 | yes |
| **C** | *Stenotrophomonas sp.* DQ984206 | γ-proteobacteria | 700 | 7 | yes |
| **G** | *Alcaligenes sp.* EU37500 | β-proteobacteria | 700 | 7 | yes |
| **AM** | *Pseudomonas sp* EF491969. | γ-proteobacteria | 700 | 7 | No |
| **AN** | *Pseudomonas chlororaphis* EF620458 | γ-proteobacteria | 700 | 7 | No |
| **AP** | *Pseudomonas sp.* DQ984204 | γ-proteobacteria | 500 | 7 | No |
| **AO** | *Sinorhizobium sp.* EU571251 | α-proteobacteria | 500 | 6 | yes |
| **AQ** | *Bacillus sp.* EU182839 | Firmicutes | 700 | 5 | No |
| **RA** | *Stenotrophomonas maltophilia* EU622536 | γ-proteobacteria | >1000 | 5 | yes |

**Tab 3.5** Mic values for the most represented OTUs from direct isolation

| OTU | Enrichm. culture | Taxonomic reference ID | Phylog. group | MIC TEL mg/kg | MIC In. Pb mM | Precipitate formation |
|---|---|---|---|---|---|---|
| **A1** | DM | *Pseudomonas aeruginosa* FJ009393 | γ-proteobacteria - | >1000 | 5 | yes |
| **A2** | DM | *Cupriavidus sp.* AB266611 | β-proteobacteria | >1000 | 7 | yes |
| **A3** | DM-DMY | *Pseudoxanthomonas sp.* AY635897 | γ-proteobacteria | >1000 | 7 | no |
| **A4** | DM-DMY | *Ochrobactrum sp.* GU248309 | α-proteobacteria | >1000 | 8 | yes |
| **A5** | DM-DMY | *Delftia sp* FJ594443 | β-proteobacteria | >1000 | 8 | yes |
| **A6** | DM-DMY | *Pseudoxanthomonas sp.* EU276093 | γ-proteobacteria | >1000 | 6 | yes |
| **A7** | DM-DMY | *Ochrobactrum sp.* FJ598332 | α-proteobacteria | >1000 | 8 | yes |
| **A8** | DM | *Variovorax sp* EU734636 | β-proteobacteria | >1000 | 8 | yes |
| **A9** | DM | *Agrobacterium sp.* EF189105 | α-proteobacteria | >1000 | 7 | yes |
| **A10** | DMY | *Pseudomona putida* DQ833753 | γ-proteobacteria | >1000 | 5 | no |
| **A11** | DMY | *Pseudomonas sp. EU375660* | γ-proteobacteria | >1000 | 7 | yes |
| **A12** | DMY | *Microbacterium sp.* DQ227343 | Actinobacteria | >1000 | 7 | yes |
| **A13** | DMY | *Variovorax sp.* AM285013 | β-proteobacteria | >1000 | 7 | yes |
| **A14** | DM | *Alcaligenes sp.* EU37500 | β-proteobacteria | >1000 | 7 | yes |

| | | | | | | |
|---|---|---|---|---|---|---|
| **A15** | DM | *Variovorax sp* AY689053 | β-proteobacteria | >1000 | 8 | yes |
| **A16** | DMY | *Stenotrophomonas maltophilia* EF620462 | γ-proteobacteria | >1000 | 8 | yes |
| **A18** | DMY | *Microbacterium sp.* EF612319 | Actinobacteria | >1000 | 5 | yes |
| **A19** | DMY | *Pseudomonas sp.* GQ180165 | γ-proteobacteria | >1000 | 7 | yes |
| **A20** | DMY | *Arthrobacter sp.* EU342346 | Actinobacteria | >1000 | 8 | yes |
| **A21** | DMY | *Microbacterium sp.* AB461019 | Actinobacteria | >1000 | 2 | no |

**Tab 3.6** MIC values for the Enrichment OTUs

### 3.1.4.2 Primers-specific PCR for Heavy metal resistances and Hydrocarbon degradation determinants

A molecular study in relation to Pb and heavy metal resistances and to degrading capabilities of hydrocarbons has been performed to evaluate the resistance and degrading potential within the indigenous microflora.

This study was performed by Primer-specific PCR amplifications on total genomic DNA, targeting resistance determinants for Pb and other heavy metals – resistances which are often associated on plasmids or transposons - and degrading capabilities towards hydrocarbons: polycyclic aromatic hydrocarbons (PAH) - components of petrol - and aliphatic hydrocarbon. Petrol spills were in fact reported in the Ex-SLOI area, and a degrading capability of linear aliphatic hydrocarbon could be connected to a degradation potential of alkyl groups in organic lead.

The heavy metal determinants in study were *pbr, czc, chr, ncc* and *mer* genes respectively responsible for resistance to Pb, Cd-Zn-Co, Cr, Ni and Hg (par2.6) (Borremans *et al.*, 2001; Abou-Shanab *et al.*, 2007).

To study the hydrocarbon degrading potential within the examined OTUs, the analysis was performed targeting the genes encoding the large (α) subunits of PAH dioxygenases in *nah*-like and *phn* catabolic operons for the degradation of PAH, and encoding the alkane hydroxylases involved in the first step of the aliphatic alkanes' degradation (par 2.6) (Smits *et al.*, 1999; Laurie *et* LLoyd-Jones, 2000).

After visualization by gel electrophoresis, the PCR amplicons of the expected size (par 2.6, tab 2.2 and 2.4) were chosen for sequence analysis. The results thus obtained showed no detection of any targeted determinant in OTUs by direct isolation.

Considering Enrichment cultures, as far as the heavy metals resistances are concerned, in 5 OTUs of the Enrichments microflora the resistance to Hg was detected and at the same time in 3 of them the resistance to Pb, while in one OTU the concomitant detection of Ni resistance was obtained too (Tab 3.7). In this context it has to be reminded that mercury contamination was detected in the sampling point III located next to $C_2H_5Cl$ production system, which implied Hg in its processes.

Visualization in agarose gel electrophoresis of amplicons of the expected size, which was subsequently confirmed by sequencing, for *pbr, mer* and *ncc loci* is evidenced in Fig 3.24, 3.25, 3.26.

Of these 5 OTUs in particular 3 - respectively 1 *Stenotrophomonas maltophilia* and 2 strains of *Pseudomonas* genus - belong to Gamma-proteobacteria: the class predominantly identified in the strains directly isolated from soil and in the enrichments DMY performed with the addition of another carbon source. As previously described to *Pseudomonas* and *Stenotrophomonas* genera belong species resistant to heavy metals, able to degrade aromatic and aliphatic compounds and PGPR - able therefore to promote plant growth - interesting in a bioremediation/phytoremediation approach (Bloemberg *et* Lugtenberg, 2001; Samanta *et al.*, 2002; Compant *et al.*, 2005).

In particular the OTU characterized by the concomitant presence of all the three resistance detected belong to the *Stenotrophomonas maltophilia* species. Strains of *Stenotrophomonas maltophilia* have moreover an extraordinary range of activities that include beneficial effects for plant growth and health, the breakdown of natural and man-made pollutants that are central to bioremediation and phytoremediation strategies (Ryan *et al.*, 2009). The 2 other OTUs which display both resistances to heavy Pb and Hg belong to *Delftia* and *Cupriavidus* genera of the *Beta-proteobacteria,* the predominant class among the Enrichment OTUs selected in presence of TEL as the sole carbon source. This proteobacteria class is interesting even in relation to a phytoremediation approach, genus *Delftia* in fact includes PGPR species and a strain of *Cupriavidus* was reported in literature to enhance heavy metals uptake in the host plant (Chen *et al.*, 2008; Jing *et al.*, 2007).

Considering the targeted genetic determinants for the degradation of hydrocarbons, as previously reported for strains by direct isolation no one was detected in any member of the enrichment cultures, neither for PHA nor for n-alkane degradation. However this result can be connected to the presence in the examined strains of sequences differing from the primers used and in any case it does not exclude the existence of different catabolic genotypes than the targeted ones.

The lack of success in amplification of hydrocarbon degrading genes or a low detection of heavy metals resistances can underestimate the actual microflora potential due to the limited number of determinants examined, and in connection to biases implied in the molecular study performed, or to the existence of alternative resistance or tolerance mechanisms used to cope with the contamination (Cavalca *et al.*, 2010). Hence a further study targeting distinct determinants would be interesting to better explore the degrading and resistance potential of the examined strains.

Despite this, it is interesting to notice that the determinants for heavy metal resistances have been detected in strains originally isolated from all the 3 sampling points under study, suggesting the spreading and overall distribution within the Ex-SLOI area of an indigenous microbial community of rich biodiversity and including members of higher resistance potential, mainly associated to the most represented phylogenetic classes of gram-negative Proteobacteria. In this context the horizontal transmission of plasmids with metal resistance determinants could be a mechanism involved in the expansion from a small number of metal-resistant strains, possibly present in the soil when it was originally contaminated over 50 years ago, towards a higher biodiversity here detected in the indigenous community (Becker *et al.*, 2006).

| OTU | Enrichm. culture | Sampling point | Taxonomic Reference ID | Phylogeny Group | Pb | Hg | Cd Zn Co | Cr | Ni |
|---|---|---|---|---|---|---|---|---|---|
| **A1** | DM | I,III | *Pseudomonas aeruginosa FJ009393* | γ-Proteobacteria | - | + | - | - | - |
| **A2** | DM | II,III | *Cupriavidus* sp. AB266611 | β-Proteobacteria | + | + | - | - | - |
| **A5** | DM-DMY | I,II | *Delftia* sp. FJ594443 | β-Proteobacteria | + | + | - | - | - |
| **A10** | DMY | II | *Pseudomonas putida* DQ833753 | γ-Proteobacteria | - | + | - | - | - |
| **A16** | DMY | II | *Stenotrophomonas maltophilia* EF620462 | γ-Proteobacteria | + | + | - | - | + |

**Tab 3.7** Examined OTUs reporting heavy metal resistances in the molecular study performed

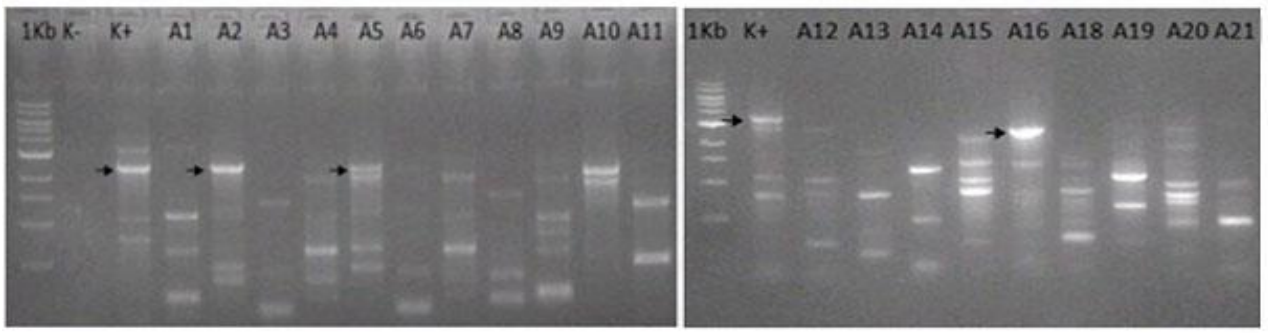



**Fig 3.24** Agarose gel electrophoresis of *pbr* PCR products. Exact lenght of amplified region:2300 bp. Lanes: 1Kb DNA size marker; K- negative control, K+ *Cupriavidus metallidurans* CH34

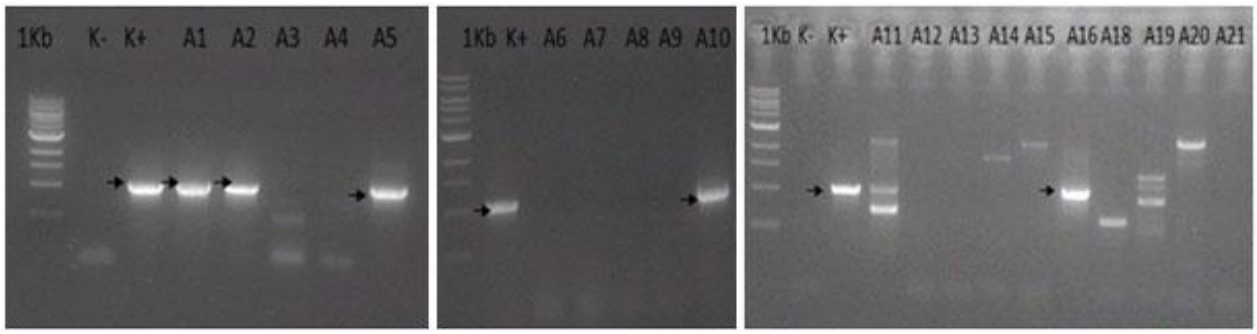



**Fig 3.25** Agarose gel electrophoresis of *mer* PCR products. Exact lenght of amplified region:1011 bp Lanes: 1Kb DNAsize marker; K- negative control, K+ *Cupriavidus metallidurans* CH34

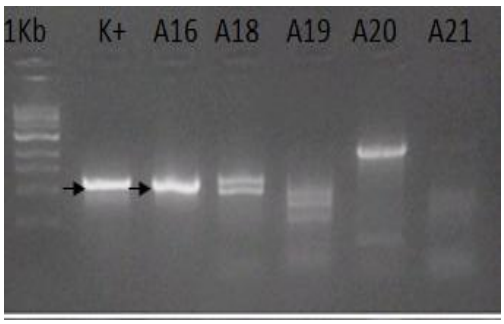



**Fig 3.26** Agarose gel electrophoresis of *ncc* PCR products. Exact lenght of amplified region:1141 bp.Lanes: 1Kb DNAsize marker; K- negative control, K+ *Cupriavidus metallidurans* CH34

## 3.1.5 Direct and Enrichment isolation of bacterial strains from a Hot Spot identified within Ex-SLOI area

In this study, focused on the soil indigenous micro flora, has been included the analysis of the Hot spot – the most contaminated spot identified within the Ex-SLOI area - to better characterize and evaluate the biodiversity, structure and potential associated to the autochthonous micro flora selected by decades of Pb contamination within the area.

The micro flora established at this spot is in fact of particular interest because of the extremely high concentration of organic and inorganic Pb imposed - one order of magnitude higher than the other 3 sampling points - with values >1000 mg/kg for organic Pb and reaching the maximum concentration detected in the whole Ex-SLOI area of 23.000 mg/kg for Inorganic Pb.

Members of the bacterial community were therefore isolated by direct isolation as for the 3 other 3 sampling points from Nutrient and R2a media, while the Enrichment cultures where performed in the strictest condition with 125 mg/L TEL as sole carbon source, to select the most resistant and metabolically interesting strains.

### 3.1.5.1 Enumeration of culturable bacterial strains at the Hot spot

Enumeration of total heterotrophic aerobic Eubacteria was performed by using Nutrient and R2a media while in the meantime Actinobacteria were enumerated with Waksman medium and Fungi with Malt medium. As reported in Fig. 3.27, data obtained showed a $Log_{10}$ value of almost 5 for heterotrophic aerobic Eubacteria, while lightly lower values for both Fungi and Actinobacteria populations were detected. The last gram-positive Actinobacteria and Fungi community components showed almost equal count values.

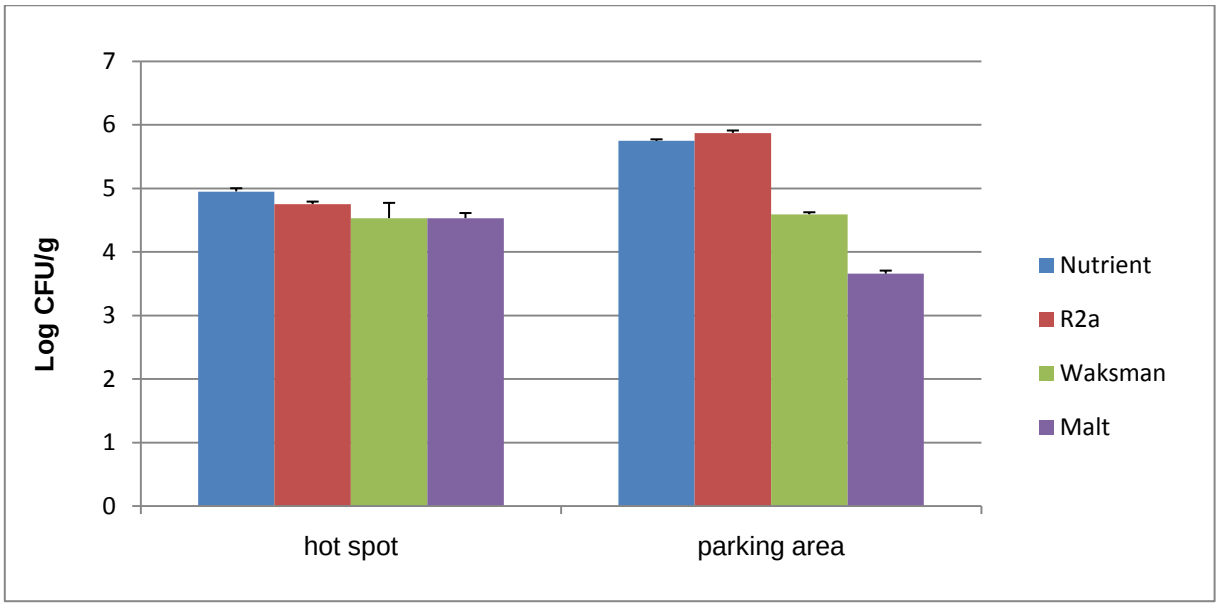



**Fig 3.27:** Culturable counts at the hot spot

In comparison to the soil from the 3 sampling points and of the parking area used as control - as previously described in fig 3.1 in par 3.1.1 - in the Hot spot a lower eubacterial population and in the meantime a higher Fungi component were detected. As far as the Actinobacteria are concerned, a value similar to the control was registered, while in comparison to the 3 sampling points, both gram-positive Actinobacteria and Fungi showed a much higher detection.

From these first data a lower eubacterial charge is observed in the Hot spot which reflects the higher contamination level, on the other side a lower antagonistic action was probably exerted by the eubacterial population on the gram-positive components, which showed a much higher charge in comparison to the other 3 samplings points. The value of gram-positive microorganisms detected in the selective conditions of the Hot spot hence indicates a high resistance to the contamination also within these generally less represented bacteria.

### 3.1.5.2 Phylogenetic analysis of OTUs isolated from the Hot spot identified within the Ex-SLOI area

As previously described for soil samples of the 3 sampling points in analysis, axenic cultures of morphologically different bacteria were obtained by direct isolation on Nutrient and R2a media, and screened by the molecular technique ARDRA. For the enrichment culture, after 8 weeks of incubation in

DM with TEL as sole carbon source the isolation of resistant microbial species was obtained by plating serial dilutions on agar plates of the same selective medium.
From the 23 and 12 isolates obtained by direct isolation and Enrichment culture, respectively 11 and 3 OTUs were detected and taxonomically identified by sequencing of 16S rDNA (tab 3.8,3.9).

| OTU | Taxonomic Reference ID | Phylogeny Group | Homology% |
|---|---|---|---|
| **Isol1** | *Cupriavidus sp.* EU65287 | β-proteobacteria | 100% |
| **Isol2** | *Ralstonia* sp. AB299603 | β-proteobacteria | 99% |
| **Isol3** | *Microbacterium testaceum* AF474330 | Actinobacteria | 99% |
| **Isol4** | *Ralstonia sp. FJ535673* | β-proteobacteria | 99% |
| **Isol5** | *Stenotrophomonas maltophilia* AF390080 | γ-proteobacteria | 100% |
| **Isol6** | *Arthrobacter oxydans* HQ331125 | Actinobacteria | 100% |
| **Isol7** | *Cupriavidus campinensis* NR025137 | β-proteobacteria | 100% |
| **Isol8** | *Arthrobacter* sp. HM151732 | Actinobacteria | 100% |
| **Isol9** | *Ralstonia* sp. AB088545 | β-proteobacteria | 99% |
| **Isol10** | *Arthrobacter sp., DQ158001* | Actinobacteria | 99% |
| **Isol11** | *Pseudomonas putida* AY918068 | γ-proteobacteria | 100% |

**Table 3.8**: Taxonomic collocation of OTUs directly isolated from Hot spot

| OTU | Enrichm. culture | Taxonomic Reference ID | Phylogeny Group | Homology% |
|---|---|---|---|---|
| **HA1** | DM | *Pseudomonas sp.* FJ791165 | γ-proteobacteria | 100% |
| **HA2** | DM | *Stenotrophomonas maltophilia* FJ405363 | γ-proteobacteria | 100% |
| **HA3** | DM | *Rhizobium sp.* HM008950 | *α-proteobacteria* | 100% |

**Table 3.9**: Taxonomic collocation of enrichment OTUs from Hot spot

It is interesting to notice (as shown in the phylogenetic tree Fig 3.29) even at the Hot spot in analysis the prevalence - previously detected at the other 3 sampling points - of the gram-negative Proteobacteria, which here respectively include the 64% of OTUs isolated by direct isolation and the totality of enrichment OTUs obteined in presence of TEL as only carbon source (Fig 3.28), just as observed for the DM enrichment cultures from the other 3 sampling points (par 3.1.3.1).

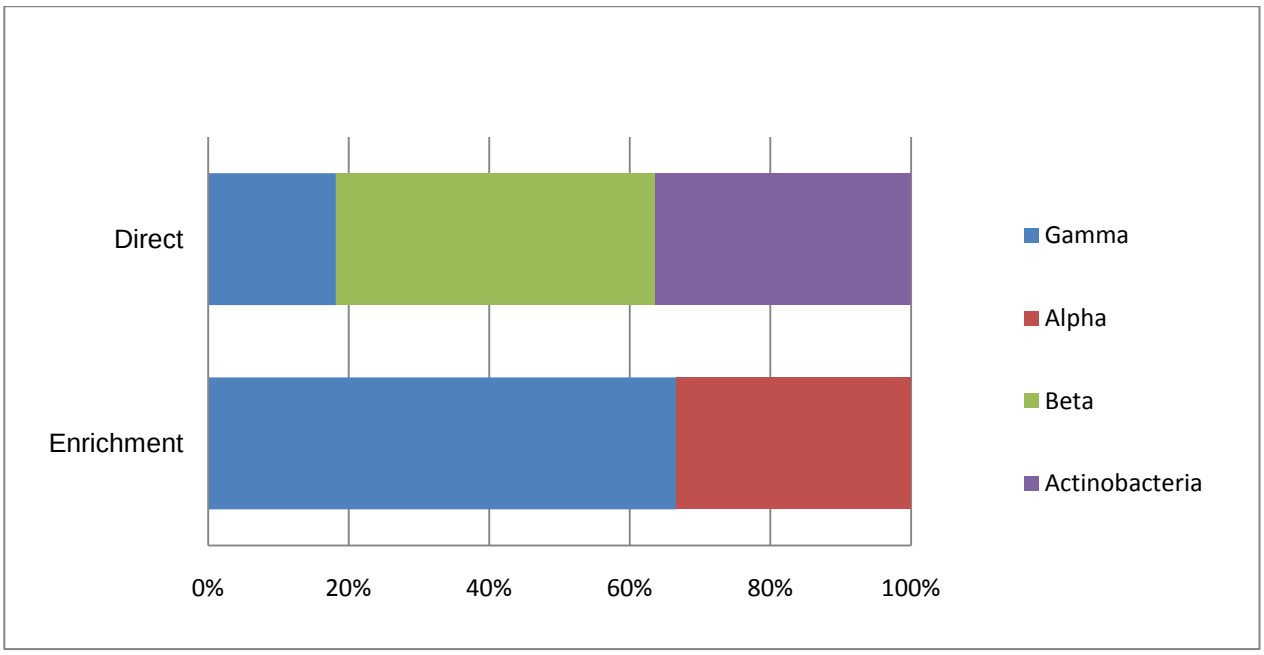



**Fig 3.28**: Taxa distribution of Hot spot OTUs isolated by direct isolation and enrichment culture

Considering the OTUs directly isolated from the Hot spot, the class of Gamma-proteobacteria - mostly represented both in the direct isolation and in DMY enrichment culture from the 3 sampling points - accounts only for 2 of the 11 OTUs isolated, including the 2 genera most detected by direct isolation at the tree sampling point, namely *Pseudomonas* and S*tenotrophomonas.*

On the other side the 45% of OTUs relate to the Beta-proteobacteria class, the most represented class in DM enrichments from the 3 sampling points, with 2 OTUs belonging to *Cupriavidus* and 3 to *Ralstonia* genera. No member of the Alpha class was detected by direct isolation, while the 36% of OTUs are Actinobacteria. Considering this gram-positive *phylum*, one of the isolated OTUs belongs to *Microbacterium* and 3 to *Arthrobacter,* genera both detected in direct isolation and in the Enrichment cultures with yeast extract performed from the other 3 sampling points (par 3.1.2, 3.1.3). No *Firmicutes* were isolated from Hot spot, neither directly nor by enrichment culture.

Considering the Enrichment OTUs isolated with TEL as only carbon source, 2 of the 3 OTUs obtained belong to the Gamma-proteobacteria class, in particular to the *Pseudomonas* genus and *Stenotrophomonas malthophila* species, while one relates to the genus *Rhizobium* of the Alpha-proteobacteria, class here not detected by direct isolation.

These results therefore confirm a high potential within the three isolated class of Proteobacteria, which account for the majority of culturable bacterial community established not only at the three sampling points, but even at the most contaminated hot spot identified within the Ex-SLOI area and characterized by the highest contamination values registered within the area. Although these results can be also connected to a higher culturability and to the media chosen for the isolation of strains, the Proteobacteria *phylum* also accounted for the totality of OTUs isolated in presence of TEL as only carbon source from all soil samplings in examination, suggesting a high resistance and metabolic potential towards organic lead associated to this *taxum*. It is although worth noting a high resistance within the lower represented gram-positive *phylum* of Actinobacteria, isolated not only directly and in the enrichment with yeast extract by the three sampling points, but by direct isolation even in the strict conditions of this Hot Spot.

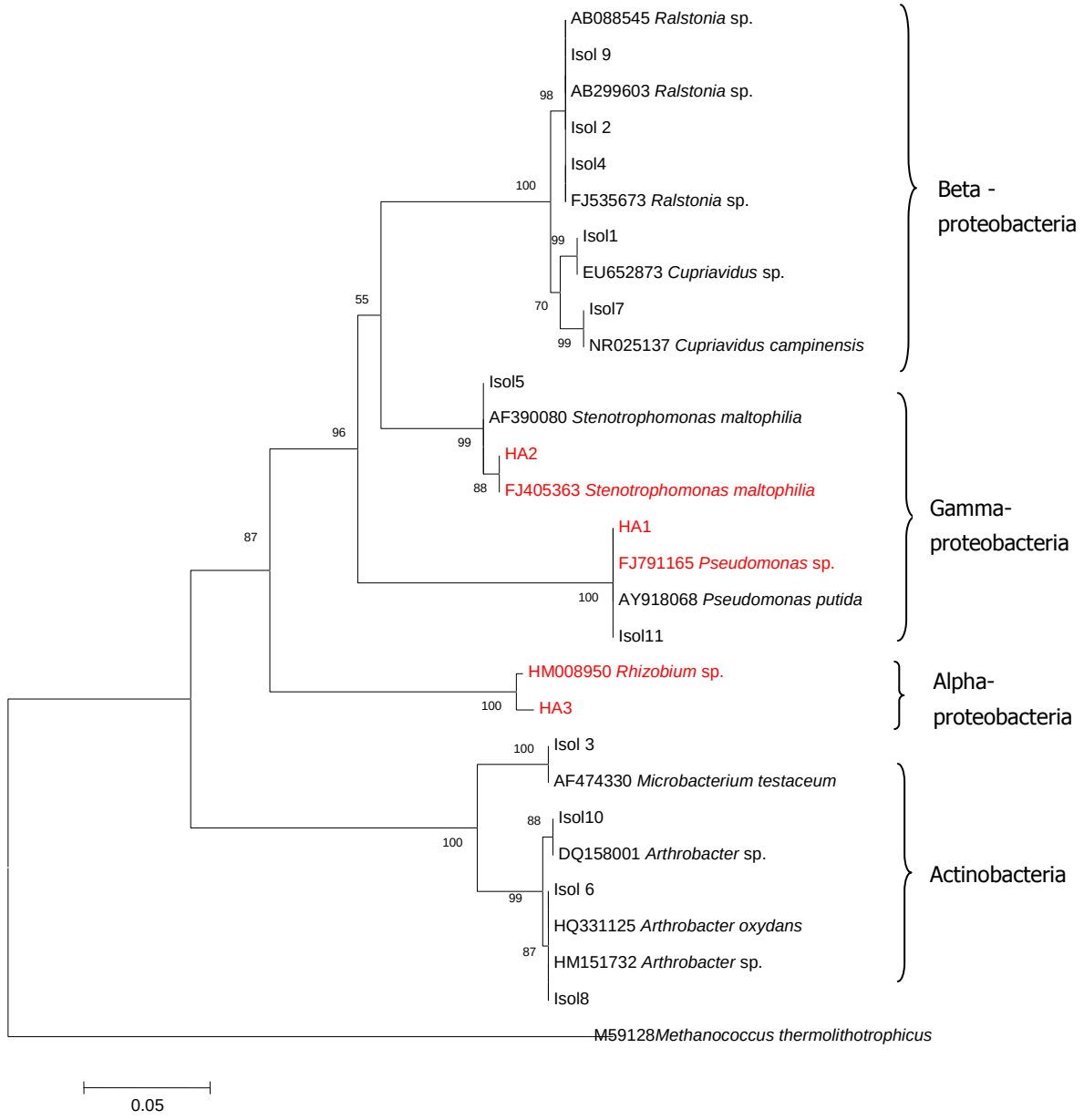



**Fig 3.29:** Phylogenetic neighbour-joining tree of the 16S rRNA gene sequences obtained from OTUs directly isolated (Isol1, Isol 2, Isol 3, Isol 4, Isol 5, Isol 6, Isol7, Isol8, Isol9, Isol10, Isol11) and Enrichment OTUs (HA1,HA2,HA3) - highlighted in red colour - and their closest database relatives 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).

### 3.1.5.3 MIC (Minimum inhibitory concentration) for organic and inorganic Pb of Hot spot OTUs

The minimum inhibitory concentration (MIC) of inorganic $Pb(NO_3)_2$ and organic Pb (TEL) was determined for all OTUs isolated from the Hot spot, obtained by both direct isolation and through the Enrichment culture with TEL as sole carbon source. As reported in tab. 3.10, elevate MIC were obtained with values >7 mM $Pb(NO_3)_2$ and >1000 mg/kg (=3mM) TEL in the majority of OTUs identified.

Only 2 OTUs - Isol 5 of *Stenotrophomonas* genus and the only Alpha member (HA3) of *Rhizobium* genus selected by Enrichment culture - showed in fact MIC value of 5 mM for inorganic Pb, but in the meantime MIC for organic Pb >1000 mg/kg, thus suggesting the presence of multiple and independent resistance-detoxification mechanisms. It has to be mentioned, as previously indicated in par. 3.1.4.1, that the lowest

MIC value here registered of 5 mM are higher than MIC values reported in literature for Pb-resistant strains, thus pointing out the high resistance associated to the autochthonous microbial community selected within the Ex-SLOI area (Pacheco *et al*., 1995; Konopka *et* Zakharova, 1999).

Only 3 OTUs, belonging to the Proteobacteria Alpha and Beta classes and to the gram-positive Actinobacteria, did not produce the brown precipitate on agar plates with inorganic Pb which attests Pb precipitation. However, as noted for OTUs from the 3 sampling points, this seems not to correlate with the MIC value registered, suggesting the concomitant presence of various resistance mechanisms.

| OTU | Isolation | Taxonomic reference ID | Phylogenetic group | MIC TEL mg/kg | MIC inorg. Pb mM | Precipitate formation |
|---|---|---|---|---|---|---|
| **Isol1** | direct | *Cupriavidus sp.* EU65287 | β-proteobacteria | 800 | 7 | yes |
| **Isol2** | direct | *Ralstonia* sp. AB299603 | β-proteobacteria | 1000 | 8 | yes |
| **Isol3** | direct | *Microbacterium testaceum* AF474330 | Actinobacteria | 500 | 7 | yes |
| **Isol4** | direct | *Ralstonia sp.* FJ535673 | β-proteobacteria | 800 | 10 | yes |
| **Isol5** | direct | *Stenotrophomonas maltophilia* AF390080 | γ-proteobacteria | >1000 | 5 | yes |
| **Isol6** | direct | *Arthrobacter oxydans* HQ331125 | Actinobacteria | 700 | 7 | no |
| **Isol7** | direct | *Cupriavidus campinensis* NR025137 | β-proteobacteria | >1000 | 10 | yes |
| **Isol8** | direct | *Arthrobacter* sp. HM151732 | Actinobacteria | >1000 | 7 | yes |
| **Isol9** | direct | *Ralstonia* sp. AB088545 | β-proteobacteria | >1000 | 7 | no |
| **Isol10** | direct | *Arthrobacter sp. DQ158001* | Actinobacteria | >1000 | 8 | yes |
| **Isol11** | direct | *Pseudomonas putida* AY918068 | γ-proteobacteria | >1000 | 7 | yes |
| **HA1** | DM | *Pseudomonas sp. FJ791165* | γ-proteobacteria | >1000 | >10 | yes |
| **HA2** | DM | *Stenotrophomonas maltophilia FJ405363* | γ-proteobacteria | >1000 | 7 | yes |
| **HA3** | DM | *Rhizobium sp. HM008950* | α-proteobacteria | >1000 | 5 | no |

**Tab 3.10** MIC values for the Hot spot OTUs by direct isolation and Enrichment culture DM, with TEL as only carbon source

In particular 3 OTUs - the directly isolated Isol4 and isol7 of Beta-proteobacteria and HA1 of *Pseudomonas* genus selected by Enrichment culture - showed the highest MIC for inorganic Pb registered in all OTUs analyzed in this study, with values of 10mM.

The gram-positive OTU Isol10 of the genus *Arthrobacter* showed MIC of 8mM inorganic Pb and >1000 mg/kg TEL, denoting a high resistance and, together with the *Artrobacter* OTU 20 isolated by enrichment from the 3 sampling points (par 3.1.4.1), has the highest MIC values obtained for the analysed gram-positive members of the Actinobacteria *phylum.*

### 3.1.5.4 Primers-specific PCR for Heavy metal resistances and Hydrocarbon degradation determinants for Hot spot OTUs

After the determination of MIC values, all the 3 OTUs isolated by TEL enrichment and 3 from the 11 OTUs by direct isolation showing the highest MIC values - namely Isol4, Isol7 and Isol10 – were chosen to be characterized in relation to Pb and heavy metal resistances and hydrocarbon degradation potential by PCR analysis.

| OTU | Isolation | Taxonomic Reference ID | Phylogeny Group | *pbr* | *mer* | *czc* | *chr* | *ncc* |
|---|---|---|---|---|---|---|---|---|
| **Isol4** | direct | *Ralstonia sp.* FJ535673 | β-proteobacteria | + | + | - | - | - |
| **Isol7** | direct | *Cupriavidus campinensis* NR025137 | β-proteobacteria | + | + | + | + | - |
| **Isol10** | direct | *Arthrobacter sp.* DQ158001 | *Actnibacteria* | - | - | - | - | - |
| **HA1** | DM | *Pseudomonas sp.* FJ791165 | γ-proteobacteria | - | + | - | - | - |
| **HA2** | DM | *Stenotrophomonas maltophilia* FJ405363 | γ-proteobacteria | - | - | - | - | - |
| **HA3** | DM | *Rhizobium sp.* HM008950 | α-proteobacteria | - | - | - | - | - |

**Tab 3.11** Results of the molecular analysis for heavy metal resistance determinants on Hot spot OTUs – isolated by direct isolation and Enrichment culture DM, with TEL as only carbon source.

Even for these strains no determinants associated to hydrocarbon degradation were detected.

As in the molecular study for the analyzed OTUs from the 3 sampling points, the determinants for Hg resistance was the most represented, detected in 3 OTUs: 2 from direct isolation - belonging to the *Cupriavidus* and *Ralstonia* genera of the Beta Class - and 1 from TEL Enrichment culture belonging to the *Pseudomonas* genus. At the same time in the 2 OTUs of the Beta-proteobacteria the determinant for Pb-resistance was also detected.

For the first time in the study so far performed it is interesting to notice, in the OTUs Isol 7 *Cupriavidus campinensis,* the detection of both determinants for Cr (*chr*) and Cd-Zn-Co (*czc*) resistances, never previously detected in this study. This OTU results therefore of particular resistance and interest, to this genus in fact belong *Cupriavidus metallidurans* CH34*,* famous and studied for its multiple heavy metal resistence (Borremans *et al*., 2001).

Visualization in agarose gel electrophoresis of amplicons of the expected size for the presence of the *pbr*, *mer, czc* and *chr* loci, which were subsequently confirmed by sequencing, is evidenced in Fig 3.30-3.33.

Thus the data obtained confirmed the expected presence of Pb resistance determinant and the previously noticed higher detection of Hg resistance within the analyzed community members. On the other side the results obtained for the Hot Spot, as for the examined OTUs from the 3 sampling points in analysis (par. 3.1.4.2), report the detection of heavy metal resistance determinants exclusively within the Beta and Gamma-proteobactera classes. Proteobacteria are in fact the most represented within the culturable bacterial population and to these classes many metal-resistant strains have been reported in literature (Chen *et al*. 2008; Pages *et al*, 2008; Cànovas *et al.* 2003*)*.

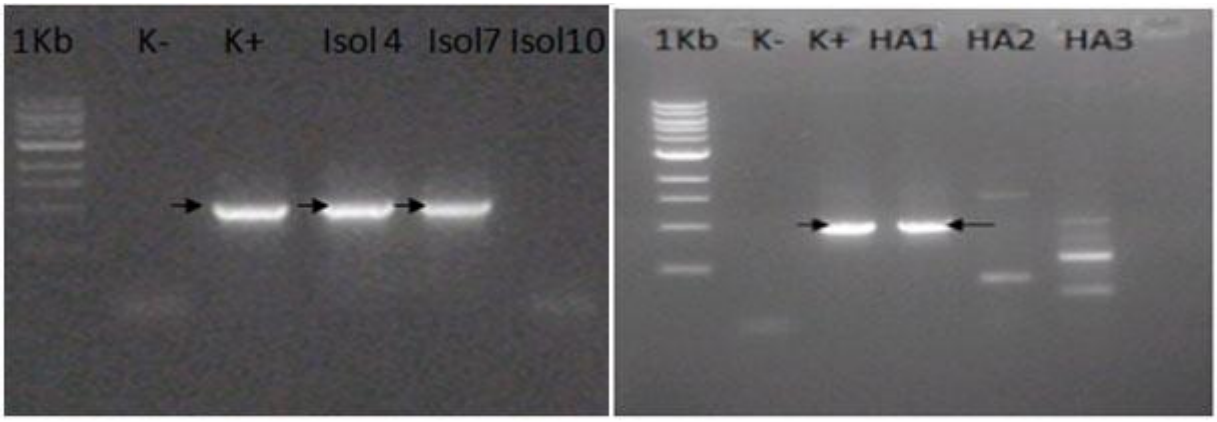



**Fig 3.30** Agarose gel electrophoresis of *mer* PCR products. Exact lenght of amplified region:1011 bp Lanes: 1Kb DNAsize marker; K- negative control, K+ *Cupriavidus metallidurans* CH34

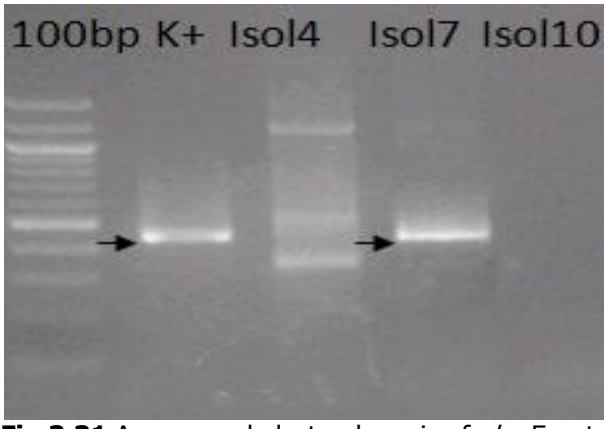



**Fig 3.31** Agarose gel electrophoresis of *chr*. Exact lenght of amplified region:450 bp Lanes: 100 bp DNA size marker; K- negative control, K+ *Cupriavidus metallidurans* CH34

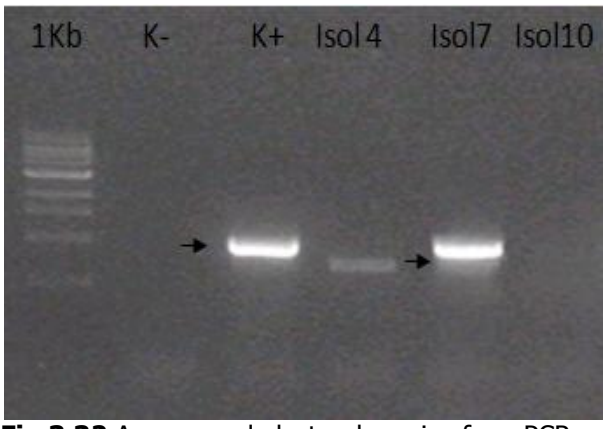



**Fig 3.32** Agarose gel electrophoresis of *czc* PCR products. Exact lenght of amplified region:1000 bp Lanes: 1Kb DNA size marker; K- negative control, K+ *Cupriavidus metallidurans* CH34

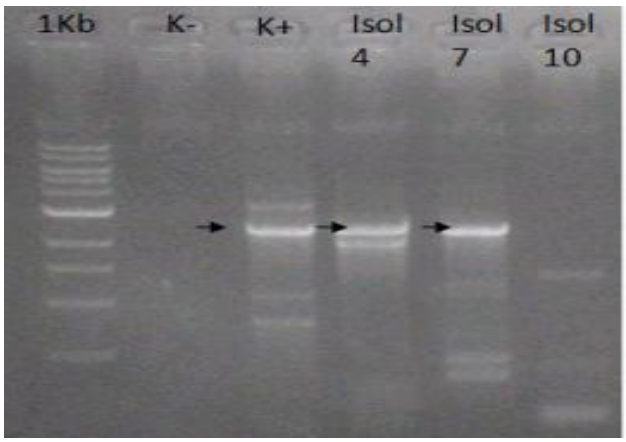



**Fig 3.33** Agarose gel electrophoresis of *pbr* PCR products. Exact lenght of amplified region:2300 bp. Lanes: 1Kb DNAsize marker; K- negative control, K+ Cupriavidus metallidurans CH34

## 3.1.6 Characterization of the strains isolated in the most restricted conditions of Enrichment cultures and of the Hot spot

Strains isolated in the strictest conditions imposed in laboratory through enrichment cultures or *in-situ* by the Hot spot contamination levels - adapted therefore to particularly hard conditions - have been the object of further characterization and study in relation from one side to metabolic potential towards organic Pb, and on the other side to PGP (Plant Growth Promoting) traits, interesting in the prospective of a bioremediation study or application.

In particular this study was performed on all OTUs isolated through Enrichment cultures from the 3 sampling points (I,II,III) and, as far as the Hot spot is concerned, on all the 3 OTUs isolated by Enrichment culture and on the 3 OTUs by direct isolation reporting the highest MIC values registered, namely Isol4, Isol7 and Isol10.

### 3.1.6.1 PGP (Plant Growth Promoting) traits study

The study of PGP (Plant Growth Promoting) traits is of particular interest in the perspective of a Phytoremediation study or application, in fact in stressed environments such as Pb contamination bacteria with PGP characteristics could play an important role in plant growth and therefore positively affect the Phytoremediation process.

To identify potential PGP bacteria, the OTUs in examination were qualitatively screened for ability to produce the auxin indoleacetic acid (IAA) and to utilize 1-aminocyclopropane-1-carboxylic acid (ACC) as the sole N source.

Actually one of the major mechanisms by which plant growth promoting rhizobacteria (PGPR) facilitate plant growth and development is the lowering of ethylene levels by deamination of ACC, the immediate precursor of ethylene in plants (Penrose *et* Glick, 2003). A number of PGPR contain the enzyme ACC-deaminase, which can cleave the plant ethylene precursor ACC, and thereby lower the level of ethylene in a developing or stressed plant. For many plants a burst of ethylene is required to break seed dormancy but, following germination, a sustained high level of ethylene - associated to environmental stress as heavy metals - would inhibit root elongation (Penrose *et* Glick, 2003).

Production of phytohormones, such as the auxin indoleacetic acid (IAA), is also widespread among plant-associated bacteria. IAA is a common metabolite of tryptophan by several microorganisms, including PGPR, and is absorbed to the roots as a plant growth regulator (Koo *et* Kyung-Suk, 2009). Promotion of root growth is in fact one of the major markers by which the beneficial effect of plant growth-promoting bacteria is measured. Whereas lateral and adventitious roots are induced by high concentrations of exogenous IAA, primary root growth is stimulated by application of relatively low levels of IAA, typically between $10^{9}$ and $10^{12}$ M and is inhibited by higher IAA concentrations, likely via auxin-induced ethylene (Patten *et* Glick, 2002).

Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their chances for survival even in perturbed soils (Patten *et* Glick, 2002).

Although numerous free-living soil bacteria are considered to be PGPR, not all bacterial strains of a particular genus and species have identical metabolic capabilities and interactions with plants (Penrose *et* Glick, 2003), therefore it is interesting a screening in relation to PGP traits.

#### *Acc deaminase and IAA production tests*

The OTUs under study were screened for the ability to use ACC as a sole nitrogen source, a trait that is a consequence of the presence of the activity of the enzyme ACC deaminase. 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 3.34 the development of a pink color indicates in fact IAA production (Cavalca *et al.*, 2010).

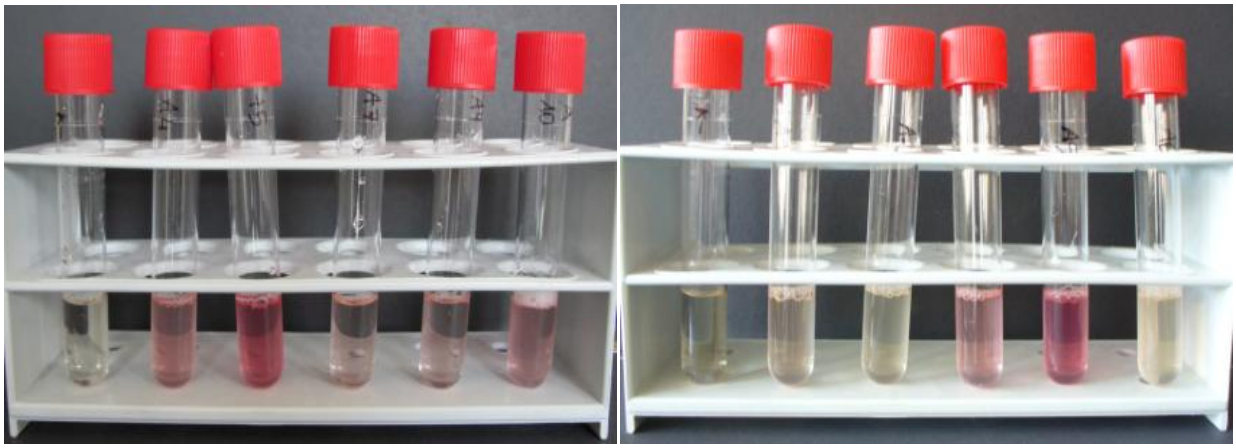


**Fig 3.34** Test for IAA production adding the Salkowski's reagent to the bacterial culture grown in presence of tryptophan

First considering the OTUs reporting Heavy metal determinants, no PGP traits was detected in Hot spot strains, whereas it is interesting to notice that 3 of the 5 OTUs selected through Enrichment culture from the 3 sampling points, also display PGP traits (tab.3.12).

| OTU | Sampling point | Taxonomic Reference ID | Phylogeny Group | Metal Resistances | | | | | PGP traits | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *pbr* | *mer* | *ncc* | *czc* | *chr* | IAA | ACC |
| **A1** | I,III | *Pseudomonas aeruginosa* FJ009393 | γ-Proteobacteria | - | + | - | - | - | - | - |
| **A2** | II,III | *Cupriavidus sp.* AB266611 | β-Proteobacteria | + | + | - | - | - | - | - |
| **A5** | I,II | *Delftia sp.* Fj594443 | β-Proteobacteria | + | + | - | - | - | + | - |
| **A10** | II | *Pseudomonas putida* DQ833753 | γ-Proteobacteria | - | + | - | - | - | + | - |
| **A16** | II | *Stenotrophomonas maltophilia* EF620462 | γ-Proteobacteria | + | + | + | - | - | + | + |
| **Isol4** | Hot spot | *Ralstonia sp. FJ535673* | β-Proteobacteria | + | + | - | - | - | - | - |
| **Isol 7** | Hot spot | *Cupriavidus campinensis* NR025137 | β-Proteobacteria | + | + | - | + | + | - | - |
| **HA1** | Hot spot | *Pseudomonas sp.* FJ791165 | γ-Proteobacteria | - | + | - | | - | - | - |

**Tab 3.12** Results of the PGP traits study for OTUs reporting heavy metal resistance determinants

In particular 2 OTUs, respectively belonging to *Delftia* sp. of Beta-proteobacteria and to the *Pseudomonas putida* species of Gamma-proteobacteria, showed positive results to IAA test, while the *Stenotrophomonas maltophilia* OTU - reporting at the same time 3 heavy metal resistance determinants – resulted to possess

both the IAA production and ACC deaminase traits – as shown in Fig 3.34. The ACC-deaminase activity was detected by comparing the strain growth in DF salts minimal medium, DF added with Ammonium Sulfate and DF with ACC as sole nitrogen source (par 2.4.1). 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 3.35).

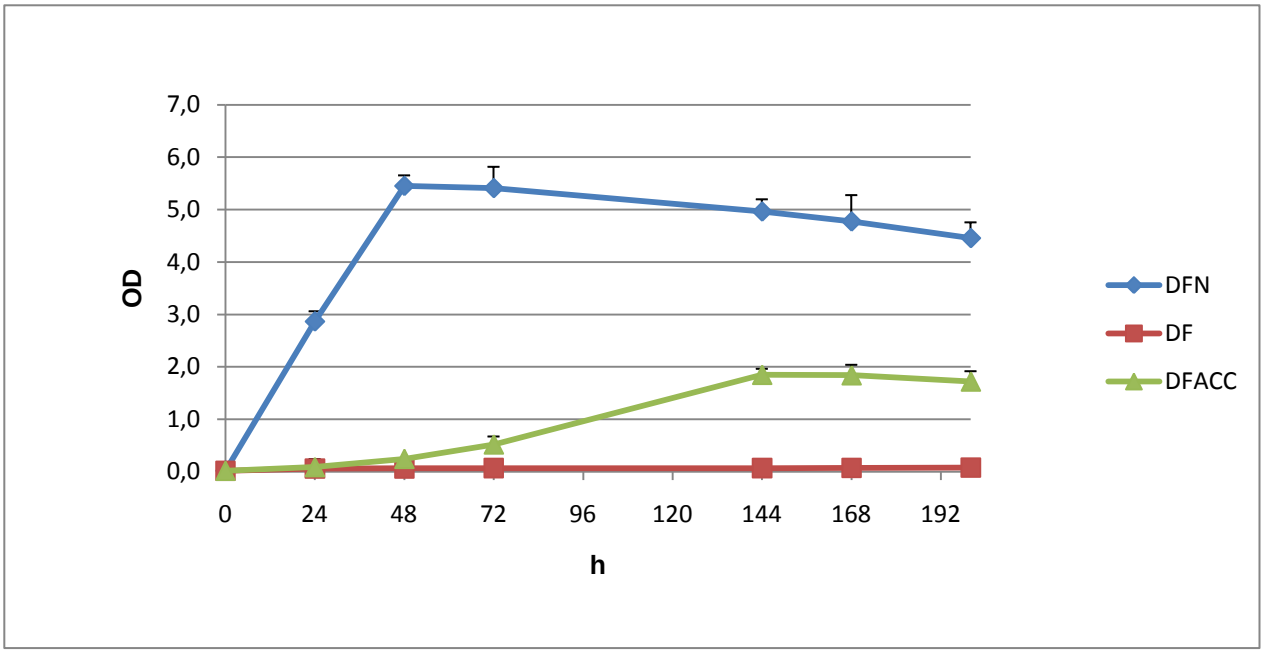



**Fig 3.35** Growth of the ACC-deaminase positive strain A16 in DF, DF with Ammonium Sulphate and DF with ACC as sole nitrogen source.

This OTU is therefore of particular interest and - as previously described in par 3.1.2.2 - to this species is reported an extraordinary range of activities including both resistance to heavy metal as Pb and association to various plants as PGPR (Seo *et al.*, 2007; Ben Said *et al.*, 2008; Pages *et al.*, 2008). *S. maltophilia* is in fact often associated with the rhizosphere of a variety of plants such as maize (*Zea mays*), rice (*Oryza sativa)*, wheat (*Triticum astivum*), cucumber (*Cucumis sativus*), potato (*Solanum tuberosum*), oilseed rape (*Brassica napus*) and Poplar (*Populus*) (Ryan *et al.*, 2009). Moreover, the genus *Stenotrophomonas* is currently studied for its potential application in bioremediation (Liu *et al.*, 2007) as well as in biological control of plant pathogens (Ryan *et al.*, 2009).

Besides even to the *Pseudomonas putida* species and *Delftia* genus, to which belong the 2 OTUs positive to IAA production, are in fact reported PGPR strains (Bloemberg *et* Lugtenberg, 2001; Belimov *et al.*, 2001; Jing *et al.*, 2007).

Considering all OTUs screened for PGP traits, including Enrichment OTUs from the 3 sampling points and Hot spot strains, results obtained showed that the 68% of OTUs possessed at least one potential PGP trait (tab3.13).

| OTUs | Isolation | Taxonomic reference ID | Phylogeny group | IAA test | ACC test |
|---|---|---|---|---|---|
| **A1** | Enrichm.(I,II,IIII) | *Pseudomonas aeruginosa* FJ009393 | γ-proteobacteria | - | - |
| **A2** | Enrichm.(I,II,IIII) | *Cupriavidus sp.* AB266611 | β-proteobacteria | - | - |

| | | | | | |
|---|---|---|---|---|---|
| **A3** | Enrichm.(I,II,IIII) | *Pseudoxanthomonas sp.* AY635897 | γ-proteobacteria | - | - |
| **A4** | Enrichm.(I,II,IIII) | *Ochrobactrum sp.* GU248309 | α-proteobacteria | + | - |
| **A5** | Enrichm.(I,II,IIII) | *Delftia sp* FJ594443 | β-proteobacteria | + | - |
| **A6** | Enrichm.(I,II,IIII) | *Pseudoxanthomonas sp.* EU276093 | γ-proteobacteria | - | - |
| **A7** | Enrichm.(I,II,IIII) | *Ochrobactrum sp.* FJ598332 | α-proteobacteria | + | - |
| **A8** | Enrichm.(I,II,IIII) | *Variovorax sp* EU734636 | β-proteobacteria | - | + |
| **A9** | Enrichm.(I,II,IIII) | *Agrobacterium sp.* EF189105 | α-proteobacteria | + | - |
| **A10** | Enrichm.(I,II,IIII) | *Pseudomona putida* DQ833753 | γ-proteobacteria | + | - |
| **A11** | Enrichm.(I,II,IIII) | *Pseudomonas sp.* EU375660 | γ-proteobacteria | + | + |
| **A12** | Enrichm.(I,II,IIII) | *Microbacterium sp.* DQ227343 | Actinobacteria | + | - |
| **A13** | Enrichm.(I,II,IIII) | *Variovorax sp.* AM285013 | β-prpoteobacteria | - | + |
| **A14** | Enrichm.(I,II,IIII) | *Alcaligenes sp.* EU37500 | β-proteobacteria | - | + |
| **A15** | Enrichm.(I,II,IIII) | *Variovorax sp* AY689053 | β-proteobacteria | - | - |
| **A16** | Enrichm.(I,II,IIII) | *Stenotrophomonas maltophilia* EF620462 | γ-proteobacteria | + | + |
| **A18** | Enrichm.(I,II,IIII) | *Microbacterium sp.* EF612319 | Actinobacteria | + | - |
| **A19** | Enrichm.(I,II,IIII) | *Pseudomonas sp.* GQ180165 | γ-proteobacteria | + | - |
| **A20** | Enrichm.(I,II,IIII) | *Arthrobacter sp.* EU342346 | Actinobacteria | - | - |
| **A21** | Enrichm.(I,II,IIII) | *Microbacterium sp.* AB461019 | Actinobacteria | + | - |
| **Isol4** | Direct (hot spot) | *Ralstonia sp.* FJ535673 | β-proteobacteria | - | - |
| **Isol7** | Direct (hot spot) | *Cupriavidus campinensis* NR025137 | β-proteobacteria | - | - |
| **Isol10** | Direct (hot spot) | *Arthrobacter sp.* DQ158001 | *Actnibacteria* | + | - |
| **HA1** | Enrichm. (Hot spot) | *Pseudomonas sp.* FJ791165 | γ-proteobacteria | - | - |
| **HA2** | Enrichm. (Hot spot) | *Stenotrophomonas maltophilia* FJ405363 | γ-proteobacteria | + | + |
| **Ha3** | Enrichm.(Hot spot) | *Rhizobium sp.* HM008950 | α-proteobacteria | + | - |

**Tab 3.13** Results of the PGP traits study for the analyzed OTUs, isolated by enrichment culture from the 3 sampling points (I,II,III) or from Hot spot

In particular the production of IAA was detected in 56% of the tested strains, allocated to 8 genera including gram-negative and gram-positive taxa and distributed, as shown in Fig 3.36(A), among Alphaproteobacteria (2 *Ochrobactrum,* 1 *Rhizobium* and 1 *Agrobacterium*), Betaproteobacteria (1 *Delftia*), Gammaproteobacteria (*3 Pseudomonas* and 2 *Stenotrophomonas*) and the gram-positive Actinobacteria *phylum* (*3 Microbacterium* and 1 *Arthrobacter*). It is interesting to notice as IAA production has been

detected in members of all *Taxa* identified by Enrichment from the 3 sampling points and in the Hot spot, this PGP traits is in fact reported to be a widespread characteristic (Patten *et* Glick, 2002).

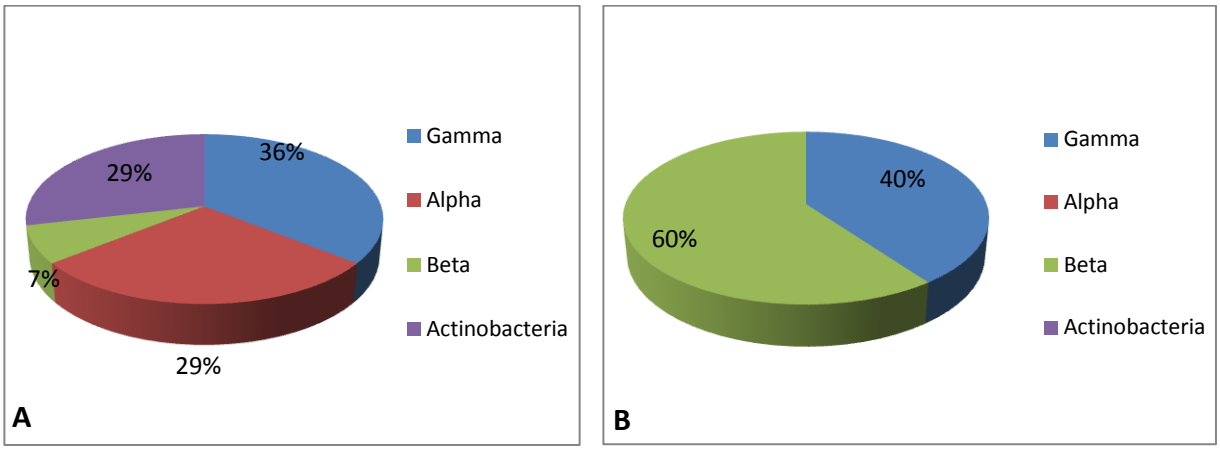



**Fig 3.36:** Taxa distribution of tested OTUs positive to IAA (A) and ACC (B) tests

A lower percentage of 28% of tested strains was detected to have ACC-deaminase activity and therefore to be able to reduce plant ethylene level. Their ability to utilize ACC as a source on nitrogen was detected by comparing strain growth in DF minimal medium, DF supplied with Ammonium Sulfate and DF with ACC as sole N source, as reported in fig 3.37-3.39 for strains positive to ACC tests.

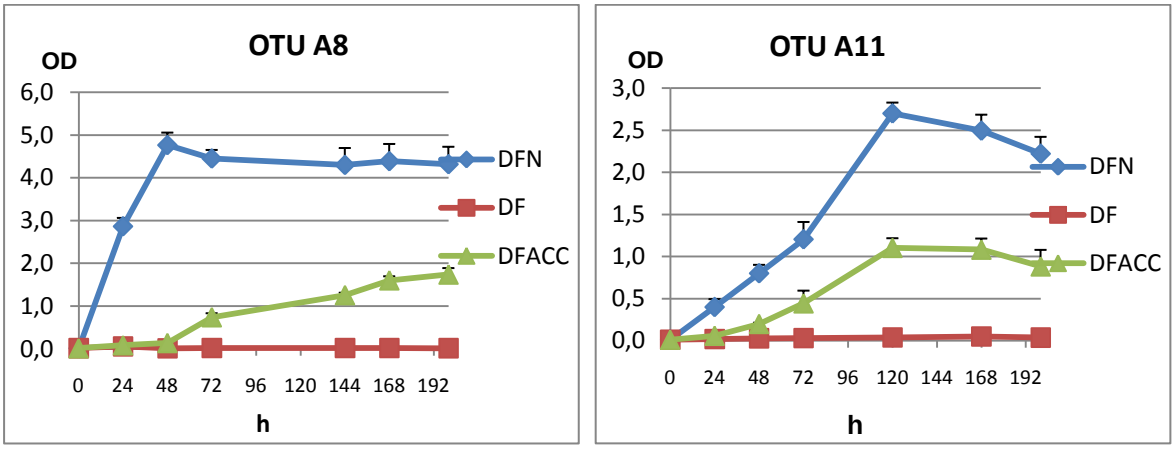



**Fig 3.37** Growth of the ACC-deaminase positive strains A8 and A11 in DF, DF with Ammonium Sulphate and DF with ACC as sole nitrogen source

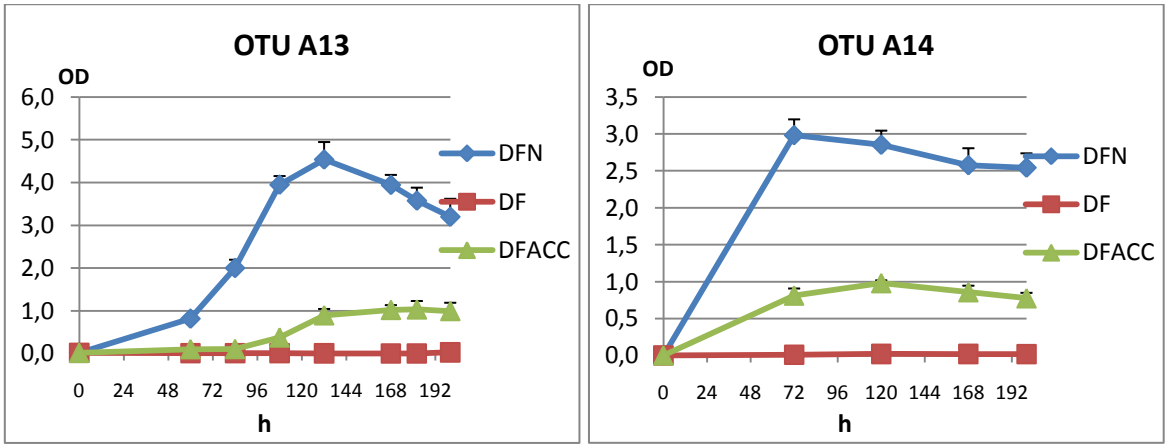


**Fig 3.38** Growth of the ACC-deaminase positive strains A13 and A14 in DF, DF with Ammonium Sulphate and DF with ACC as sole nitrogen source

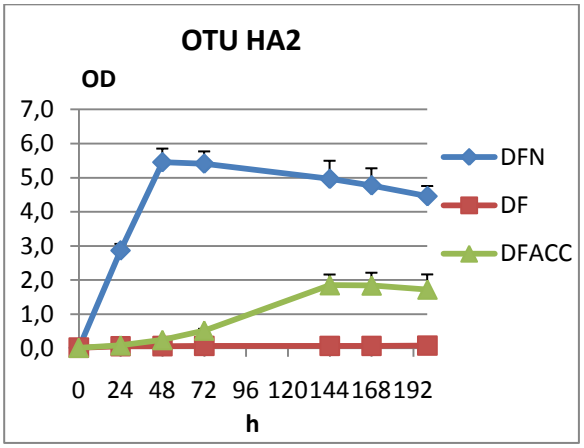



**Fig 3.39** Growth of the ACC-deaminase positive strain HA2 in DF, DF with Ammonium Sulphate and DF with ACC as sole nitrogen source.

These strains are all gram-negative bacteria (Fig 3.36B, Tab 3.13), in particular 2 OTUs belong to the *Variovorax* genus (OTUs A8 and A13, Fig.3.37, 3.38) – detected exclusively by Enrichment culture - 1 to *Acaligene*s genus (OTU A14, Fig 3.37) of Beta Class, 1 to *Pseudomonas* (OTU A11, Fig. 3.37) and 2 to the *Stenotrophomonas* genus of the Gamma-proteobacteria (OTUs A16 and HA2, Fig. 3.35, 3.39). These strains are therefore interesting in a phytoremediation perspective. Plants that are treated with ACC deaminase-containing PGPR are in fact much more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful conditions such as flooding, the presence of phytopathogens, drought and heavy metals (Penrose *et* Glick, 2003; Zhuang *et al.*, 2007; Husen *et al.*, 2009).

In particular 3 OTUs reported both PGP traits, i.e. one *Pseudomonas sp.* (A11) and 2 *Stenotrophomonas malthophilia* (A16 and HA2). It is worth noting that these OTUs are all Gamma-proteobacteria and this draws further attention on the genera *Pseudomonas* and *Stenotrophomonas* which are, as previously mentioned, dominant members of the autochthonous bacterial cenosis and reported in literature for PGP activities (Bloemberg *et* Lugtenberg, 2001; Pages *et al.*, 2008).

Considering that in stressed environment bacteria with PGP characteristics can play an important role in plant growth, a consortium of Proteobacteria with PGP traits has been chosen - on the bases of the data here presented - to be used in a Biaugmentation protocol in a lab-scale Phytoremediation trial as further described in par 4.3.6.

### 3.1.6.2 Growth test with TEL as sole carbon source

Growth test with TEL as sole carbon source were performed to identify, between the strains isolated in the strictest conditions of enrichment cultures and Hot spot, possible microorganisms able degrade organic Pb.

Cultures were therefore set up in minimum medium with TEL as sole carbon source and incubated at 27°C on an orbital shaker in the dark for 10 days, but no positive results were obtained as no tested strains was able to grow using TEL as sole carbon source.

Even OTUs selected through enrichment with TEL as sole carbon source were not able to grow in these conditions. Probably in Enrichment cultures the concomitant presence of soil components and of a microbial community enabled the strain to find nutrient or to degrade organic Pb in subsequent steps integrated by different components of the community. Indeed although many bacteria are able to metabolize organic pollutants, a single bacterium does not possess the enzymatic capability to degrade all

or even most of the organic compounds in a polluted soil. Mixed microbial communities have the most powerful biodegradative potential because the genetic information of more than one organism complements increasing the overall biodegrading potential (Fritsche *et* Hofrichter, 2008).

### *Future work: Transformation Trial of TEL by co-metabolism*

As a metabolic degradation of organic Pb as only carbon source had not been detected, it will be interesting to explore the possible degradation of organic Pb in co-metabolism. Biodegradation of organic compounds is in fact based on two processes: growth and co-metabolism. In the case of growth organic pollutants are used as sole source of carbon and energy, resulting in their complete degradation and therefore mineralization. On the other side co-metabolism is defined as the metabolism of an organic compound in the presence of a growth substrate which is used as the primary carbon and energy source (Fritsche *et* Hofrichter, 2008), and as far as high molecular weight PAHs are concerned, a co-metabolic degradation is mainly involved (Gong *et al.*, 2001).

The cultures will be therefore supplemented with both TEL and an additional carbon source such as yeast extract, and inoculated with the strains previously grown in the same medium supplemented with a lower concentration of TEL - in case inducible genetic systems were involved in organic Pb degradation. It will be also interesting to performe the trial with a consortium of strains, which could degrade organic Pb in subsequent steps integrated by the distinc components of the consortium.

In a first step this trial will be performed with those strains particularly interesting in the prospective of a bioremediation approach (Tab.3.14), i.e. strains showing heavy metal determinants, such as OTUs A2 *Cupriavidus sp.* and A5 *Delftia sp.* showing Hg and Pb resistance determinants, the letter also positive for IAA production; OTU A14 *Alcaligenes* sp. reporting ACC-deaminase activity and the 3 OTUs isolated through enrichment from the highest contaminated Hot spot (OTU HA1 *Pseudomonas sp,* HA2 *Stenotrophomonas maltophilia* and HA2 *Rhizobium sp.)* and for which Heavy metal resistance determinants or PGP traits had been detected. In this trial OTU A16 *Stenotrophomonas maltophilia* will be also included. These strains in fact, reporting PGP traits and determinants for the resistances to Pb, Hg and Ni, are particularly interesting.

| OTU | Taxonomic reference ID | Phylogenetic group | Metal resistences | | | PGPR traits | |
|---|---|---|---|---|---|---|---|
| | | | Pb | Mer | Ncc | ACC | IAA |
| **A2** | *Cupriavidus sp.* AB266611 | β-proteobacteria | + | + | - | - | - |
| **A5** | *Delftia sp* Fj594443 | β-proteobacteria | + | + | - | - | + |
| **A14** | *Alcaligenes sp.* EU37500 | β-proteobacteria | - | - | - | + | - |
| **A16** | *Stenotrophomonas maltophilia* EF620462 | γ-proteobacteria | + | + | + | + | + |
| **HA1** | *Pseudomonas sp.* FJ791165 | γ-proteobacteria | - | + | - | - | - |
| **HA2** | *Stenotrophomonas maltophilia* FJ405363 | γ-proteobacteria | - | - | - | + | + |
| **HA3** | *Rhizobium sp.* HM008950 | α-proteobacteria | - | - | - | - | + |

**Tab 3.14** OTus to be tested for the transformation of TEL by co-metabolism

### 3.1.7 Most interesting microorganisms isolated within the Ex-SLOI area

An overall analysis of the results so far obtained suggest the selection within the Ex-SLOI area of a tolerant soil bacterial community, selected by and adapted to the contamination present in the area for over half century and characterized by an heterogenic structure, a rich biodiversity - for a perturbed soil - and resistance potential.

All the isolated strains showed in fact high homologies with bacteria diffused within different environmental niches including contaminated soils, capable of degrading organic pollutants and hydrocarbon and, as expected, reporting high resistances to heavy metals.

A detailed analysis pointed out the preponderance of gram-negative Proteobacteria, which are highly represented within the indigenous community and moreover account for the totality of strains isolated in the strictest condition of enrichment culture with TEL as sole carbon source, performed from all the 3 sampling point in analysis and from the Hot spot, i.e. the highest contaminated spot detected within the whole Ex-SLOI area.

Besides 8 isolated strains - all belonging to the Proteobacteria *phylum,* namely to *Cupriavidus, Ralstonia* and *Delftia* genera of Beta class, and to *Pseudomonas* and *Stenotrophomonas* of the Gamm class - displayed at least one heavy metal resistance determinant, while 5 of them presented the concomitant presence of multiple resistances. In particular a strain of *Cupriavidus campinensis* – isolated from the most contaminated Hot Spot - displayed at the same time 4 resistance determinants: for Pb, Hg, Cr and Cd-Zn-Co.

Considering also the microbial ability of promoting plant growth, the high represented class of Gamma-proteobacteria included the 2 genera - *Pseudomonas* and *Stenotrophomonas* - whose members within the autochthonous community displayed both examined PGP (Plant growth promoting) traits.

In particular to these Gamma-proteobacteria genera, and to the *Delftia* genus of the Beta class, belong the 3 OTUs repoting both Heavy metal resistence determinants and PGP traits: namely OTU A5 *Delftia* sp., OTU A10 *Pseudomonas putida* and OTU A16 *Stenotrophomonas maltophilia.*

In particular the OTU A16 *Stenotrophomonas maltophilia* - selected in the Enrichment culture DMY supplemented with another carbon source, from the most contaminated sampling point II - is characterized by the concomitant presence of the three determinants for the resistances to Pb, Hg and Ni and reports at the same time both potential PGP traits of IAA production and ACC-deaminase activity. Strains of *Stenotrophomonas maltophilia* have in fact an extraordinary range of activities that include beneficial effects for plant growth and health and degradation capabilities central to bioremediation and phytoremediation strategies (Samanta *et al.*, 2002; Compant *et al.*, 2005; Ryan *et al.*, 2009).

In other members of the Proteobacteria, such as *Cupriavidus, Delftia, Alcaligenes* and *Variovorax* of the Beta class and *Ochrobactrum* and *Agrobacterium* of the Alpha class, have been also detected heavy metal resistances or PGP traits and are therefore interesting in a bioremediation/phytoremediation approach.

This confirms the actual resistance and richness associated to the autochthonous microbial community and the selection towards a resistant microbial cenosis, with members of particular high resistance belonging to the most represented gram-negative Proteobacteria classes but even to the gram-positive Actinobacteria component. In fact, although the gram-positive Actinobacteria have been directly detected mainly in the less contaminated of the 3 sampling points, they have also been selected in the Enrichment culture with yeast extract. Moreover to *Microbacterium* and to *Arthrobacter* genera - to which multiple heavy metal resistances have been reported in literature (Abou-Shanab *et al.*, 2007) – belong members of the indigenous culturable community selected within the most contaminated Hot spot and OTUs reporting PGP traits. This confirms the hypothesis that the Actinobacteria class, although less represented within the cultivable fraction of the indigenous micro flora, includes community members of high resistance and bioremediation potential.

# 3.2 Culture-independent characterization: molecular-based evaluation of the soil autochthonous bacterial community

The genetic complexity of microbial soil communities can be estimated by reassociation of community DNA, which depends on the amount of homogeneous DNA present in a sample (Torsvik *et al.*, 2002). The analysis so performed on the total bacterial DNA in a 30 g soil sample reported the detection of more than 500 000 species, and revealed that the entirety of the microbial genomes found in soil samples, termed the soil metagenome, harbors more genetic information than is contained in the approximately 5000 to 6000 individual prokaryotic microorganisms available in the different culture collections (Daniel, 2004). Thus, the genetic diversity of the soil metagenome can be a rich and interesting object of study and source of information on its microbial community.

Environmental DNA-based methods for accessing the soil metagenome as the extraction of nucleic acids and PCR amplification do not rely on isolation and cultivation of single microorganisms reducing the bias associated with it (Daniel, 2004). It has in fact been estimated that only a small subset of the actual soil population can be cultured by applying cultivation techniques, while the majority of the genetic diversity present within the population is lost owing to difficulties in growing and isolating the microorganisms (Daniel, 2004).

Molecular techniques were therefore applied for analyzing the entire soil bacterial community, including both the cultivated and non-cultivated fraction.

A metagenomic study was performed in relation to heavy metal resistances and degrading capabilities towards hydrocarbons on soil samples from the sampling points in analysis (I,II,III and Hot spot). This is indeed an interesting mean of study to explore the resistance and degrading potential within the autochthonous soil microbial community.

Moreover fingerprint patterns based on the separation of 16S rDNA PCR products by denaturing gradient gel electrophoresis (PCR-DGGE) were applied to explore the structure and biodiversity within the bacterial community comprehensive of the unculturable fraction. PCR-DGGE technique has been used in several studies to compare microbial communities, because multiple samples can be simultaneously analyzed discriminating different communities (Joynt *et al.*, 2006).

## 3.2.1 Soil metagenomic molecular analysis

The molecular study targeting genetic determinants for heavy metal resistances and hydrocarbon degradation - previously performed on members of the culturable microbial community and described in par 3.1.4.2 and 3.1.5.4 - was performed in duplicate directly on the total genomic DNA extracted from sub-superficial soil samples for the different sampling points.

For the molecular analysis on the Hot spot two sampling from a probing were included: Hot spot A collected from 0 to 1m depth and and Hot Spot B collected at a higher depth of 3 meters. This allowed a wider culture-independent analysis of the most contaminated spot within the whole Ex-SLOI area.

After visualization by gel electrophoresis, the PCR amplicons of the expected size were chosen for sequence analysis, and the results obtained showed at all the 3 sampling points (I,II,III) the detection of Hg (*mer*) and Ni (*ncc*) resistance determinants, the latter detected even in both Hot spot samples (Tab.3.15). Visualization in agarose gel electrophoresis of positive amplicons of the expected size which were subsequently confirmed by sequencing is evidenced in Fig 3.40-3.45.

| Soil sample | Heavy metal resistence determinants | | | | | Hydrocarbon degradation det. | | |
|---|---|---|---|---|---|---|---|---|
| | ***pbr*** | ***mer*** | ***czc*** | ***chr*** | ***ncc*** | ***alk*** | ***nah*** | ***phn*** |
| **Point I** | + | + | - | - | + | - | + | - |
| **Point II** | - | + | - | - | + | - | - | - |
| **Point III** | - | + | - | - | + | - | - | - |
| **Hot Spot Pb A** | + | - | + | + | + | - | - | - |
| **Hot Spot Pb B** | - | - | | - | + | - | - | - |

**Tab 3.15** Results of the molecular study targeting Heavy metal resistance and hydrocarbon degradation determinants on metagenomic soil DNA

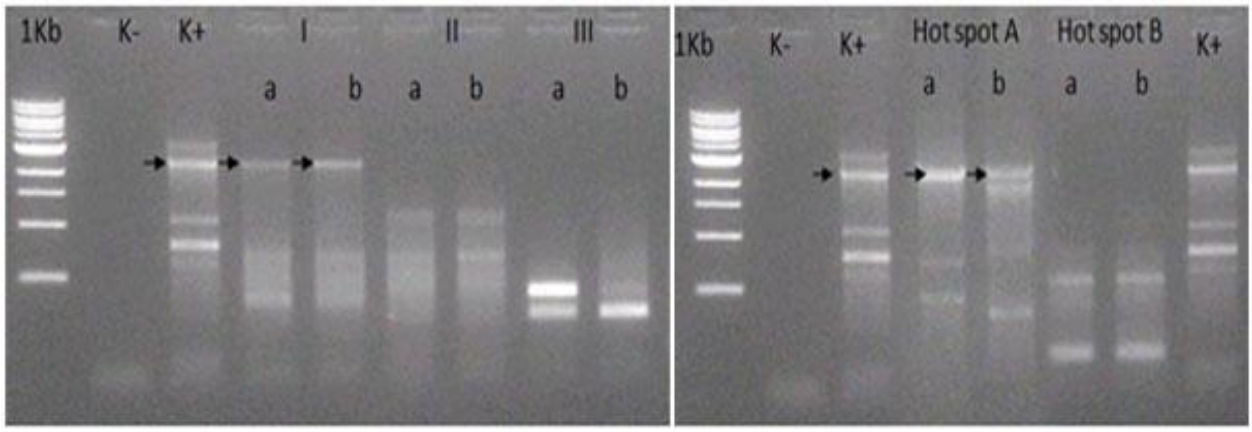



**Fig 3.40** Agarose gel electrophoresis for *pbr* PCR products. Exact lenght of amplified region:2300 bp Lanes: 1Kb DNA size marker; K- negative control, K+ *Cupriavidus metallidurans* CH34. A and B refers to duplicate analysis

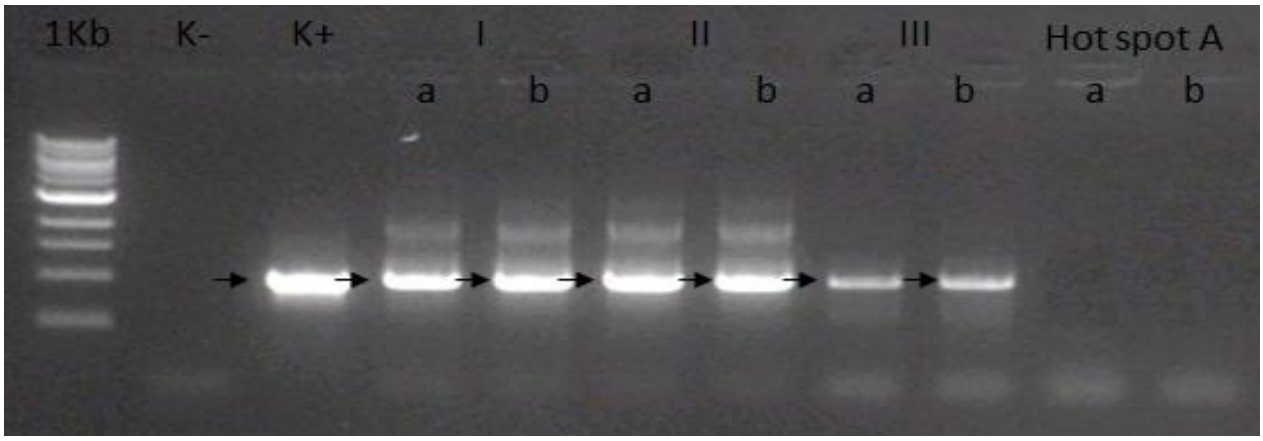



**Fig 3.41** Agarose gel electrophoresis for *mer* PCR products. Exact lenght of amplified region:1011 bp Lanes: 1Kb DNA size marker; K- negative control, K+ *Cupriavidus metallidurans* CH34. A and B refers to duplicate analysis

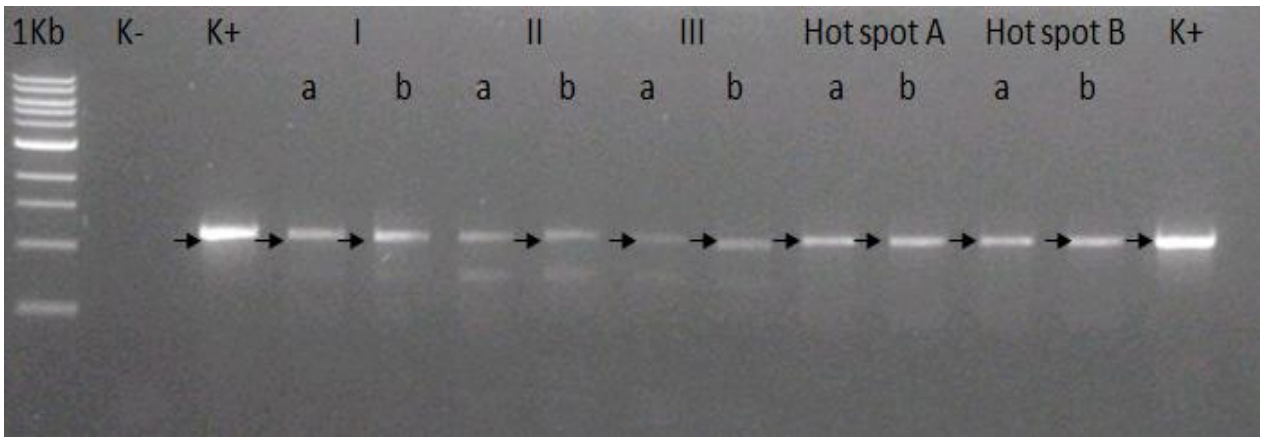



**Fig 3.42** Agarose gel electrophoresis for *ncc* PCR products. Exact lenght of amplified region:1141 bp Lanes: 1Kb DNA size marker; K- negative control, K+ *Cupriavidus metallidurans* CH34. A and B refers to duplicate analysis

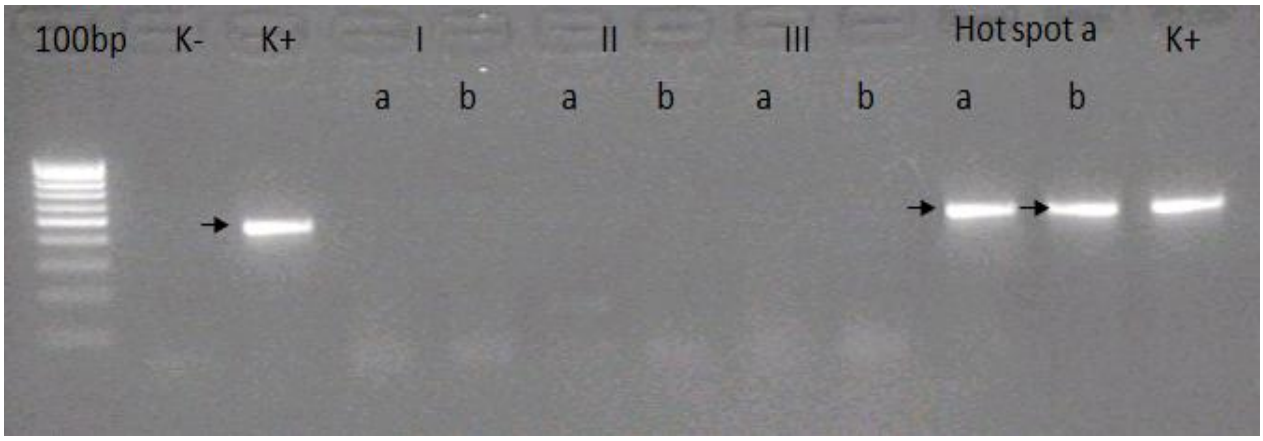



**Fig 3.43** Agarose gel electrophoresis of *chr* PCR products. Exact lenght of amplified region:450 bp Lanes: 100 bp DNA size marker; K- negative control, K+ *Cupriavidus metallidurans* CH34. A and B refers to duplicate analysis

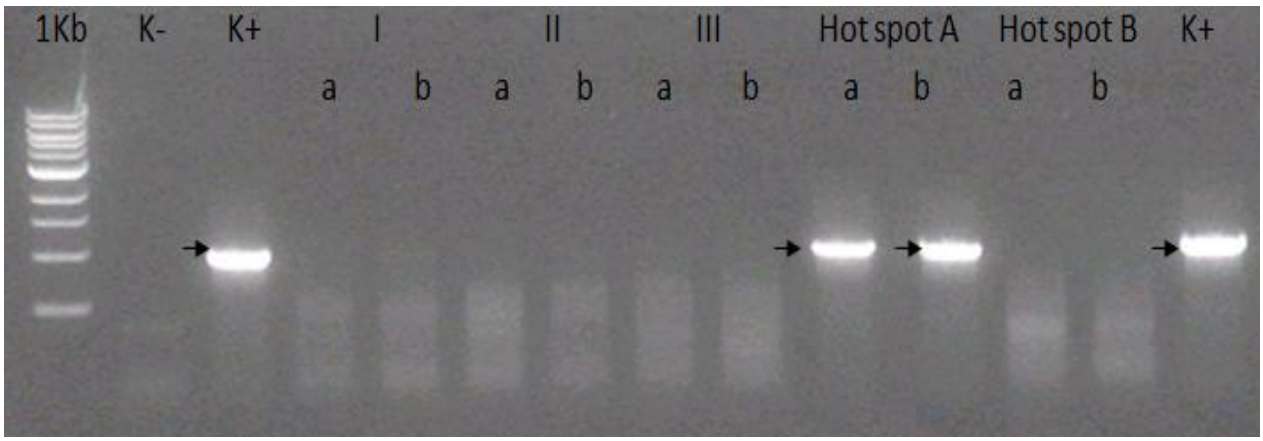



**Fig 3.44** Agarose gel electrophoresis of *czc* PCR products. Exact lenght of amplified region:1000 bp Lanes: 1Kb DNA size marker; K- negative control, + *Cupriavidus metallidurans* CH34. A and B refers to duplicate analysis

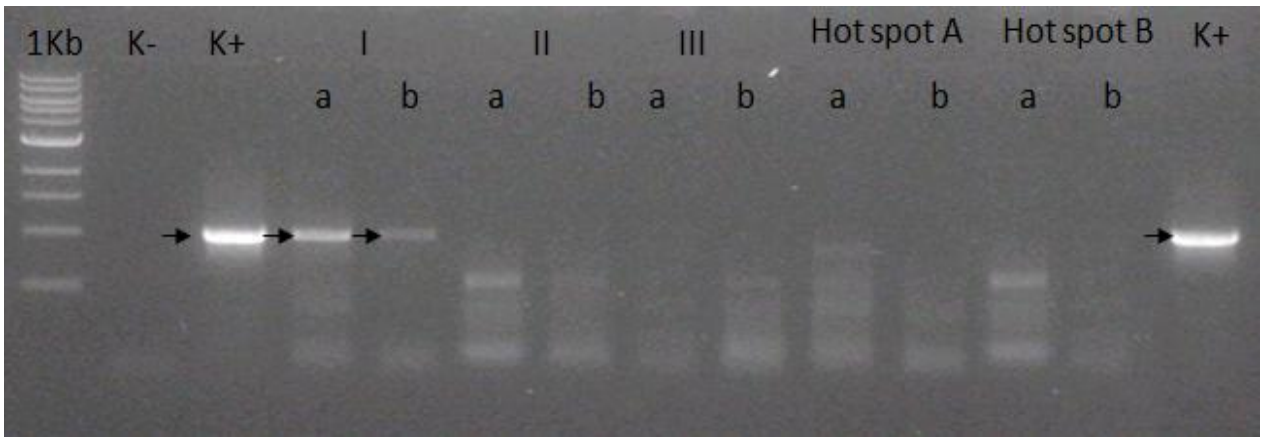



**Fig 3.45** Agarose gel electrophoresis of *nah* PCR products. Exact lenght of amplified region: 992 bp Lanes: 1Kb DNA size marker; K- negative control, K+ *Pseudomonas putida G7 nahAc.* A and B refers to duplicate analysis

The Hot spot A sample, therefore corresponding to the sub-superficial soil layers also used in the culture study, was the only one reporting the detection of the determinants *czc* and *chr*, respectively encoding for the resistances to Cd-Co-Cz and Cr. These two resistance determinants in fact had been previously detected only in one single OTU isolated just from the Hot spot (par 3.1.5.4).

At the Hot spot A sample and Point I were also detected the Pb-resistance determinant. Moreover in Point I it is interesting to notice for the first time the detection of a determinant for the degradation of PAH: the *nah* determinant. This sampling point was in fact located near the mixer and spills of petrol contaminated the area also with hydrocarbons.

This datum testifies a hydrocarbon degrading potential within the microbial community and a probable association to the unculturable fraction, as the same determinant was not detected in any analyzed strain.

Even the determinant for the resistance to Ni, reported in the previous molecular study just in 1 OTU (par 3.1.4.2), was here detected at all sampling points in analysis, suggesting a high diffusion of this resistance determinant within the soil microflora.

Considering the Hg resistance determinant, it is interesting to notice its detection at all the 3 sampling points (I,II,III). Moreover it was previously detected in the all OTUs reporting at least one heavy metal determinant (par 3.1.4.2 and 3.1.5.4). This determinant is in fact reported in a wide range of both gram-negative and gram-positive bacteria and is characterized by ubiquity with respect to geographical location, environment and species range. It is moreover hypothesized that *mer* determinant is an ancient system, carried by ancient bacteria in response to increased levels of mercury in natural environments, perhaps resulting from volcanic activity (Osborn *et al.*, 1997).

Unexpectedly the Pb-resistance determinants was not detected in the most contaminated point II and Hot spot, although from these sampling points strains reporting the Pb-resistance determinant have been isolated (par 3.1.4.2). The lack of success in amplification of Pb resistance determinants could in fact be connected to biases implied in the molecular study performed (Cavalca *et al.*, 2010).

These data confirm therefore, for the soil autochthonous microbial community in exame, the heavy metal resistance previously detected in some of its members and at the same time indicate a degrading potential towards hydrocarbons. These results also support the value of a combined approach integrating culture-dependent and independent analysis chosen in this PhD study.

## 3.2.2 Analysis of soil bacterial communities by PCR-Denaturing Gradient Gel Electrophoresis analyses (PCR-DGGE)

In order to better evaluate the microbial community structure at each examined sampling point, PCR-DGGE analyses were carried out on V3-16S rDNA PCR products, amplified from soil total DNA extracted in duplicate.

The analysis was performed on the 3 sampling points and on 2 distinct samples from the Hot spot as in the soil metagenomic molecular analysis (par 3.2.1): Hot spot A collected from 0 to 1m depth and Hot Spot B collected at a higher depth of 3 meters.

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 unculturable 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.

Previous research had shown that a shift in the microbial community towards a metal-tolerant or metal resistant community occurs in heavy metal contaminated soils (Becker *et al.*, 2006). These microbial responses tend to be dose-dependent, with increasing metal amendments causing a larger shift in the microbial response compared to the uncontaminated control. Few studies have although attempted to address chronically contaminated sites, as this PhD study, where natural selection is supposed to have selected for a metal-tolerant community (Becker *et al.*, 2006).

Considering the DGGE profile shown in fig 3.46, for each sampling point analyzed a first comparison of the profiles performed in duplicate confirmed the reproducibility and significance of the analysis performed. Actually duplicate samples were found to match closely showing similar profile complexity and band composition.

Visual comparison of the bacterial communities at the different sampling points (Fig 3.46) showed the presence of few bands of major intensity – from 3 to about 10 - corresponding to dominant components of the microbial community, indicating the selection imposed by the contaminant towards a resistant community.

Despite distinct profiles were associated to each sampling point, a higher similarity was observed between point I and II, which in fact are characterized by Pb contamination in the same order of magnitude. In particular 3 major bands were discernable at point I and 2 of them were also highly detected in point II.

Considering the 3 sampling points examined (I, II and III), only one band (band 1) was detected in all of them; the detection of the same band in different profiles suggests the presence of a bacterial member able to face Pb contamination at the different concentrations and co-contaminations present in the 3 sampling points. This band was therefore cloned and the sequencing results related it to the *Alcaligenes* genus. For this genus are in fact reported both heavy metal resistant and alkane degrading strains (Periello, 2000), moreover this was one of the major genus of the Beta class identified by culture techniques within the indigenous culturable micro flora at the 3 sampling points (par 3.1.2.3). However in the culture analysis it was detected only in point I and III, indicating the value of combining both culture and molecular approaches.

In point II two more major bands were detected (band 2 and 3), both belonging to the Proteobacteria, the first in particular to the Gamma class (Table 3.16), in agreement with the major representation of this *phylum* detected by the culture study previously described (par 3.1).

Another band less intense (band 4) belongs to the *Pedobacter* genus of the Bacteroidetes *phylum*. To this genus has been described a cold-adapted strain with remarkable oil hydrocarbon biodegradation and recently even a strain which can produce IAA (Margesin *et al.*, 2003; Chao-Lei *et al.*, 2009).

Bacteroidetes have indeed 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 however two OTUs of this *phylum*, belonging to the Flavobacteria class, were obtained in pure culture and isolated from the most polluted Point II (par 3.1.2).

Comparing the most and least contaminated point II and III, two bands - band 1 and 2 corresponding to Beta and Gamma-proteobacteria - highly intense in point II were faintly discernable even in point III. However a global distinct profile was associated to the latter which is the only one with Hg contamination and with a lower Pb contamination level in comparison to the other 2 points (I,II). The sequencing of two bands (5 and 6) identified in this sampling point related both of them to the Alpha class (Table 3.16). The latter band belongs to *Sphingomonas,* genus of major interest for the degradation of organic contaminants, bioremediation protocols (Vanbroekhoven *et al.*, 2004; Seo *et al.*, 2007; Kertesz *et* Kawasaki, 2010) and also detected in the rhizospheric microbial communities associated with plants grown in metal rich soils (Kamaludeen *et* Ramasamy, 2008).

Comparing point III to the other samplings points in analysis, although it is the less contaminated point a low number of bands was detected along the first part of the profile. This could reflect a lower bacterial population or more probably be connected to biases implied in the molecular analysis, such as limited DNA extraction and purity, damage or samples loss along the various phases of the analysis.

However in point III profile it is interesting to notice bands in the lower part of the gel, this could be connected to a high population with high G + C content which migrated to the lower part of the gel characterized by higher denaturant concentrations (Joynt *et al.*, 2006). Actually at point III a higher percentage of high G+C gram-positive bacteria was detected in the culturable fraction such as *Nocardia, Streptomyces* and *Microbaterium* of the Actinobacteria *phylum* (par 3.1.2.2). Besides in the sequencing results of the band 7, an alpha proteobacterium component was identified belonging to the genus *Rhodoplanes* – which includes species with G+C content of almost 70% (Okamura *et al.*, 2009). This genus*,* not isolated in the previous culture analysis performed, it is reported in molecular study on heavy metal contaminated soils (Ellis *et al.*, 2003; Hui *et al.*, 2009).

Profiles differing from those obtained from the 3 sampling points were associated to the Hot spot, pointing out the specific bacterial community selected at its higher contamination level.

For the Hot spot sample A a lower number of bands of particular high intensity were evident. This suggests a higher selection towards few resistant members dominating the bacterial community, probably in a situation of lower competition towards other species much inhibited by the high contamination. The Hot spot is in fact characterized by a contamination level one order of magnitude higher than the other 3 sampling points.

The two major bands (8 and 9) corresponded respectively to the *Acidovorax* genus of the Beta-proteobacteria and to the *Xanthobacter* genus of the Alpha class (Tab.3.16). Both bands were also detected in the deeper Hot spot sample B, even if with a much lower intensity. To strains of *Acidovorax* are reported both resistance to heavy metals (Abou-Shanab *et al.*, 2007) and PAH degrading capacities (Singleton *et al.*, 2009). However no member of this genus was detected in the culture study performed on the Ex-SLOI sampling points, nor for *Xanthobacter* genus.

In particular the homologue for band 9 sequence - the uncultured *Xanthobacter sp*. FN594647 - was isolated in a study on bacteria diversity and arsenic mobilization in rock biofilms from an ancient gold mine, suggesting for this genus resistance and maybe influence on metal mobilization. To this genus relate also facultative methylotrophic species and a remarkable degree of metabolic versatility, including substituted of the heterocyclic aromatic compound thiophenes (Padden *et al.*, 1998).

Even if in a much fainter band (band 10), the sequencing identified a community component of the *Rhodopseudomonas* genus of the *Rhizobiales* family - genus not detected by the culture analysis - for which PGP activity such as enhancement of plant growth and yield is known (Lee *et al.*, 2008). This band however was much more intense in Hot spot B profile, indicating its higher representation.

Comparing the two hot spot samples A and B it is interesting to notice a redistribution of intensity between the bands in similar profiles. Actually in the Hot spot sample B, in addition to the major bands detected at the shallower sample A, 5 more bands highly intense were evident. On the other side with the only exception of one band, they were all also discernable in the Hot spot A profile even if with low intensity. In particular two bands highly intense in Hot spot B (band 10 and 11) are both member of the gram-negative Proteobacteria, respectively of Alpha and Beta classes.

Moreover it is interesting to notice as the sequencing of the band 12, detected in the Hot spot B and faintly discernable in Hot spot A, corresponded to the Actinobacteria order of *Actinomycetales*, denoting a higher resistance even for this gram-positive *phylum.*

The results from the DGGE profiles evidenced a selective pressure exerted by the contamination with the selection of dominant members within the autochthonous bacterial community. The concomitant presence of distinct ribotype at the analyzed sampling points reflected the contamination type, in particular the Hot spot showed a peculiar profile indicating the selection for a specific microbial community. As shown in the phylogenetic tree (Fig.3.47), it is noteworthy the prevalence of proteobacteria and of Alpha class in particular. Actually to this class of Proteobacteria a high biodiversity of genera connected to high degrading capabilities and resistances to metals was also detected in the culture analysis (par.3.1.2.1).

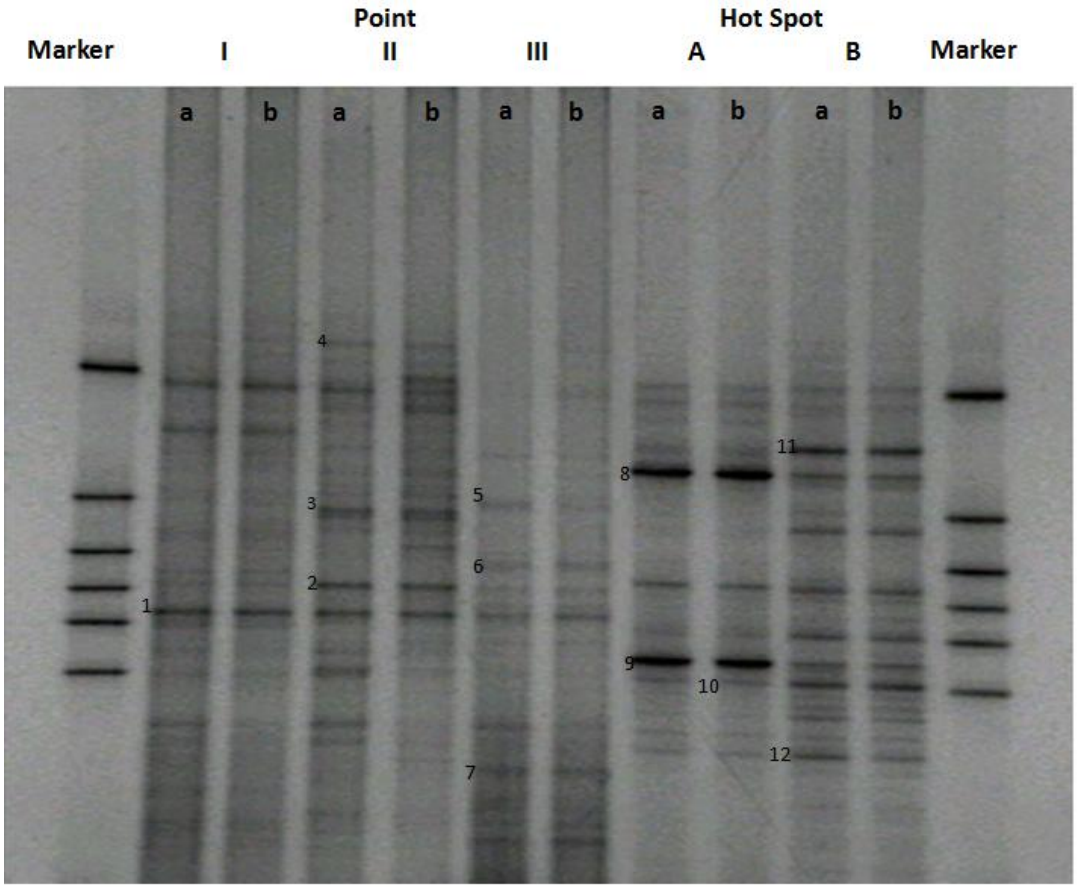



**Fig 3.46** Community profiles based on DGGE for soil samples of the points under study (I,II,III,Hot spot). A and B refer to the repeted analysis for the same soil samples. Numbers refers to sequenced bands.

| Band | Taxonomic Reference ID | Phylogeny Group | Homology % |
|---|---|---|---|
| **1** | *Alcaligenes* sp. HQ670710 | β-proteobacteria | 100% |
| **2** | uncultured *Xanthomonadaceae* bacterium EU640674 | γ-proteobacteria | 100% |
| **3** | Uncultured bacterium EU826797 | proteobacteria | 99% |
| **4** | uncultured *Pedobacter* sp. HQ132437 | Bacteroidetes | 100% |
| **5** | Alpha proteobacterium FR691422 | α-proteobacteria | 100% |
| **6** | *Sphingomonas* sp. FM997989 | α-proteobacteria | 99% |
| **7** | *Rhodoplanes* sp. GQ369128 | α-proteobacteria | 100% |
| **8** | *Acidovorax* sp. DQ922753 | β-proteobacteria | 100% |
| **9** | uncultured *Xanthobacter* sp. FN594647 | α-proteobacteria | 99% |
| **10** | *Rhodopseudomonas* sp. FN813495 | α-proteobacteria | 100% |
| **11** | uncultured Beta proteobacterium HM534390 | β-Proteobacteria | 100% |
| **12** | Uncultured *Micrococcineae* bacterium HM346348 | Actinobacteria | 100% |

**Tab 3.16** Taxonomic characterization of the major DGGE bands

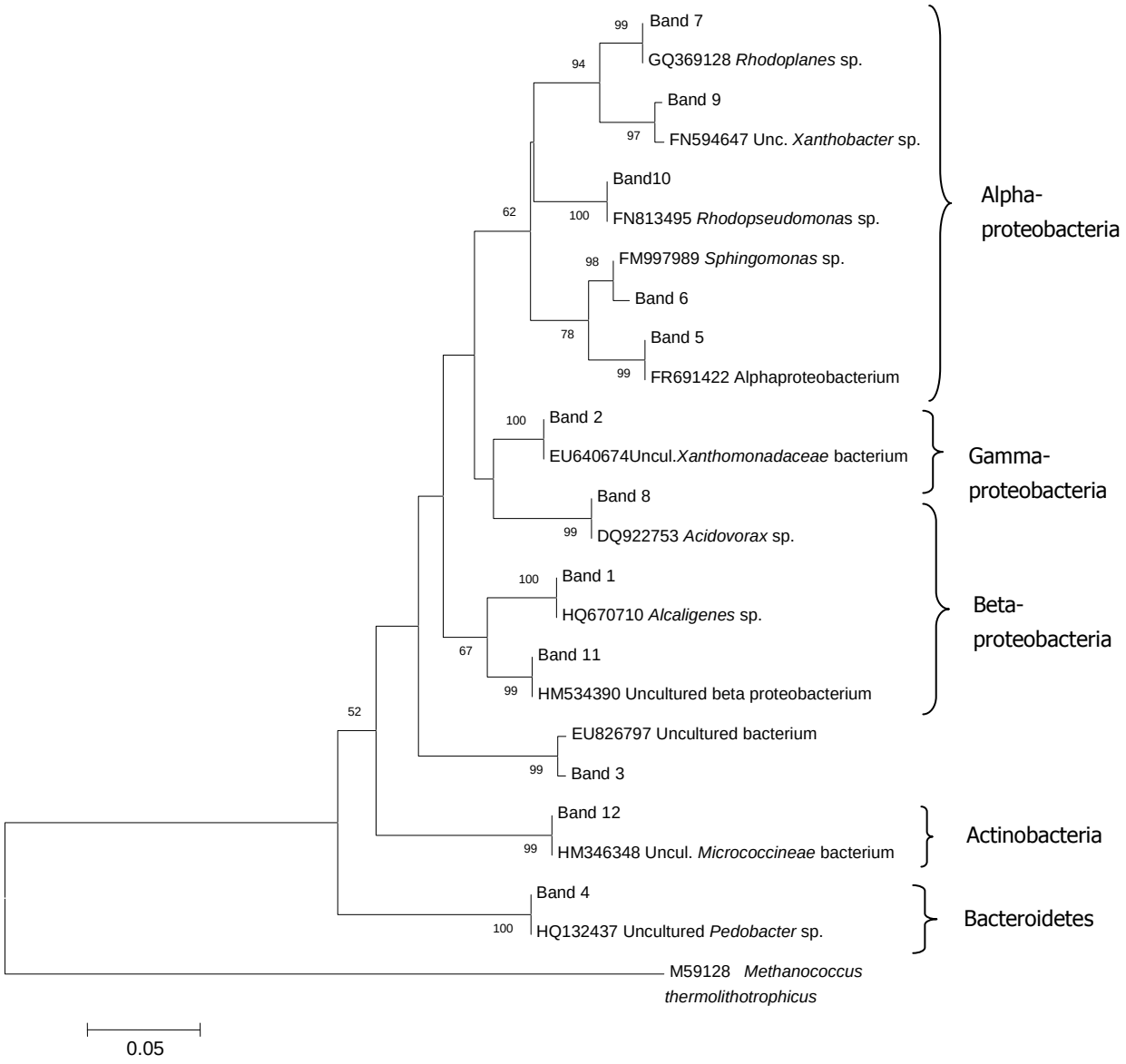



**Fig 3.47:** Phylogenetic neighbour-joining tree of the V3 sequences obtained from excised bands from DGGE analysis and their closest database relatives with Species Name and GenBank accession numbers. Bootstrap values (*n*_1,000) above 50% are indicated at the nodes. The scale bar represents genetic distance (nucleotide substitutions per site).

## 3.3 Comparison of culture-dependent and culture-independent study

Considering the fact that 0.1-10% of the total soil population is estimated to be possibly cultured by applying cultivation techniques, a molecular approach is important as it allow to target the >90% of the genetic diversity present in this population, which was lost owing to difficulties in enriching and isolating microorganisms (Daniel, 2004; Marschner *et al.*, 2004).

On the other side 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 may cause 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 minimizing chimera formation. Conversely, populations may be underestimated because all sequences are not amplified equally or because gel resolution is low (Joynt *et al.*, 2006). Nevertheless, as shown in the study here presented, DGGE is a useful screening tool that allows evaluating the selection for specific populations and distinguishing samples with diverse community structure.

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

The results from the DGGE profiles indicated the selection of dominant members within the autochthonous bacterial community and reflected the distinct contamination associated to the sampling point and the soil heterogeneity. Soil in fact is a non-uniform, heterogeneous environment in which the distribution of microorganisms is not ubiquitous (Becker *et al.*, 2006). In particular an exclusive profile was associated to the Hot spot - characterized by the highest contamination level detected within the Ex-SLOI area - therefore indicating a peculiar microbial population.

The DGGE results also indicated a reduction of ribotype richness compared to non-contaminated soil. However although the level of diversity was much lower than those detected in agricultural soils (Torsvik *et al.*, 2002), the observed metacommunity diversity was consistent with reductions observed in soils contaminated with heavy metals (Feris *et al.*, 2003; Becker *et al.*, 2006). Moreover a high biodiversity was detected within the culturable microbial community by the culture analysis performed, suggesting a good adaptation of the tolerant community established within the area.

Furthermore data obtained by the molecular analysis performed on both the metagenomic soil DNA and on isolated strains – along with the MIC determination – indicated heavy metal resistances within the autochthonous microbial community with strains reporting multiple resistances. The metagenomic molecular analysis also identified a degrading potential towards hydrocarbons within the microbial community.

It is interesting to notice as the results of the molecular analysis were consistent with those obtained by the culture approach. Indeed the sequencing results obtained for some of the major bands indicated the predominance of the Proteobacteria *phylum* at all the sampling points under study, even at the highest contaminated spot identified within the Ex-SLOI area.

Hence the results obtained by the molecular analysis - therefore independent from the microorganisms' culturability - supported the prevalence of the Proteobacteria *phylum* previously reported in the culture study.

In particular the direct sequencing from the molecular analysis detected the prevalence of Alpha class. Even if this class was less represented than the Gamma-proteobacteria class in the culture analysis,

nevertheless six different genera were isolated in the highest polluted point II by itself, confirming its great resistance and potential (par 3.1.2.2).

Moreover the molecular study allowed the detection of a member of the Bacteroides *phylum***,** detected in the most contaminate Point II, confirming a high representation and resistance of the gram-negative bacteria within the soil community under examination. This is in agreement with the prevalence of gram-negative organisms in metal-contaminated soils reported in literature (Lorenz *et al.*, 2006; Feris *et al.*, 2003; Hui *et al.*, 2009; Cavalca *et al.*, 2010; Zhang *et al.*, 2007a).

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.

At the same time the molecular study also confirmed a high resistance associated to the gram-positive Actinobacteria - even if less represented in comparison to the gram-negative Proteobacteria - with the identification of one component in the Hot spot profiles. Actually in both approaches were detected genera characterized by heavy metal resistances, reporting degrading potential even of recalcitrant organic compounds and PAH, used in bioremediation protocols and exerting plant growth promotion. These results point out therefore the selection in the Ex-SLOI area towards a soil autochthonous microbial community with high resistance and bioremediation potential even in the perspective of a Bioremediation/Phytoremediation approach.

# 4. Results and discussion Chapter II: Study of *Brassica juncea* and *Apocynum cannabinum* plants in relation to organic and inorganic Pb contamination, in the context of a Phytoremediation enhanced by microorganisms approach

Phytoremediation refers to the use of green plants to remove, contain or render the pollutants harmless. In comparison to conventional methods it is not only a cost-effective and convenient strategy, but it is also not destructive and invasive towards the soil structure and its biological activity, leading to a long-term applicability and efficacy in cases of moderate as well as extensive contaminations (Manousaki *et* Nicolas, 2009; McGuinness *et* Dowling, 2009). This technology relies indeed on natural processes by which plants and their microbial rhizospheric cenoses can degrade, transform or sequester the pollutants.
A phytoremediation study on the soil under examination is of particular interest. The contamination of the examined area combines in fact both inorganic and organic Pb, and very few investigations have been performed in relation to the latter, especially in a phytoremediation approach. Moreover even if various studies have been reported on phytoremediation trials in relation to inorganic Pb, the majority implies the addiction *ad hoc* of the contaminant rather than studying a long-term contaminated soil as the present study (Becker *et al.*, 2006).
In the context of this kind of contamination phytoextraction and phytodegradation are the main involved processes of phytoremediation. Actually, phytodegradation exploits plants to degrade - i.e. mineralize - organic pollutants by means of their own enzymatic activities or by microorganisms to them associated. In the rhizosphere in fact, rich in exudes from the roots and organic matter, there is an enhanced microbial population, 100-1000 times higher than in the bulk soil (Maier *et al.*, 2009). On the other side the presence of bacteria, in particular of PGPR, promotes the growth of plants even in stressed conditions as in contaminated soils (Reed *et al.*, 2005; Jing *et al.*, 2007; Khan *et al.*, 2009). Indeed - as previously reported in par. 1.2.2.4 - PGPR are able to synthesize compounds such as hormones, facilitate the uptake of nutrients from the environment or to prevent plant diseases (Glick, 2003; Príncipe *et al.*, 2007). Moreover soil microorganisms are also known to be able to affect metal mobility and availability to the plant, through acidification, redox changes or by the production of siderophores and/or mobilizing the metal phosphates, thus contributing to contaminant uptake by the plant and making phytoremediation more efficient (Jiang *et al.*, 2008; Jing *et al.*, 2007; Rajkumar *et al.*, 2010). Phytoextraction indeed specifically refers to the use of pollutant-accumulating plants that can extract and translocate contaminants to the harvestable portions, which can then be removed from the site.
This option however is only viable in sites with moderate contamination. Recently, the coupling of phytoextraction with other soil treatments (e.g. soil washing, soil vapor extraction) is gaining interest; this may be especially useful in cases where mixed or high level contamination necessitate the use of more than one technique to effectively remediate the site, as the examined one Ex-SLOI area (Marchiol *et al.*, 2004).
It is worth noting that in a combined approach the Phytoremediation process not only contributes to a further removal of the contaminant but even to the restoration of the soil structure and biological activity affected by the application of effective but invasive physical/chemical techniques.

## 4.1 Plants species under study

The ideal plant for use in phytoextraction should have various traits, namely the ability to accumulate the targeted metal preferably in the aboveground parts, tolerate high metal concentrations in soils, to easily grow as an agricultural crop and be fully harvestable and to be characterized by fast growth and high biomass.

The biomass of plants has in fact a critical role in the total metal removal, being directly proportional to the phytoremediation efficiency. Therefore for phytoextraction to be practical a high biomass and a very high metal accumulation in the plant tissues should be coupled, while unfortunately no wild plants or crops have yet been identified that combine both properties (Marchiol *et al.*, 2004; Karami *et* Shamsuddin, 2010).

Plants with extraordinary ability to accumulate the contaminants, known as hyperaccumulators, have usually a small above-ground biomass, slow growth and a long maturity phase (Karami *et* Shamsuddin, 2010). Moreover, even if hundreds of plants are recognized worldwide as metal hyperaccumulators, among these only few plants are reported to accumulate lead (Kapourchal *et al.*, 2009). Hence a proposed approach is the use of tolerant plants with a relatively higher accumulation ability as compared to most other plants (but with lower ability as compared to hyperaccumulators) and high biomass such as corn, rice, sunflower and Indian mustard (*Brassica juncea*) (Marchiol *et al.*, 2004; Karami *et* Shamsuddin, 2010).

Taking into account all these factors two plants have been chosen in this study: *Brassica juncea* and *Apocynum cannabinum*, reported to be able to accumulate respectively inorganic and both organic and inorganic lead.

Heavy metal accumulator species belonging to the *Brassica* family have in fact been suggested for Phytoremediation and *Brassica juncea* (Indian mustard) - annual easily cultivated high biomass crop plant - can tolerate various toxic conditions. It has been object of many literature studies and, although it is not a hyperaccumulator, it has been reported to efficiently accumulate various metals including inorganic Pb (Kumar *et al.*, 1995; Liu *et al.*, 2000; Kapourchal *et al.*, 2009). This plant can not only absorb large amount of lead in its root, but it is also reported to transport the absorbed lead from roots to its shoots, which is an important characteristic for phytoextraction purpose (Kapourchal *et al.*, 2009). On the other side at the best of our knowledge for this plant no studies have been performed in relation to organic Pb.

Considering *Apocynym cannabinum* (Indian hemp), this plant is an interesting quite new subject of study on which only few literature data are available. It is reported to be able to accumulate Pb in concentrations from about 100 mg/kg d.w in shoots and its use has been suggested to economically recover soil containing organic or inorganic lead (Cunningham, 1994; Lasat, 2000). However at the best of our knowledge no further literature data are available.

The study presented in this second part of the PhD Thesis was aimed from one side at studying the behavior and basic potential of phytoextraction of *Brassica juncea* and *Apocynym cannabinum* in relation to the combined contamination of inorganic and organic Pb. On the other side this study was undertaken - in particular in the framework of a lab-scale trial - to evaluate the interaction of the autochthonous micro flora established within the examined area and its possible synergistic role with these plants in relation to both inorganic and organic Pb contamination, in the context of a Phytoremediation enhanced by microorganisms.

Therefore a lab-scale and a field scale trials were performed with these two plant species and the soil of each of the 3 sampling points (I,II,III) chosen within the Ex-SLOI area.

Seeds of *Brassica juncea* (*L.*) Czern (Accession Number 173874 – USDA, North Central Regional Plant Introduction Station, Iowa State University, Ames, Iowa, USA) and *Apocynum cannabinum* (3920, Bonn

University - Botanical Gardens, Germany) were sown directly in pots prepared in glasshouse and in parcels set-up at the Ex-SLOI area in Trento. Data on the biomass growth of these species are reported, and to test their phytoextraction potential Pb concentrations in plant parts were determined and bioaccumulation coefficients calculated.

The study on a third plant - Hybrid poplar – has been started. This arboreal plant is characterized by a huge biomass production and an extensive root system, while at the same time it is reported to accumulate metals (Di Lonardo *et al.*, 2010). The study performed in a biaugmentation protocol with selected components of the autochthonous community is still in progress, thus no data on this experiment are reported in this document.

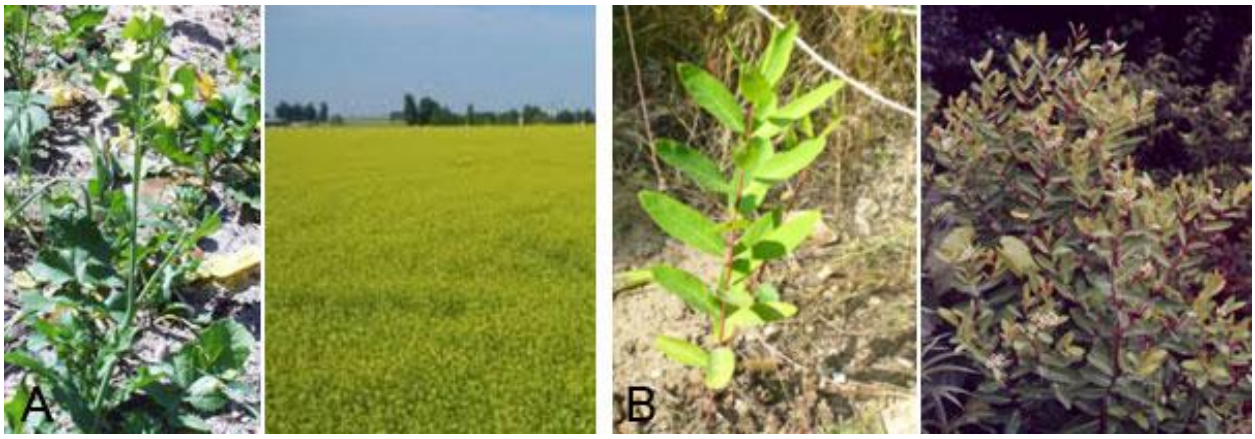



**Fig 4.1** The two examined plants: *Brassica juncea* (A) and *Apocynum cannabinum* (B)

## 4.2 Lab-scale Phytoremediation trial

A lab-scale trial was performed in glasshouse - at temperature ≥ 24°C and with a photoperiod of 12 hours - with the two plants *Brassica juncea* and *Apocynum cannabinum*. Mesocosms of 1 kg were set-up in triplicate with the soil collected within the Ex-SLOI area - at the 3 examined sampling points I, II and III (par 2.2) - and in parallel a series of controls was prepared by using the same soil sterilized, as described in par 2.7.2, and shown in Fig. 4.2.

Actually this lab-scale study focused on the interaction between the examined plants with the soil microbial autochthonous micro flora selected by and adapted to the inorganic and organic Pb contamination present in the area for over half a century, to evaluate its actual role and the possible effect on the phytoremediation processes.

Experiments set up with *B. juncea* were carried out for four months and plants were collected after 2 (T1), 3 (T2) and 4 (T3) months from sawing; on the other hand tests performed with *A. cannabinum* were carried out for six months with a unique sampling at the end of the trial (Tf), due to a slower growth rate of this plant.

Moreover for each plant controls were set-up using agricultural soil, this allowed a comparison between plant growth in the contaminated soil of the former industrial Ex-SLOI area and in an agricultural uncontaminated soil. Moreover with both untreated and sterilized soils control mesocosms with no plant were set-up in duplicate for each sampling point in analysis.

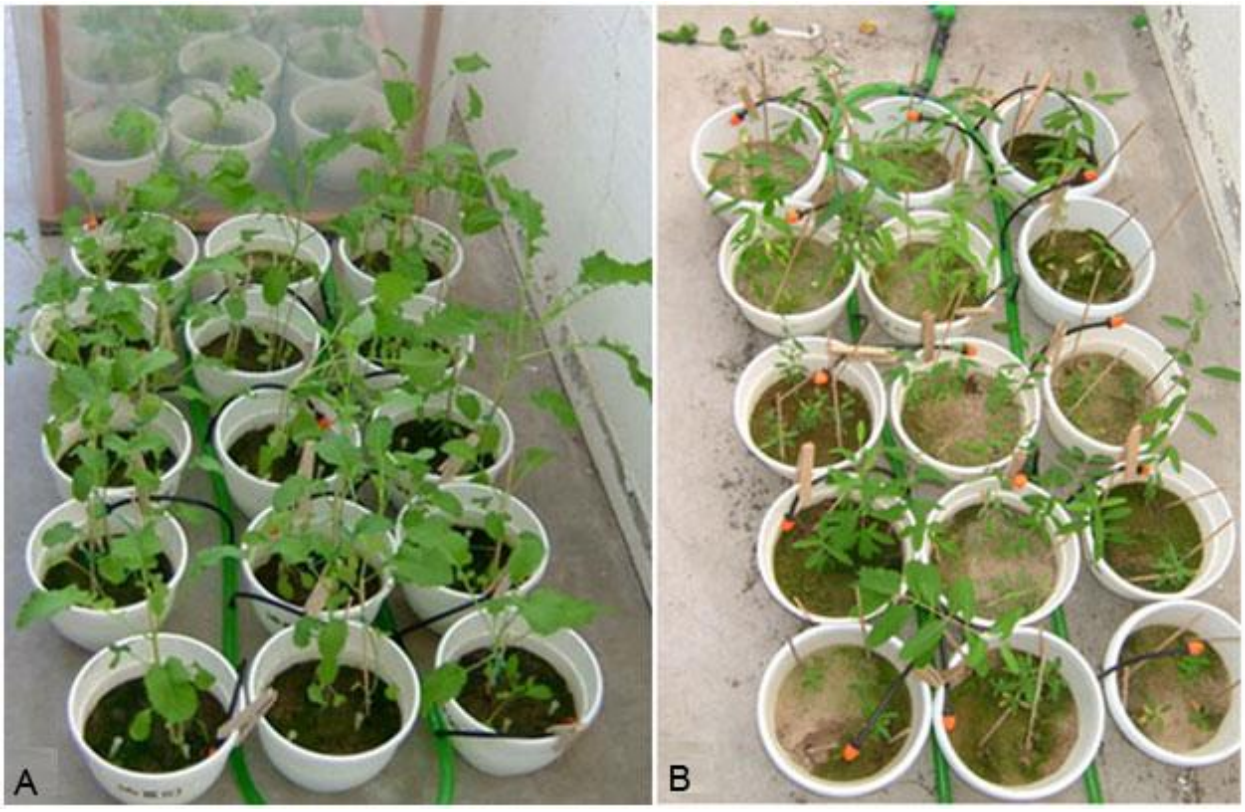



**Fig 4.2** Lab-scale mesocosms with *Brassica juncea* (A) and *Apocynum cannabinum* (B)

### 4.2.1 Enumeration of the culturable rhizosphere community

At each sampling time microbial counts were determined in Nutrient and R2a media for rhizosphere soil samples, in order to monitor the dominant eubacteria population - as detected in the previous microbial counts of the sampling points in analysis (par 3.1.1) - along the experiment and the effect of plant growth on the rhizosphere micro flora charge.

Results obtained for untreated mesocosms (fig 4.3-4.5) showed a $\log_{10}$ value for the eubacterial population of about 6, in agreement with the previously reported data of microbial charge at the 3 sampling points (I, II, III) reported in par 3.1.1.

Considering the sterilized mesocosms, the values obtained at the beginning of the experiment (T0) confirmed the almost total lowering of the microbial charge obtained by the thermal treatment applied (fig 4.3-4.5). However in autoclaved mesocosms it was observed - as expected in the unsterile glasshouse environment - an increase of the eubacterial population along the experiment. Moreover within the sterilized mesoscosms set-up with *B.juncea* a slightly higher charge was reached in comparison to the untreated mesocosms*,* probably in connection to a lower nutrient and niche competitions met by colonizing microorganisms in relation to the almost zeroing of the autochthonous micro flora. Further contribute to the observed microbial charge could be connected to volatilization events affecting organic Pb fraction and to possible alterations of metal biodisponibility implied in the thermal-sterilization (Genthe, 1998).

On the other side a microbial charge's discrepancy between sterilized and untreated mesoscosms was detected neither in *A.cannabinum* mesocosms nor in unplanted mesocosms, where a lightly overall lower charge was detected in comparison to *B.juncea* sterilized mesocosms (fig 4.3-4.5.). This suggested therefore a low influence and impact of the thermal treatment on soil characteristics and conditions.

In particular in untreated mesocosms set-up with *B.juncea,* a $\log_{10}$ value of about 7 was generally reached along the trial and it confirmed the positive influence of plant growth on rhizosphere population (Fig 4.3).

As expected a lower microbial charge was detected in the unvegetated mesocosms (fig 4.5), according the enhanced microbial population in comparison to the bulk soil reported in plant rhizosphere rich in exudes and organic matter (Lasat, 2000; Maier *et al.*, 2009). On the other side, even if in *A.cannabinum* mesocosms was observed an increase of the eubacterial population along the experiment, not significant difference was detected in comparison to that observed in unplanted mesocosms at the same sampling time (Tf in fig 4.4 and 4.5). This could be connected to the lower and slower growth of this plant, and to differences in amount and composition of root exudates, which appear to be key drivers for the differences in rhizosphere community structure (Marschner *et al.*, 2004).

In mesocosm control (CTR) – set up as mean of comparison between plant growth in the perturbed contaminated soil and in an agricultural soil - and in unplanted mesocosms, values were registered at the beginning and at the final sampling of the trials (Fig 4.3, 4.4). As expected a higher microbial charge of $10^8$ was associated to the unperturbed agricultural soil, with a light increase associated to both plants' growth.

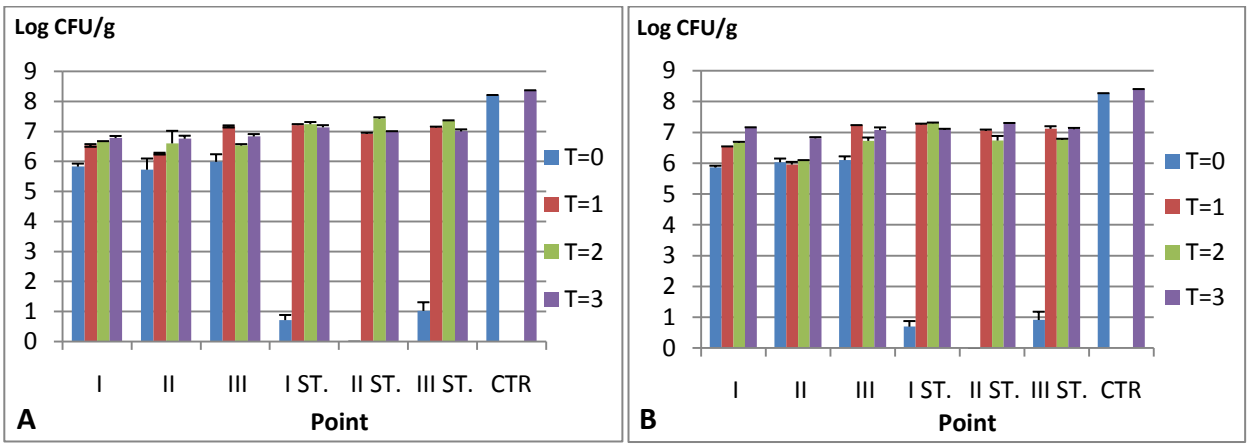



**Fig 4.3**: Microbial counts on Nutrient (A) and R2a (B) media performed at each sampling time for the *B. juncea* untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms

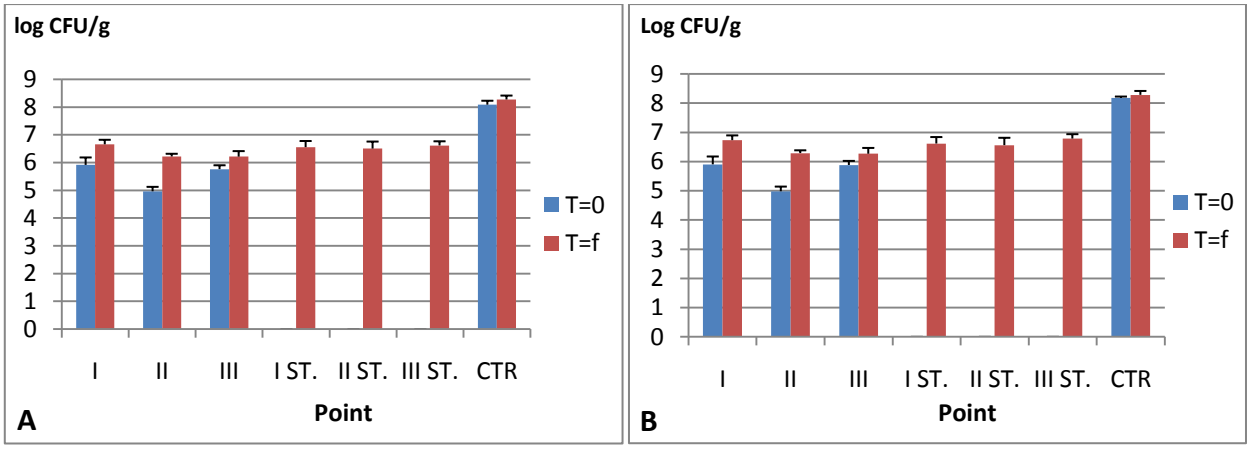



**Fig 4.4**: Microbial counts on Nutrient (A) and R2a (B) media performed at each sampling time for the *A. cannabinum* untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms

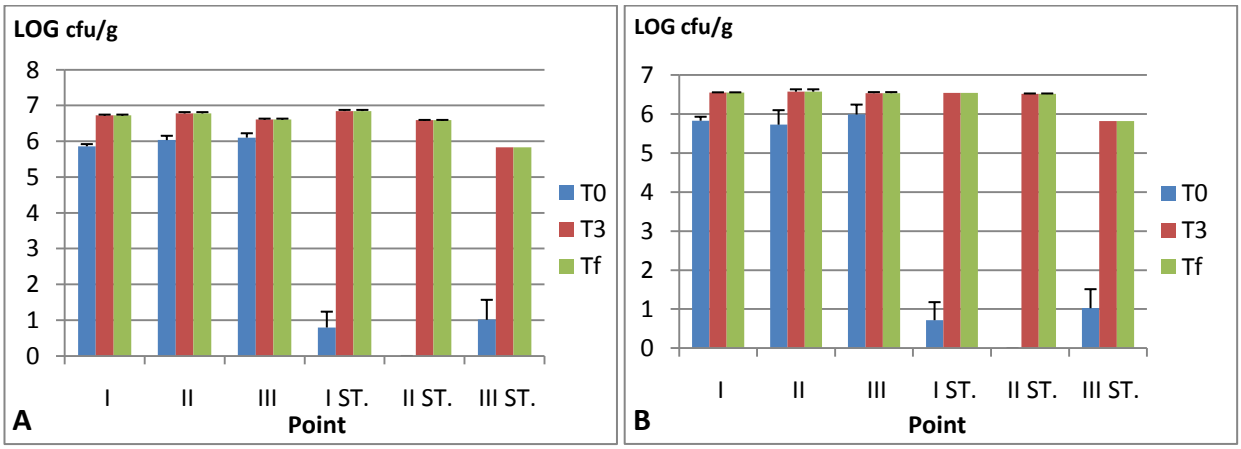



**Fig 4.5**: Microbial counts on Nutrient (A) and R2a (B) media for the unvegetated control mesocosms - untreated (I,II,III) and sterilized (IST, IIST, IIIST) - performed at T0 and in parallel with the final sampling of *B.junca* (T3) and of *A.cannabinum* trial (Tf)

## 4.2.2 Seedlings survival and plant biomass production

During the lab-scale trial for both plants a generally higher biomass production and vegetative luxuriance could clearly be noticed looking at plants of untreated mesocosms, suggesting from a first approximate comparison a positive synergy of autochthonous micro flora exerted on plant growth (Fig 4.6).

Entering into a more precise analysis and first considering the germination percentages of the 2 plants, in all mesocosms much higher values (around 80%) were observed in *B.juncea* while a much lower germination (< 40%) was detected in *A.cannabinum* (fig 4.7). This was a quite expected difference comparing crop to wild plant.

As far as the seedling survival is concerned, for both plants a high percentage was detected and higher values up to 100% were reached in untreated mesocosms; moreover no significant difference was detected in comparison to controls set-up with uncontaminated agricultural soil (fig 4.8).

It is interesting to notice as generally in both plants slightly higher values of both germination and seedlings' survival (fig 4.7, 4.8) were detected in untreated mesocosms, and therefore in association to the autochthonous micro flora estabilished in the soil at the EX-SLOI area. This can be connected to the presence of PGPR within the autochthonous micro flora - accordingly to the PGP traits analysis performed on isolated members of the indigenous community reported in par 3.1.6.1 - synergistically able to promote seed germination and plant growth in stress conditions as in heavy metal contaminated soils (Reed *et al.*, 2005).

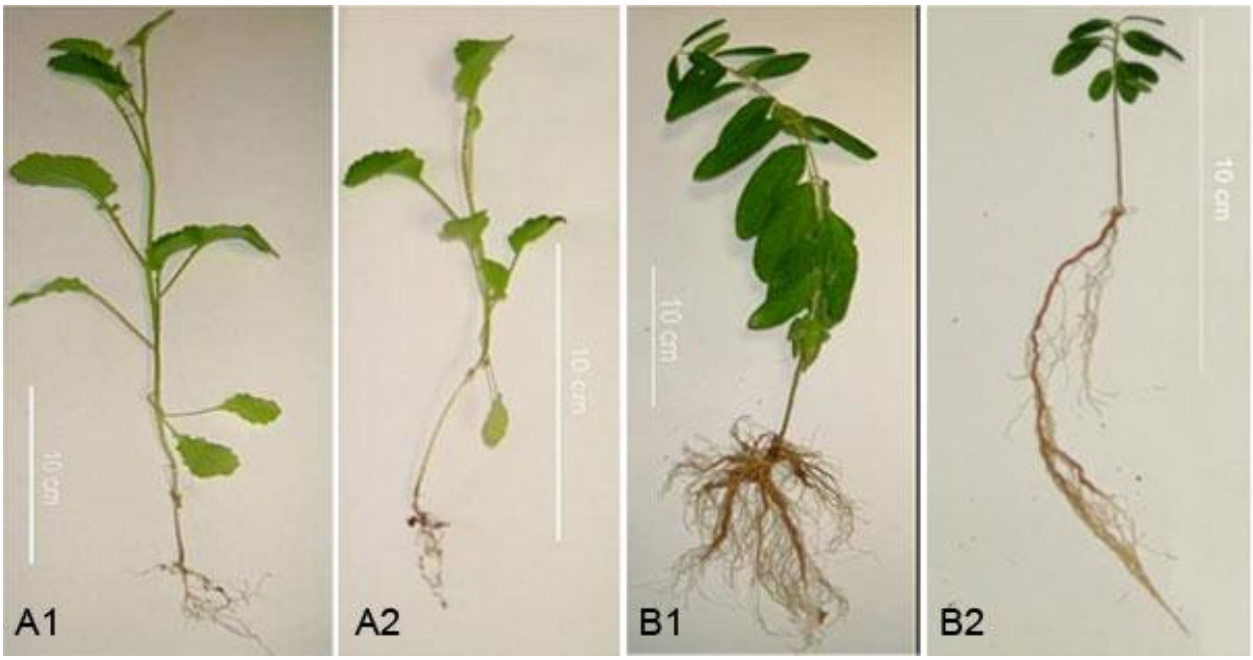



**Fig. 4.6** –*B.juncea* (A) and A.*cannabinum* (B) in untreated (1) and sterilized (2) mesocosms of the same sampling time for each plant

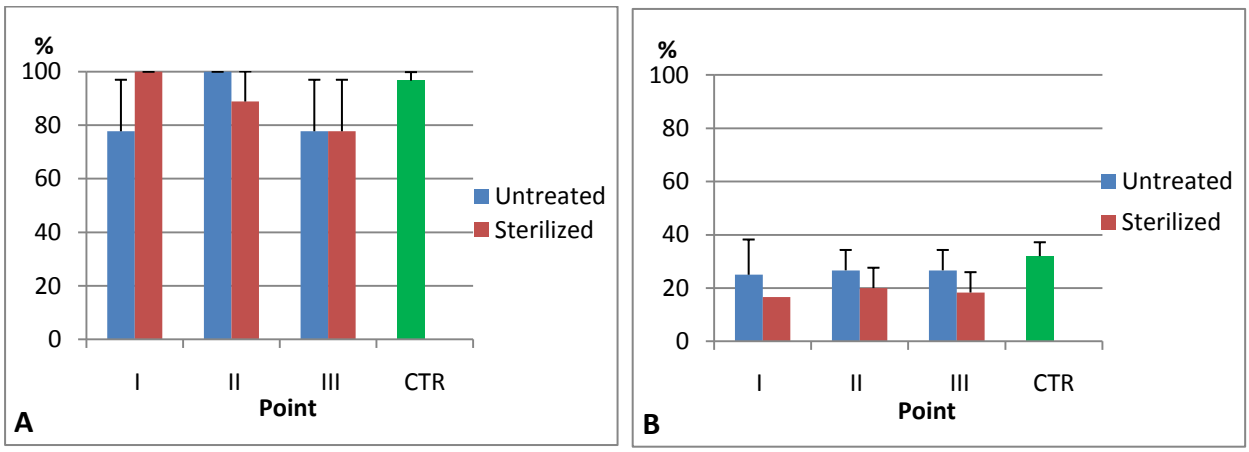



**Fig. 4.7** Germination % of *B.juncea* (A) and A.*cannabinum* (B) in untreated and sterilized mesoscosms

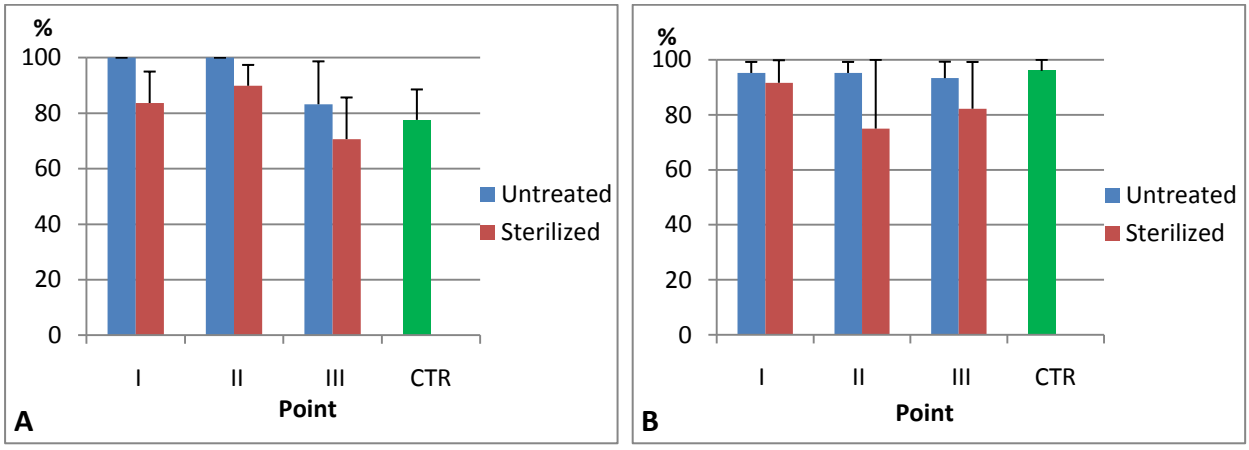



**Fig. 4.8** Seedlings survival % of *B.juncea* (A) and A.*cannabinum* (B) in untreated and sterilized mesoscosms

As far as plant biomass production is concerned, dry weight of plant tissues (roots and shoots) was recorded at the three different sampling times T1 (2 months from sawing), T2 (3 months from sawing) and T3 (4 months from sawing = the end of the trial) for *B.juncea*, and at the only final sampling (Tf) after 6 month from sawing for *A.cannabinum*. The results obtained are shown in Fig. 4.9, 4.10 and 4.11.

Considering biomass production for *B.juncea*, plants grown in untreated mesocosms seemed not to be much affected by Pb contamination (Fig 4.9), as similar biomass production was detected in untreated and control mesocosms set-up with agriculture uncontaminated soil - with the exception of the most contaminated point II.

On the other side a higher reduction of biomass production was detected in sterilized mesocosms – where a new microbial community has been establishing in the soil - in comparison to both control and corresponding untreated mesocosms. In particular for sterilized point III mesocosms, no sampling was collected at T2 as the majority of a mesocosm plants were died (Fig 4.9).

Even if the values registered for shoots and roots were respectively lower than 1,2 g d.w. and than 0,2 g d.w (fig 4.10), it has to be noted that values are expressed per plant and results obtained are in agreement to those reported in literature (Quartacci *et al.*, 2006; Sheng *et al.*, 2008a; Jiang *et al.*, 2008). In this context it has to be considered that *B.juncea* is a crop plant with a vigorous growth, for which more crops and a yield higher than 3 tones/ha/year can be obtained (Schnoor, 1997).

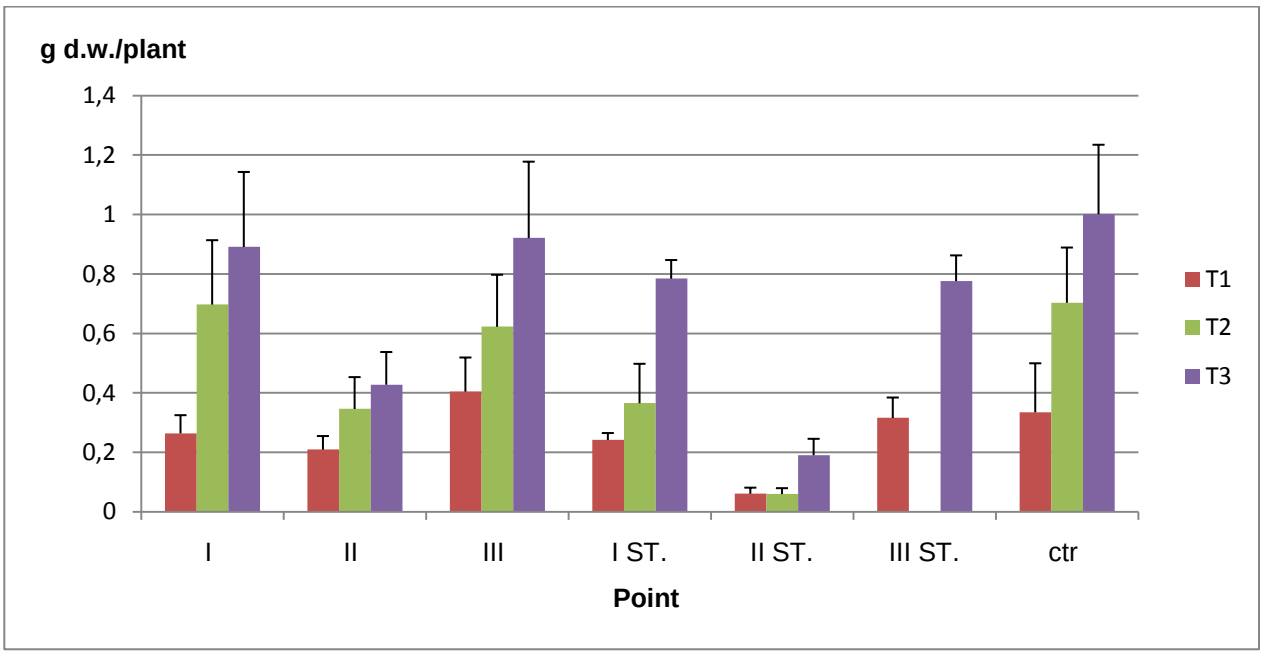



**Fig. 4.9** Total biomass production of *B.juncea* along the trial in untreated (I,II,III) and sterilized (IST, IIST, IIIST.) mesocosms

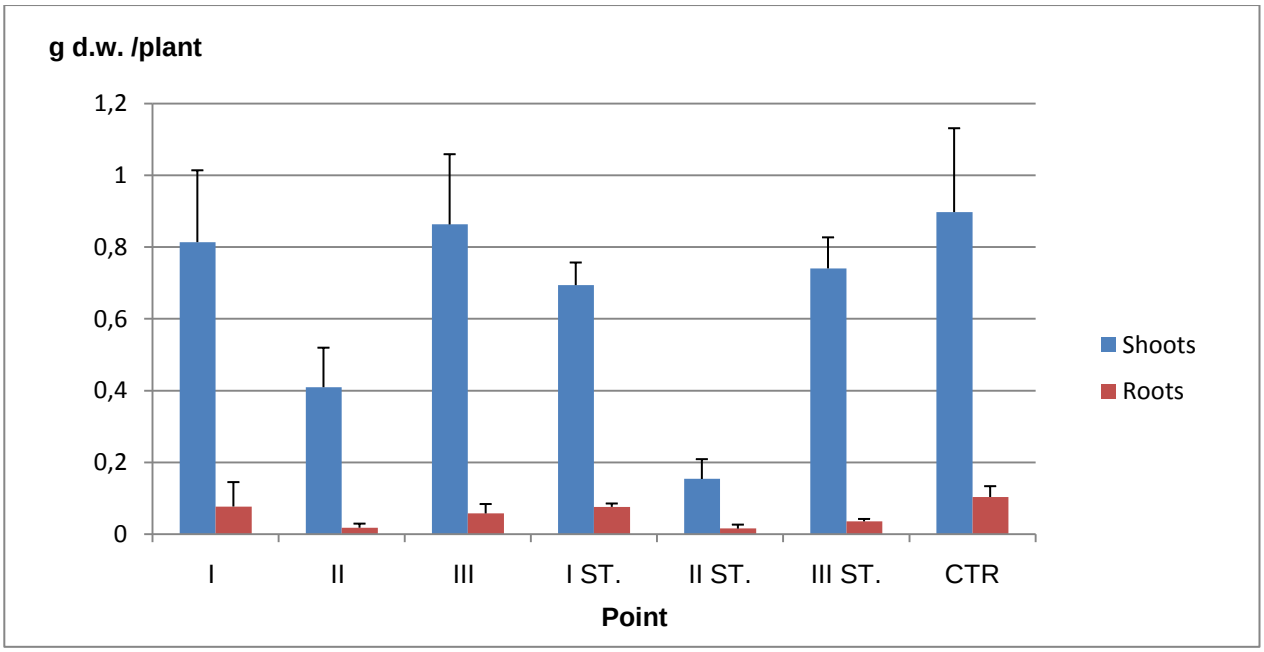



**Fig. 4.10** *B.juncea* shoot and root biomass at the final T3 sampling time in untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms

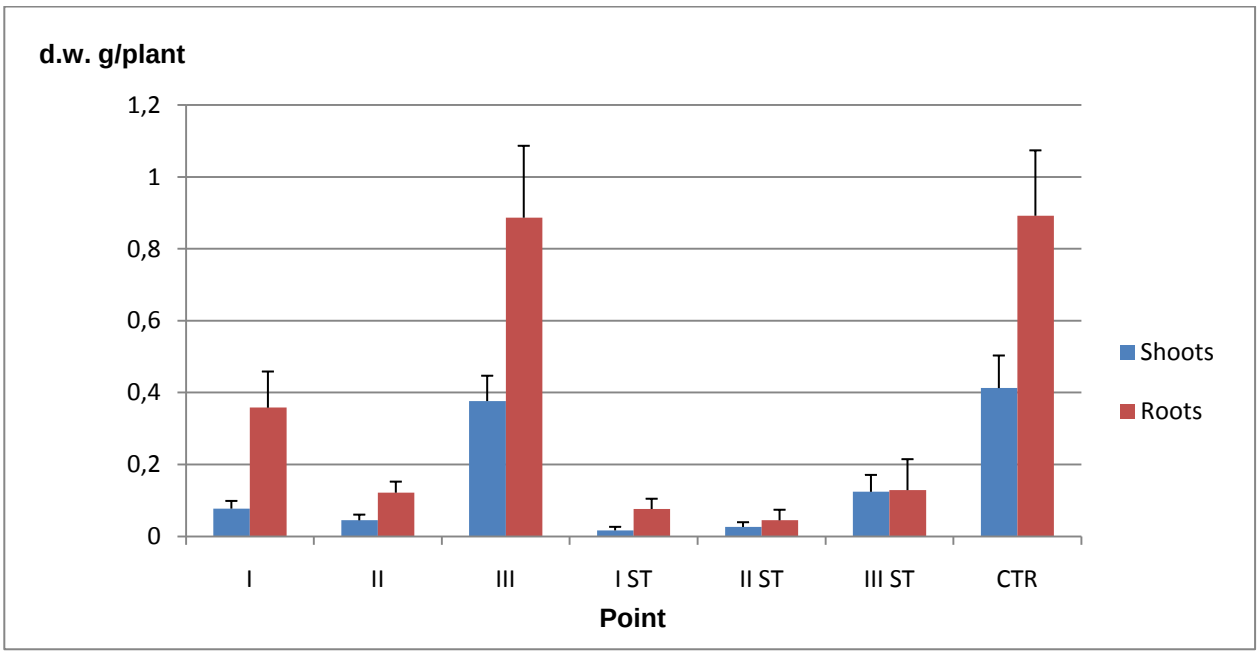



**Fig. 4.11** Shoot and root biomass production of *A.cannabinum* in untreated (I,II,III) and sterilized (IST, IIST, IIIST.) mesocosms at the sampling Tf

As far as *A.cannabinum* is concerned, a low shoot biomass was produced with values generally lower than 0,5 g Dry weight per plant measured after 6 months from the sawing (fig 4.11). Reflecting the results obtained in the germination percentages, a much higher shoot biomass production and growth rate was detected in *B.juncea* in comparison to the *A.cannabinum.*

However it is worth noting a high root production in *A.cannabinum*, not only much higher than its shoots biomass but even of *B.juncea* roots, therefore able to explore and reaching deeper soil levels.

Even in *A.cannabinum* mesocosms a higher biomass was detected in untreated mesocosms (Fig 4.11). In particular at the less contaminated point III a great reduction was detected in the sterilized mesocosms while in the untreated mesocosm it was observed a biomass production comparable to the control set-up with agriculture uncontaminated soil. As for *B.juncea* a lower biomass was detected in the mesocoms with point II soil, which is in fact the most polluted.

As previously detected at the seedling stage, for both plant species it is interesting to notice a higher biomass production value in plants grown in untreated soils rather than in the sterilized ones (Fig.4.9-4.11), where however along the trial a microbial community of the same order of magnitude has established. These results therefore point out a positive influence on plants' growth of the soil autochthonous micro flora seclected by and adapted to the contamination present in the Ex-SLOI area, which exerts a plant growth promoting action in the stressed condition of a Pb contaminated soil.

Improvement in the growth of seedling exposed to Pb contamination was in fact observed in presence of *Pseudomonas* strains, genus predominantly identified within the autochthonous micro flora under study together with various genera reporting PGPR activities (par 3.1.6.1) (Karami *et* Shamsuddin, 2010; Sheng *et al.*, 2008b).

Actually rhizobacteria belonging to different genera identified within the examined indigenous micro flora (par.3.1), such as *Pseudomonas*, *Microbacterium*, *Agrobacterium* and *Arthrobacter,* have plant growth promoting characteristics that can reduce stress symptoms in plants and even potentially support heavy metal uptake (Sheng *et al.*, 2008a). Besides plants treated with ACC deaminase-containing PGPR - also detected within the autochthonous culturable micro flora as reported in par 3.1.6.1 - are much more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful

conditions such as heavy metals (Erturk *et al.*, 2010; Kuffner *et al.*, 2010; Jing *et al.*, 2007; Penrose *et* Glick, 2003).

### 4.2.3 Lead content in plant tissues

Plant samples of at least 0,5 g D.W.(minimum sample weight for the ICP-OES analysis) were analyzed to determine total lead content. *Brassica juncea* roots analysis was not performed as enough biomass was not available. Total Pb content determinations were therefore carried out for *B.juncea* shoot samples collected at the three different sampling times (T1, T2, T3) and on both roots and shoots of *A.cannabinum* collected at the final sampling time (Tf) after 6 month from sawing. The results obtained are shown in Fig. 4.12, 4.13 and 4.14. For point II and III sterilized mesocosms set up with *B.juncea* data at the sampling time T2 are not reported, as not enough biomass was available for the analysis.

As far as Pb content in *B. juncea* shoots is concerned, it is interesting to notice a higher Pb accumulation in plants grown in untreated soils rather than in sterilized ones, with values ranging from about 50 to 250 mg Pb/kg d.w. (Fig. 4.12). Actually comparing untreated and sterilized mesocosms all value reached in the untreated ones were higher, with the only exception for the final sampling T3 of Point I, which showed a slightly higher value in the sterilized mesocosm.

In particular, it was observed that plants grown on point II resulted to accumulate the highest Pb content reaching concentrations up to about 250 mg Pb/kg of d.w., and reflecting the highest contamination level associated to this reference point (Fig. 4.12).

Moreover values obtained were higher than those observed in an experiment with *Brassica juncea* plants associated with a PGPR bacterium (Sheng *et al.*, 2008a) and of those reported in *B.juncea* shoots in presence nitrilotriacetate (NTA) and citric acid amendments to increase Pb mobility (Quartacci *et al.*, 2006). It has although to be mentioned that comparisons of different trials are affected by the influence of variables such as Pb concentration, Pb biovailability and soil characteristics peculiar to each experiment. Nevertheless these results suggest the presence in the Ex-SLOI examined soil of an autochthonous microbial community that positively affects in *B.juncea* both biomass production and Pb phytoextraction.

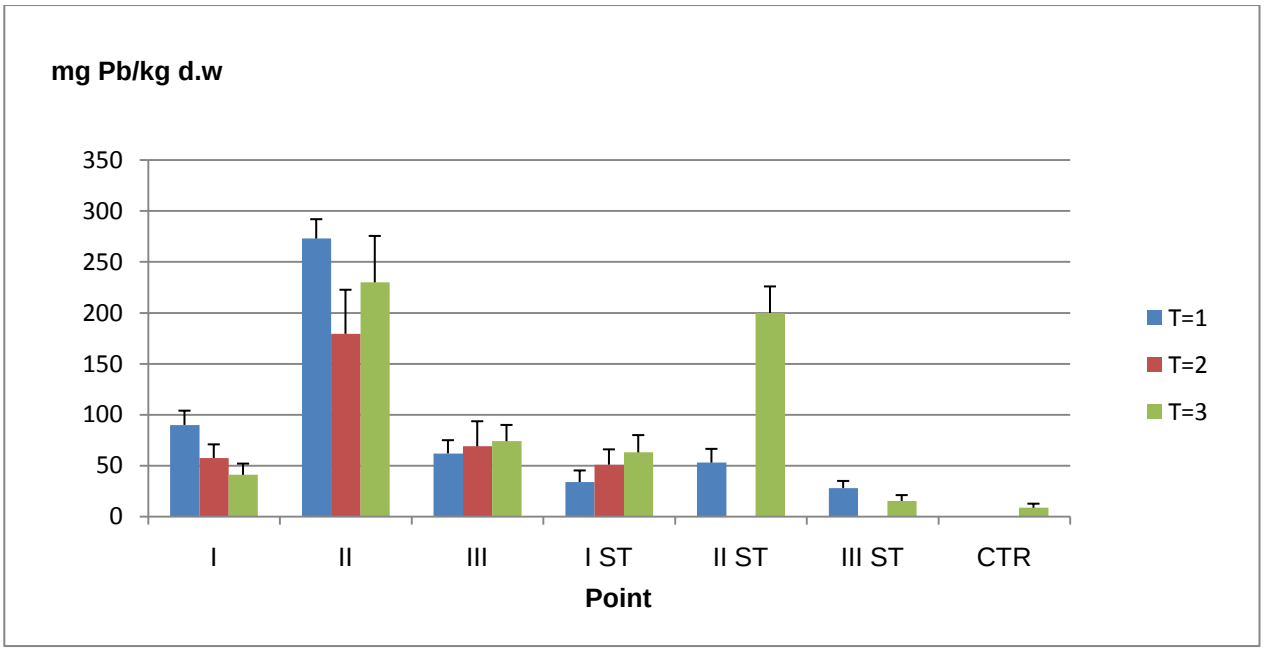



**Fig. 4.12** Total Pb content in *Brassica juncea* shoots in untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms.

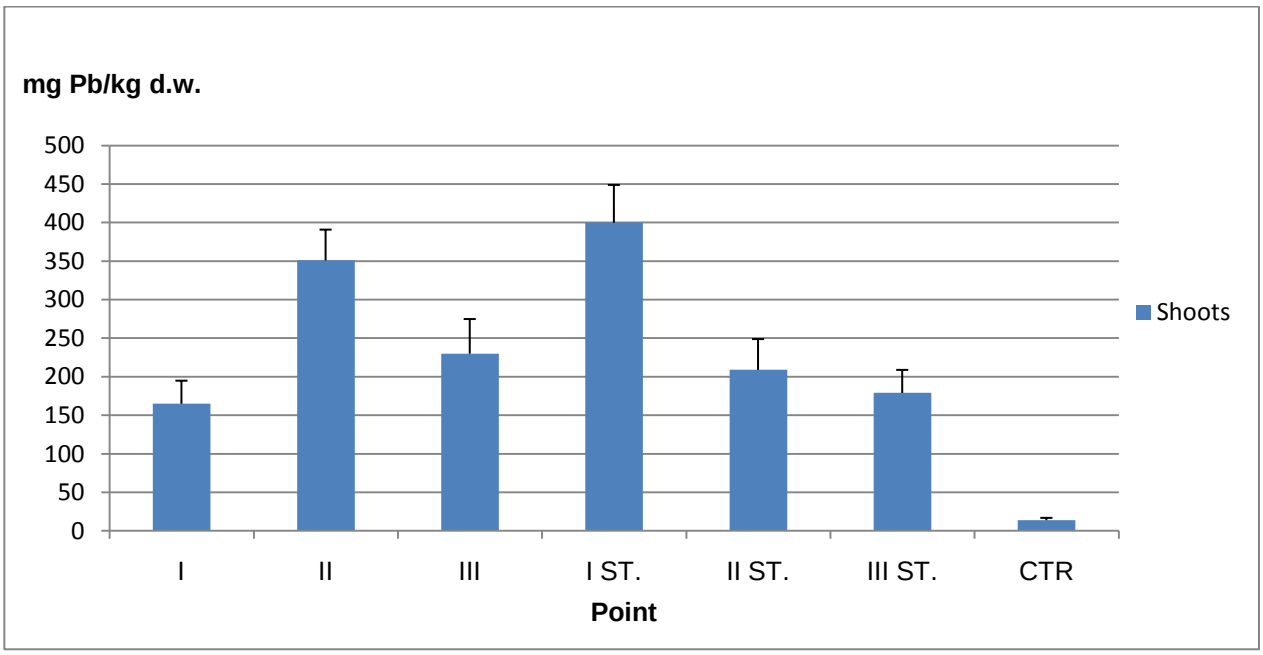



**Fig. 4.13** Total Pb content in *Apocynum cannabinum* shoots in untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms at the final sampling Tf

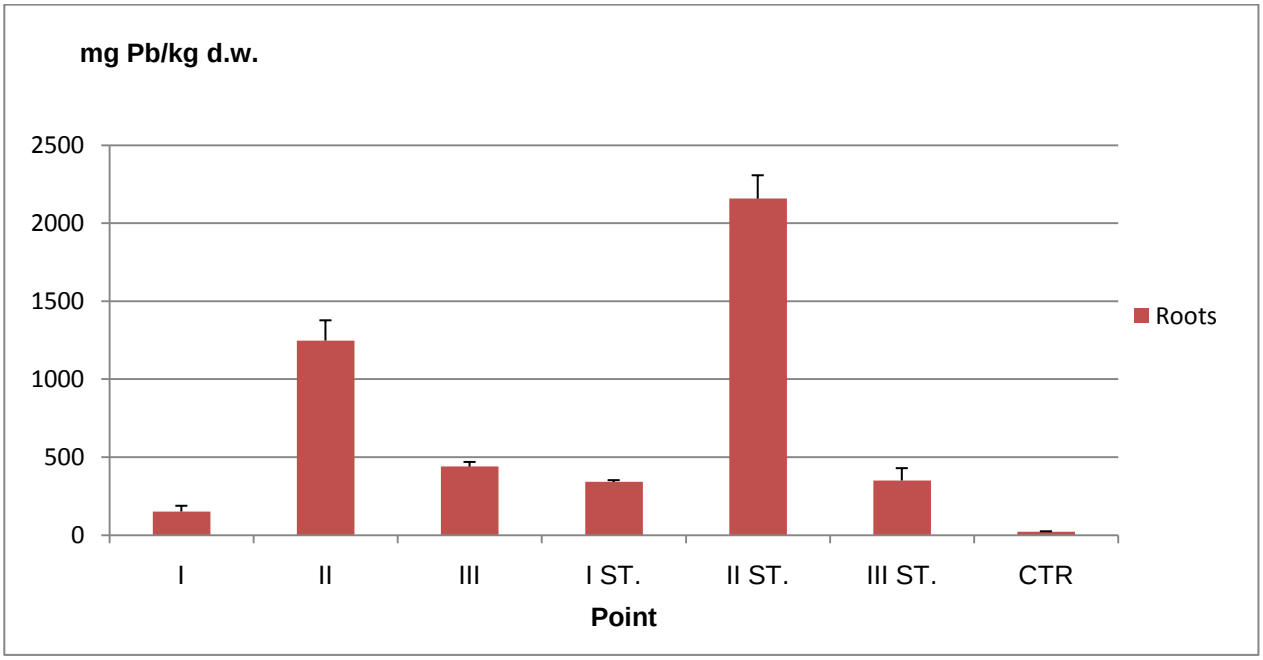



**Fig. 4.14** Total Pb content in *Apocynum cannabinum* roots in untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms at the sampling Tf

As far as *Apocynum cannabinum* is concerned, it reached in its tissues higher concentration than *B.juncea* (Fig. 4.13, 4.14). *A. cannabinum* was in fact is capable of accumulating from about 150 up to 400 mg Pb/Kg d.w. in its epigeous tissues - values especially important in phytoextraction - and higher than those reported in literature (349 mg Pb/kg) for the same plant (Cunningham, 1994).

Moreover really high concentrations were reached in its roots, with values ranging from 150 to 2000 mg Pb/kg d.w, the highest Pb concentration detected in either plant (Fig. 4.14). This evidenced in *Apocynum cannabinum* a preferential accumulation in roots. Pb absorption by roots is a passive process and Pb

translocation from roots to shoots is in fact reported to be limited as Pb binding at root surfaces and cell walls limits its translocation from roots to aerial shoots (Manousaki *et* Nicolas, 2009; Sharma *et* Dubey, 2005). This affects therefore Pb Phytoextraction process since harvesting of the shoots is the main purpose.

On the other side comparing untreated and sterilized *A.cannabinum* mesocosms, alternating results were obtained. For point II in fact a higher shoot concentration was detected in the untreated mesocosm but along with a much lower root accumulation. Conversely to *B.juncea,* for point I mesocosms higher values were registered in sterilized mesocosms while at the less contaminated point III slightly higher but similar values were detected in both mesocosms. Therefore no significant trend and difference could be detected in Pb accumulation at untreated and sterilized mesocosms of this plant species.

On the other side as in *B.juncea* mesocosms, *A.cannabinum* plants grown on Point II reached in general higher Pb concentrations, reflecting the higher contamination's level of this sampling point. In fact a positive relationship between the lead concentrations in soil and that accumulated in plant roots and shoots has been reported, and it was observed that by increasing the lead concentration in soil its accumulation in plant tissues increased (Kapourchal *et al.*, 2009).

Consequently the results obtained showed for both plants high Pb concentration reached within their tissues, moreover a positive influence of the autochthonous micro flora was evidenced on Pb uptake in *B.juncea* mesocosms.

Rhizospheric microorganisms may in fact interact symbiotically with roots to enhance the potential for metal uptake. In addition, some microorganisms may excrete organic compounds which increase bioavailability and facilitate root absorption of essential metals, such as Fe and Mn as well as nonessential metals. They can also influence metal solubility solubilizing inorganic phosphates or by directly altering their chemical properties (Lasat, 2000; Jiang *et al.*, 2008). Indeed soil microorganisms are known to be able to affect metal mobility and availability to the plants and the presence/inoculation of rhizosphere bacteria was reported to increase the uptake of Cd in *Brassica napus* (Sheng *et* Xia, 2006), of Ni in *Alyssum murale* (Abou-Shanab *et al.*, 2007), and significantly improved Cu uptake by *B. juncea* (Ma *et al.*, 2009).

### 4.2.4 Evaluation of Pb content in soil of lab-scale mesocosms

For each sampling time of the mesocosm trial with *Brassica juncea,* soil samples (i.e. the total soil of a mesocosm) were analyzed to evaluate both total Pb content - including both inorganic and organic compounds - and speciation of the organic Pb compounds (Fig. 4.15, 4.17), in order to better study and monitor Pb along the trial. For *A.cannabinum* mesocosms the total Pb content was also determined (Fig. 4.16).

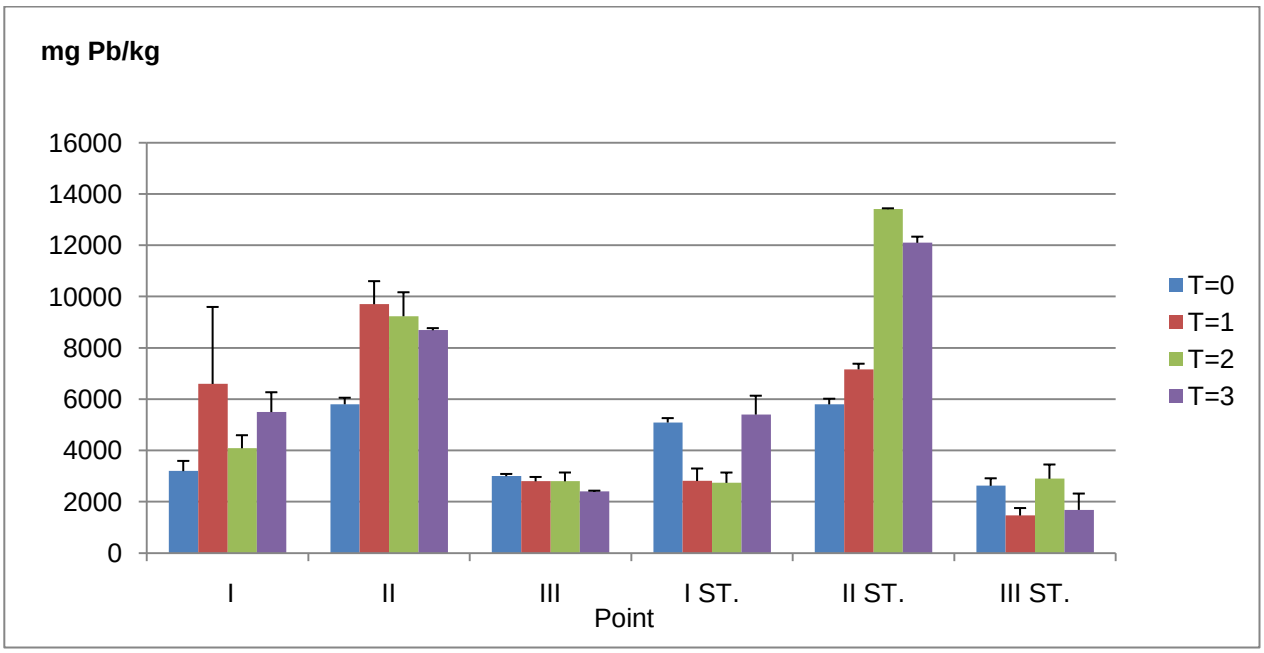



**Fig. 4.15** Total Pb content in soil of *Brassica juncea* untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms along the trial

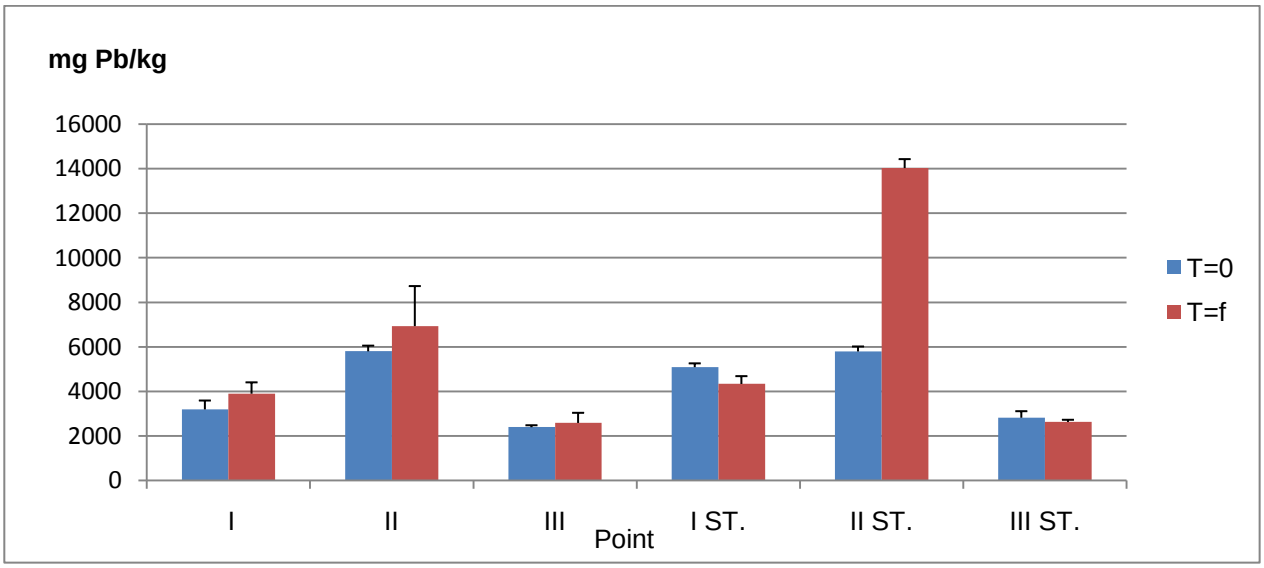



**Fig. 4.16** Total Pb content in soil of *A. cannabinum* untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms

The results obtained showed a highly heterogeneous distribution of soil contamination, reflecting the heterogeneity associated to a complex matrix as soil. Moreover it has to be considered that at each sampling time along the trial all plants and soil from distinct mesocosms were collected. The heterogeneity of soil components led to uncorrelated distinct samplings as the distribution of the different Pb compounds, characterized but different solubility and partitioning, resulted to be highly variable within the soil. TEL is in fact associated and adsorbed to areas of higher organic and humic content, while the soluble alkyl compounds due to water infiltration such as from rainfall can be associated to deeper soil layers of lower humic content (Genthe, 1998).

From the results of total Pb content registered in mesocosms of both plants, Pb uptaken from plants seemed not to affect total Pb content in the soil due to the really high contamination associated to the soil

under examination. Moreover these data reflected the above-mentioned heterogeneity of soil composition and of contaminant distribution.

It has also to be taken in account that a single crop was performed, while one of the most important drawbacks in phytoextraction is the long time that may be required. Neugschwandtner et al. (2008) estimated in fact that in order to obtain the Czech threshold values for Pb (220 mg/kg soil) in contaminated soils under maximum obtained remediation factors, 300 cropping seasons were required (Neugschwandtner *et al.*, 2008). Obviously, operating the huge number of cropping seasons is definitely very costly and time-consuming, thus explaining the interest on a Phytoremediation assisted by microorganisms and on PGPR applications to increase biomass production and Phytoextraction efficiency, and on combined approach with physical-chemical techniques (Karami *et* Shamsuddin, 2010).

As far as the soil concentration of alkyl-lead related compounds in *B.juncea* mesocosms is concerned, data obtained evidenced the presence of alkyl-lead compounds - especially tetraethyl-, triethyl- and diethyl-lead - only in point II (Fig. 4.17), although in the sterilized mesocosm lower levels were detected in connection to the thermal treatment. Actually Point II was located behind the reactor where TEL was produced and is characterized by a higher organic Pb contamination level.

The detection of low organic Pb concentrations - much lower than expected on the basis of data reported from previous analysis and surveys performed by the local authorities (*Provincia Autonoma di Trento*) - is probably connected to TEL volatilization and to chemical-biological degradation processes. TEL in fact is volatile and can be decomposed photocatalytically by UV irradiation to the water-soluble ionic tri- and dialkylated species; this has probably led to the lower concentrations registered in the soil samples collected from sub-surface soil layers. As above-mentioned, probably a main organic Pb accumulation is stocked at lower layers, thus confirming the interest for the study of arboreal species - such as poplar under examination - and of a combined approach of chemical/physical methods for the specific remediation of this area.

On the other side the data obtained confirmed the presence of a really high Pb contamination at three sampling points in exame. Concentration of total Pb even higher than those reported by the previous surveys can in fact be observed, with values from about 5.000 mg/kg to >12.0000 mg/kg deteced in Point II, which is confirmed to be the most contaminated examined point (fig 4.15, 4.16). Values in the range of 1.000-3.000 mg/kg were also detected in the less contaminated Point III.

Considering the results obtained it is interesting to notice for point II - the reference location reporting the higher and detectable organic Pb contamination - a decrease in TEL, triethyl-Pb and diethyl-lead content during the experimental time, along with an increase of ethyl-lead levels (Fig 4.17).

Moreover the presence of ethyl-lead (Fig. 4.17) - the compound of minor alchylation - was registered not only in Point II but also in Point I and III except for T=0. The ethyl-lead accumulation during the experimental time could be due to a progressive degradation that organic lead compounds of major alkylation's grade underwent during the phytoremediation process, probably connected to both transformation promoted by the plant/micro flora system and by chemical/physical processes (Fig.4.17).

The absence of monoalkyl-lead compound at the T=0 and its increase along the experiment together with the di- and trialkyl-compounds confirmed therefore the proposed mechanism of TEL degradation through a series of sequential dealkylations to eventually inorganic lead (Collins *et al.*, 2004).

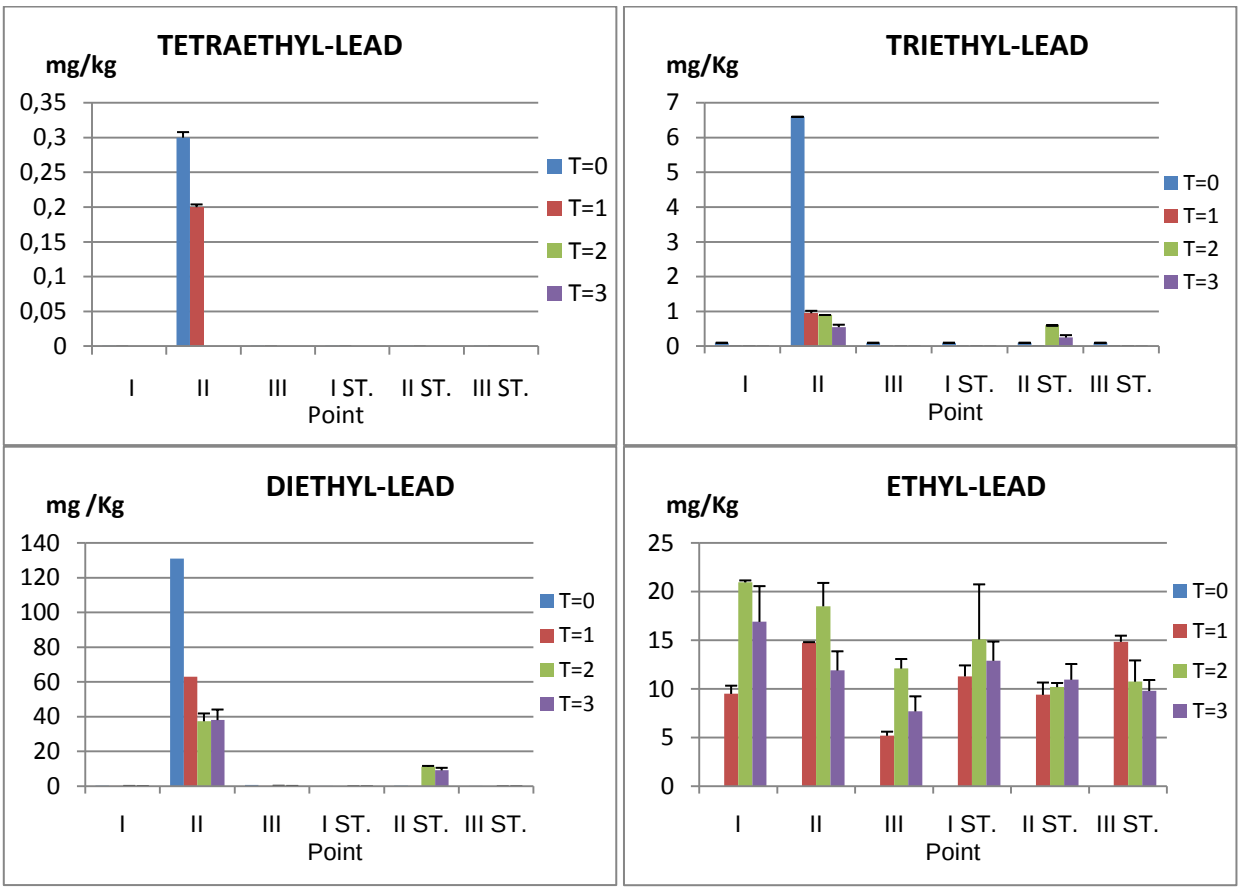



**Fig. 4.17** Speciation of alkyl-lead compounds along the trial in soil of *B. juncea* untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms.

## 4.2.5 Evaluation of Phytoextraction efficiency

With the purpose of investigating the efficiency of the examined plants in relation to this kind of contamination, different parameters were monitored.

Lead phytoextracted in shoots tissues per plant - defined as µg Pb Accumulated/plant = tissue [Pb] x dried biomass - gives a measure of the contaminant actually extracted and therefore of the system efficiency. It was determined at the distinct sampling times along the process of *Brassica juncea* mesocosms and at the final sampling of *A.cannabinum,* to monitor Pb uptake in the harvestable part of the plant. Moreover for *A.cannabinum* plants lead phytoextracted was also calculated for roots tissues.

The Bioconcentration Factor (BF) that is the concentration of Pb in tissues divided by that in soil - defined as $C_{TISSUE}/C_{SOIL}$ - measures the plant capability to bioconcentrate the toxic element (Pb) into its tissues.

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 Pb 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 (Xiong, 1998; Karami *et* Shamsuddin, 2010).

BF and TF factors were calculated as above described and are displayed in Table 4.1 and 4.2. As far as *Brassica juncea* mesocosms are concerned, as only shoot Pb concentration data were available, TF could not be determined and BF was calculated only for shoots tissues.

Concerning Bioaccumulation Factor (BF) (Tab 4.1, 4.2), very low values of BF were obtained for both plants even in the untreated mesocosms were higher tissues concentrations were detected. BF is an

interesting parameter to consider as it measures the plant capability to bioconcentrate the toxic element (Pb) into its tissues, taking also in account the concentration level within the contaminated matrix. However these values can be misleading, as they take into account total Pb content in soil, whereas only a low part is mobile and bioavailable. Therefore the extremely high concentrations of total Pb registered in the trial soil led to really low BF values despite the high concentrations reached within the plant tissues, pointing out the necessity of increasing Pb biovailability by chelating agents and/or use a combined remediation approach in relation to the really high contamination of the area under study.

| | **Soil [Pb]** | **BF** |
|---|---|---|
| **Point I** | 5500±774 | 0,007±0,002 |
| **Point II** | 8689±84 | 0,026±0,005 |
| **Point III** | 2400±37 | 0,030±0,006 |
| **Point I steril.** | 5403±736 | 0,012±0,003 |
| **Point II steril.** | 12099±240 | 0,016±0,002 |
| **Point III steril.** | 1687±637 | 0,009±0,003 |

**Table 4.1** Soil Pb concentrations and Bioconcentration Factor (BF) values for untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms of *B. juncea* at the final sampling T3

| | **Soil [Pb]** | **BF** | | **TF** |
|---|---|---|---|---|
| | | **Shoot** | **Root** | |
| **Point I** | 3903±509 | 0,042±0,008 | 0,038±0,009 | 1,092±0,037 |
| **Point II** | 6934±1800 | 0,051±0,005 | 0,179±0,018 | 0,281±0,003 |
| **Point III** | 2591±455 | 0,088±0,017 | 0,169±0,011 | 0,490±0,067 |
| **Point I steril.** | 4340±352 | 0,090±0,010 | 0,079±0,003 | 1,171±0,091 |
| **Point II steril.** | 14027±403 | 0,015±0,002 | 0,150±0,007 | 0,096±0,012 |
| **Point III steril.** | 2637±97 | 0,067±0,011 | 0,133±0,029 | 0,568±0,030 |

**Table 4.2** Bioconcentration Factor (BF) and Translocation Factor (TF) values for untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms of *A.cannabinum* at the final sampling Tf

Considering the Translocation Factor (TF) determined for *A.cannabinum* mesocosms (Tab.4.2), values of TF>1, which indicate a preferential accumulation of Pb in shoot rather than in root portion, were detected at point I in both untreated and sterilized mesosoms. This cannot therefore be connected to the autochthonous micro flora influence.

Much lower TF values determined at point II are connected to the much higher root concentrations reached at this reference location, almost one order of magnitude higher than the other 2 sampling points, as shown in par 4.2.3. For this plant TF values of about 0.5 were also calculated in both untreated and sterilized mesocosms of point III, suggesting quite a good phytoextraction potential of this plant despite the preferential accumulation at root level. It has in fact to be mentioned that for lead hyperaccumulating plants, which usually have a higher shoot/root ratio of lead content in plant than the non-hyperaccumulators, are reported values of 0.04 - 0.1 TF (Kumar *et al.*, 1995; Xiong, 1998)

It is interesting to point out that the phytoremediation efficiency depends directly on both contaminant concentration reached in plant tissues and biomass production. The development of large-scale phytoextraction techniques have in fact considered crop species as bioaccumulators of heavy metals as

some of them can accumulate heavy metals while producing high biomass in response to established agricultural management (Marchiol *et al.*, 2004).

The evaluation of total lead phytoextracted in shoots tissues has therefore been performed for both plants (fig 4.18-4.20). As for the biomass determination values were calculated per plant and single values are generally lower than 200µg Pb/plant, pointing out the need of high plant density and several cropping seasons in a Phytoremediation application.

On the other side considering *Brassica juncea* mesocosm, shown in Fig. 4.18, accumulation values obtained were not only comparable but higher than those obtained for the same species in presence of a PGPR strain (Sheng *et al.*, 2008a) and with amendments of chelating agents, reporting in fact values lower than 25 µg Pb/plant and lower than 20 µgPb /plant in *B.juncea* shoots in presence nitrilotriacetate (NTA) and citric acid amendments (Quartacci *et al.*, 2006).

In *B.juncea* trial all obtained values were much higher in the untreated mesocosms rather than the sterilized ones. As only exception in point I a higher value was detected in the sterilized mesocosm at the final sampling, reflecting the slightly higher Pb concentration in shoots to it associated (Fig. 4.18). On the other side even for point I the two first samplings T1 and T2 showed a higher accumulation in untreated mesocosms, confirming a general synergistic role of the indigenous soil micro flora in exame (Fig. 4.18).

As far as *A.cannabinum* is concerned, a general higher Pb accumulation was obtained in roots – ranging from 25 to about 400 µg Pb/plant - due to the much higher biomass and Pb concentrations reached in this plant tissues (Fig 4.19). Comparing untreated and sterilized mesocosms, in the latter much lower values of Pb accumulation were obtained reflecting the much lower biomass production in absence of the indigenous micro flora (Fig 4.19).

Considering the higher shoot values obtained in untreated mesocosms of both plants (Fig 4.18, 4.19), shoots accumulation reached in *Apocynum cannabinum* was comparable and in the same order of magnitude of *B.juncea* results. Nevertheless *A.cannabinum* showed lower Pb accumulation in shoots per plant in point I and II - with values < 20 µg Pb/plant - despite the much longer growth time of *A.cannabinum* trial.

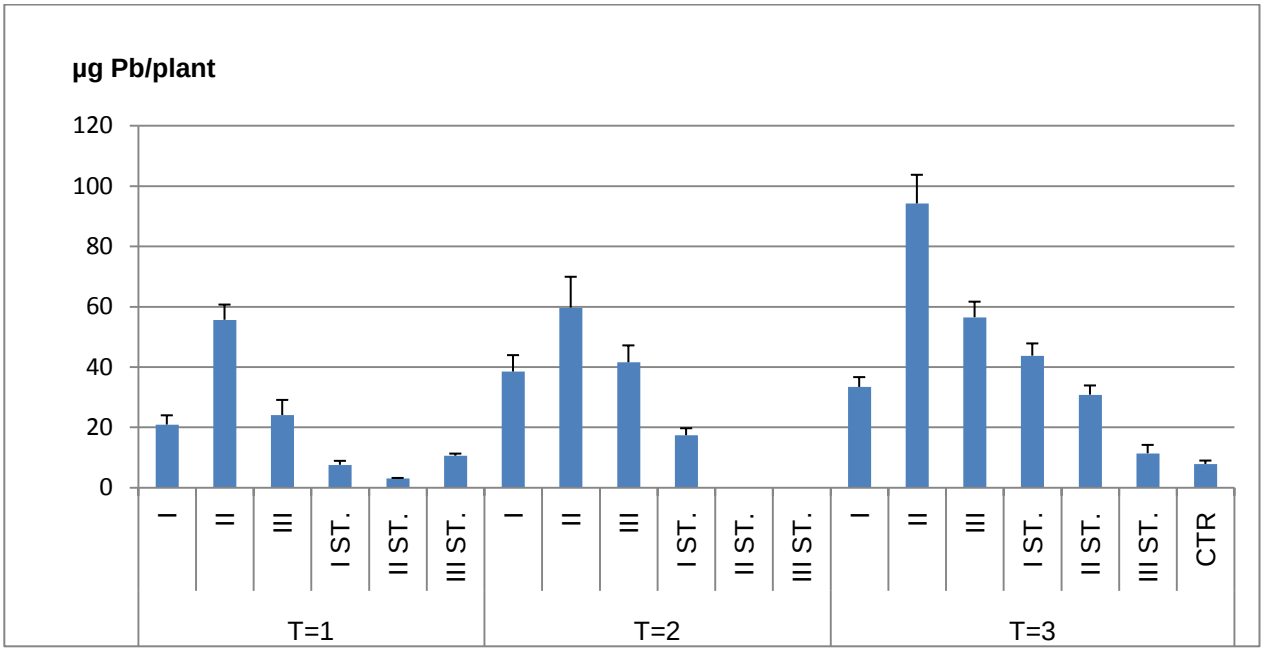



**Fig. 4.18** Pb phtoextracted per plant in shoots of *B. juncea* along the lab-scale trial in untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms.

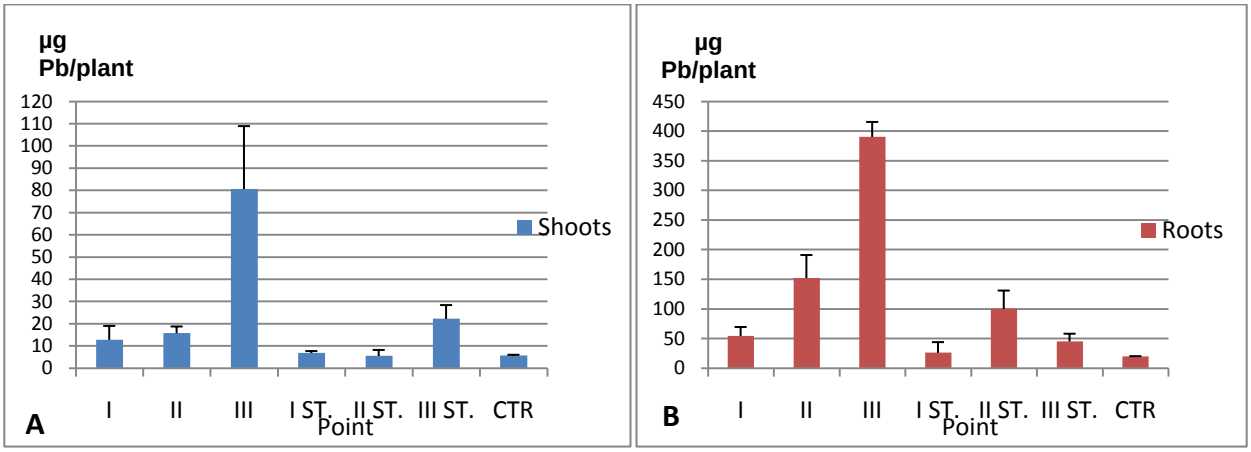



**Fig. 4.19** Pb phtoextracted per plant in shoots (A) and roots (B) of *A.cannabinum* in untreated (I,II,III) and sterilized (IST, IIST, IIIST) mesocosms at the sampling time Tf

| ***Brassica juncea*** | **micro flora effect (%)* on µg Pb shoot/plant** | | |
|---|---|---|---|
| | **T1** | **T2** | **T3** |
| **Point I/IST** | + 177% | +121% | -24% |
| **Point II/IIST** | + 1712 % | - | +206 |
| **Point III/IIIST** | + 127 % | - | +397% |

**Table 4.3** Effect of the autochthonous micro flora on µg Pb/plant in shoots of *B.juncea,* comparing untreated and sterilized mesocosms' values obtained along the lab-scale trial.
*means the effect on Pb accumulation per plant, calculated from the ratio between µg Pb shoot/plant mean values obtained in untreated and sterilized *Brassica juncea* mesocosms.

| ***Apocynum cannabinum*** | **micro flora effect (%)* on µg Pb shoot/plant** | **micro flora effect (%)* on µg Pb root/plant** |
|---|---|---|
| **Point I/IST** | +88% | +108% |
| **Point II/IIST** | +185 % | +52 % |
| **Point III/IIIST** | +262% | +761% |

**Table 4.4** Effect of the autochthonous micro flora on µg Pb/plant in shoots and roots of *A.cannabinum,* comparing untreated and sterilized mesocosms' values obtained at the sampling time Tf.
*means the effect on Pb accumulation per plant in shoot and roots, calculated from the ratio between µg Pb/plant mean values obtained in untreated and sterilized *A.cannabinum* mesocosms

It is particularly interesting to notice as in general for both plants higher values of phytoextracetd Pb were reached in untreated mesocosms, pointing out the positive effect of the indigenous micro flora selected by and adapted to the the Pb contamination in the Ex-SLOI area.

For *B.juncea* in fact both biomass and Pb content were positively affected by the autochthonous micro flora. As reported in Tab 4.3, comparing untreated and sterilized *B. juncea* mesocosms, total Pb accumulated in shoots per plant generally increased from the 121 % - detected in point I at T2 - to almost 400 % - detected in point III at the final sampling time. An increased of about 1700% was

moreover detected in the most contaminated pint II at T1, due to really low biomass production obtained in the sterilized mesocosm.

As far as *A. cannabinum* is concerned (Tab 4.4), total Pb accumulated in shoots per plant increased from about 80% to 262% in presence of the autochthonous micro flora. Moreover in roots it was detected an increase per plant from the 50% in the most contaminated Point II to more than 750% in the less contaminated point III. The difference between untreated and sterilized mesocosms observed in *A.cannabinum* was mainly connected to the much higher biomass production in presence of the soil autochthonous micro flora selected at the Ex-Sloi sampling points.

These data point out the positive influence specifically exerted by the soil autochthonous micro flora in exame on both plants and on Phytoremediation efficiency. As the positive influence of the autochthonous micro flora in *B.juncea* mesocosms was evidenced on both biomass production and Pb uptake, the indigenous rhizobacterial community of *B.juncea* mesocosms was further explored in the molecular analysis hereafter reported in par 4.2.6.

### 4.2.6 Molecular analysis of rhizosphere bacterial communities by PCR-DGGE in *Brassica juncea* mesocosms

The composition of the rhizosphere microbial community of *Brassica juncea* mesocosms was analysed through PCR-DGGE technique. This analysis was carried out on rhizosphere soil samples collected at the different sampling times along the experiment, in order to monitor and characterize the composition of the bacterial community that might have been selected during the phytoremediation process.

From a global visual comparison of the rhizobacterial communities in the untreated mesocosms (Fig 4.20, 4.21, 4.22) is evident the presence of few bands of major intensity, corresponding to dominant components of the microbial community. In agreement with the results obtained by the previous molecular analysis performed on the three sampling points (par 3.2.2), this indicates the selection towards a specific community.

The profiles of each sampling were performed in double copy (named A and B) - from distinct genomic DNA extractions - matching closely one another (Fig 4.20, 4.21, 4.22).

Distinct profiles were observed in the rhizospere communities of the different sampling points (I, II, III), with the presence of stable species over time. Major bands in DGGE profiles were excised from the gels and sequenced to taxonomically identify the dominant member of the microbial community.

Considering untreated mesocoms of Point I, in the visual comparison of the bacterial community profiles few major bands were detected (Fig 4.20). Two bands in particular (A and B) were detected at all the samplings along the trial and belong respectively to *Alcaligene*s of Beta-proteobacteria and to *Oscillatoria* genus of the *Cyanobacteria phylum* (Tab 4.5).

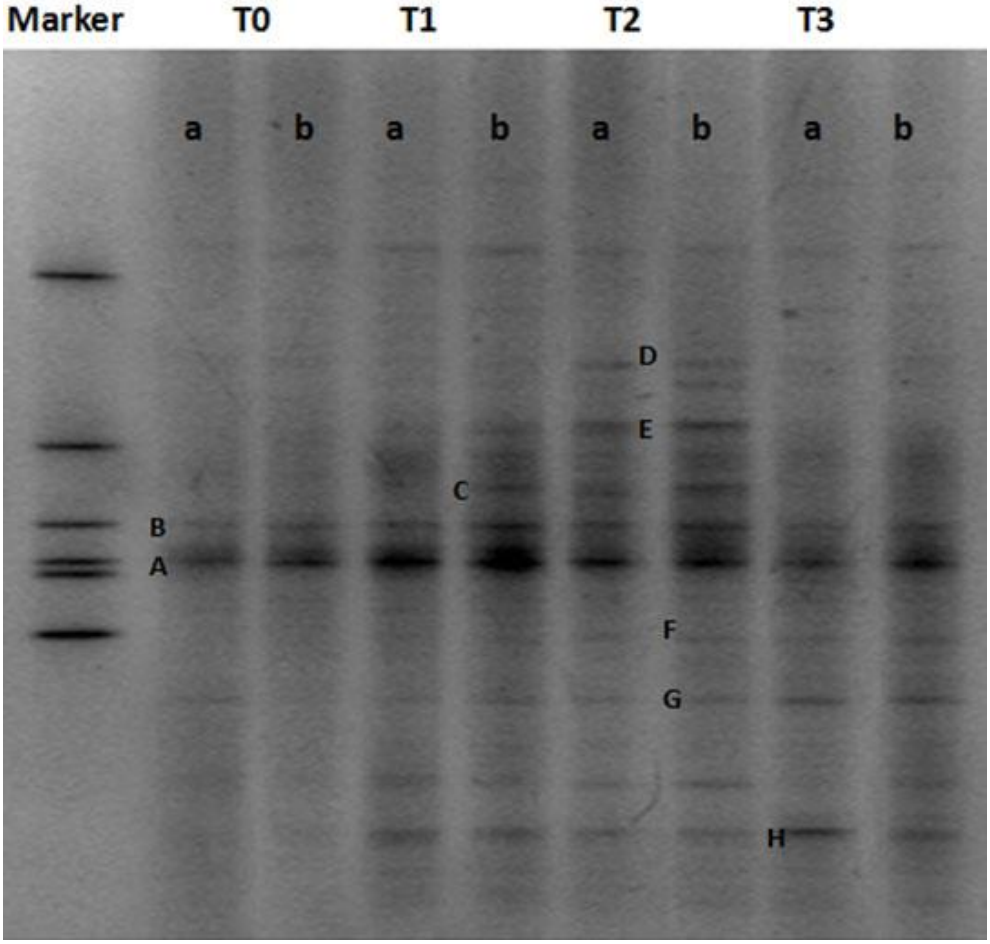



**Fig. 4.20**. DGGE analysis on rhizosphere soil of Point I *Brassica juncea* mesocosms. A and B refer to the repeated analysis for the same soil samples. T0, T1, T2, T3 refers to sampling times along the trial. Letters refer to sequenced bands.

| Bands Point I | Taxonomic Reference ID | Phylogentic group | Homology % |
|---|---|---|---|
| **A** | *Alcaligenes sp.* HQ670710 | β-proteobabacteria | 100% |
| **B** | *Oscillatoria sp. EU282434* | Cyanobacteria | 100% |
| **C** | *Uncultured Sphingomonas sp.* HQ674818 | α-proteobabacteria | 98% |
| **D** | *Uncultured Kaistobacter sp.* EU440720 | α-proteobabacteria | 100% |
| **E** | *Uncultured bacterium* AF257865 | Bacteria | 100% |
| **F** | *Sphingomonas sp.* FM997989 | α-proteobabacteria | 98% |
| **G** | *Arthrobacter sp.* FJ434129 | Actinobacteria | 99% |
| **H** | *Saccharothrix sp.* HQ588356 | Actinobacteria | 100% |

**Tab 4.5** Taxonomic characterization of the major DGGE bands identified in point I untreated *B.juncea* mesocosms

Actually *Alcaligenes* genus - previously detected both in the culture-dependent and in the molecular analysis performed on the 3 sampling points (par 3.1., 3.2.2) - includes heavy metal resistant and alkane degrading strains, and also PGPR members (Periello, 2000; Kaymak *et al.*, 2008). It is interesting to notice the detection of the Cyanobateria *phylum* - not previously detected - simple and diverse group of microorganisms with characteristics in common to both bacteria and algae. They are commonly used for the phytostimulation and biofertilization of agriculture crops due to their nitrogen-fixing ability, and moreover cyanobacterial phytohormones are a major tool for improved growth and yield in wheat (Hussain *et* Hasnain, 2011). Besides to *Oscillatoria* genus belong species with multiple heavy metal co-tolerance (Tong *et al.*, 2002).

An uncultured bacterium (band E) was exclusively detected at T1 and T2, together with two component of the *Sphingomonadacae* family of Alpha-proteobacteria, namely one related to the *Sphingomonas* (Band C) and one to the *Kaistobacter* genus (band D). Strains of the *Kaistobacter* genus - detected in T2 samples and not previously detected in the culture nor in the molecular study performed - have been also found in association with the rhizosphere of pioneer plants growing on heavy metals-contaminated soils (Navarro-Noya *et al.*, 2010).

Three more faint bands (F, G and H), showed a higher intensity at the final sampling time, suggesting an increase in their representation within the community along the trial. One of them also relates to the alpha *Sphingomonas* genus (band F), which includes strains able to use PHA as only carbon and energy source (Vanbroekhoven *et al.*, 2004; Seo *et al.*, 2007). Actually this sampling point is also contaminated by hydrocarbons.

The two other bands (G and H) both relate the gram-positive Actinobacteria *phylum*, to *Arthobacter* and *Saccharothrix* genera. For the latter degrading capabilities of PAH are reported (Yuting *et al.*, 2003), while to the *Arthrobacter* genus belong multiple metal-resistant members, strains with phosphate solubilizing ability and also demonstrating various plant growth promoting and biocontrol activities including indole acetic acid (IAA) production (Benyehuda *et al.*, 2003; Bafana *et al.*, 2010; Banerjee *et al.*, 2010). Moreover to this genus in particular is reported a strain able to completely mineralize trimethyl Pb (Macaskie *et* Dean, 1990) pointing out a high degrading potential and resistance in relation to the examined contamination.

As far as mesocosm of point II is concerned (Fig 4.21 and Tab 4.6), it is possible to detect along all the trial two major bands (A and I), belonging to the *Alcaligenes* genus of the Beta-Proteobacteria class and to the *Xanthomonodacea* family of the Gamma-proteobacteria.

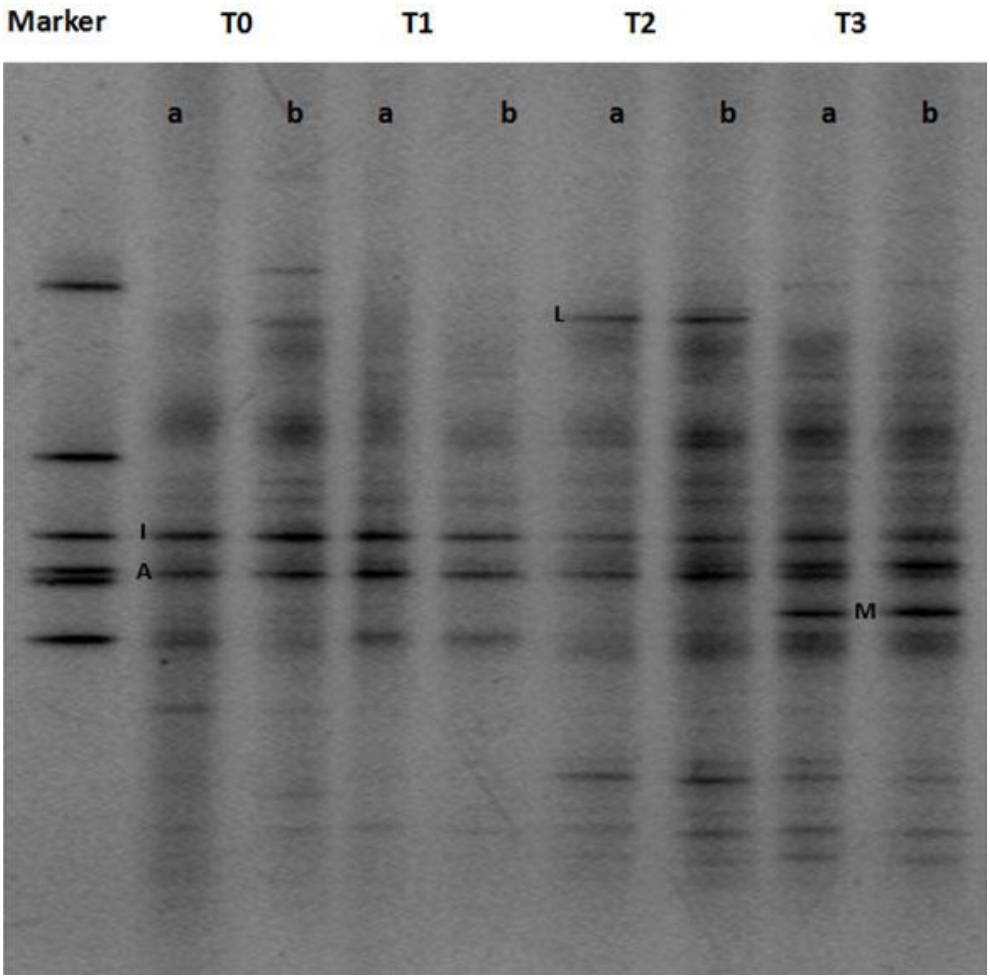



**Fig. 4.21**. DGGE on rhizosphere soil of Point II *Brassica juncea* mesocosms. A and B refer to the repeted analysis for the same soil samples. T0, T1, T2, T3 refers to sampling times along the trial. Letters refer to sequenced bands.

In T2 another component of the Gamma class (band L), belonging to *Pseudomonas* genus – of major representation in the culture study and reporting both degrading capabilities and PGP activity (Patten *et* Glick, 2002; Ben Said *et al.*, 2008) - was highly detected although no more discernable at the final sampling. Actually at the final sampling T=3 a redistribution of intensity to bands only faintly discernable in the previous samplings was observed. Besides a new major component (band M) of the rhizobacterial community at the final sampling belongs to the Alpha-proteobacteria class. This class was highly represented together with the other two Proteobacteria classes within the strains isolated in pure culture from Point II (par 3.1.2.1) and includes various members exerting plant growth promotion and belonging to genera such as *Ochrobactrum*, *Sphingomonas* and *Rhizobium* isolated from point II (Faisal *et* Hasnain, 2006; Príncipe *et al.*, 2007; Rajkumar *et al.*, 2010).

As previously observed for the untreated mesocoms of point I, one major band (band A) detected along all the trial belongs to the *Alcaligenes* genus, which includes heavy metal resistant and alkane degrading strains, and also PGPR members (Periello, 2000; Kaymak *et al.*, 2008). This is the only component of the bacterial community - as previously detected in the molecular analysis in reported in par 3.2.2 - detected at all the three sampling points I, II and III. This community member probably contributes to the positive growth promoting action observed in presence of the examined autochthonous microbial community, which allowed a higher growth in comparison to the new microbial community established along the trial in the sterilized mesocosms.

| Band Point II | Taxonomic Reference ID | Phylogentic group | Homology % |
|---|---|---|---|
| **A** | *Alcaligenes sp.* HQ670710 | β-Proteobacteria | 100% |
| **I** | Uncultered *Xanthomonadaceae* bacterium EU640674 | γ-Proteobacteria | 100% |
| **L** | *Pseudomonas* sp. HQ717394 | γ-proteobacteria | 100% |
| **M** | Uncultured Alphaproteobacterium AY144198 | α-proteobabacteria | 98% |

**Table 4.6** Taxonomic characterization of the major DGGE bands identified in point II untreated *B.juncea* mesocosms

Considering the DGGE analysis of the Point III mesocosms (fig 4.22 and Tab 4.7), three major bands (A, I and O) were detected at all the sampling times.

In particular two of them (A and I ) - belonging to *Alcaligenes* genus of the Beta-proteobacteria Class and to the *Xanthomonodacea* family of the Gamma class - were also detected along all the trial in mesocosm of point II, and in particular the first also in point I. This points out for these community members a higher representation and resistance in the soil conditions within Ex-SLOI area and a synergistic relationship with the *B.juncea* plants positively affected by the autochthonous microflora.

A redistribution of intensity was observed along the experiment in mesocosm of Point III. In particular two of the 3 major bands (A and I) were in fact much less intense in the final sampling in comparison to T1. On the other side band O - of the *Sphingomonas* genus of *Sphingomonadaceae* family - was highly descernable even at the final sampling, together with another uncultured bacterium (band P). *Sphingomonadaceae* are common gram-negative, aerobic organisms including Hydrocarbon-degrading members able to degrade a broad range of mono and polycyclic aromatic compounds (Kertesz *et* Kawasaki, 2010). Members of the *Sphingomonas* genus have also been detected in the rhizospheric microbial communities associated with plants grown in metal rich soils (Kamaludeen *et* Ramasamy, 2008) and exerting growth promoting action. Inoculation with *Sphingomonas* sp. strains resulted in fact in considerable enhancement of orchid seeds germination (Tsavkelova *et al.*, 2007). Another component (band N) of the Alpha class, even if faintly intense, was detected along the trail.

Actually in the profiles of Point III mesocoms along with few major bands highly intense, except for the T0 it is possible to detect a high number of bands even if of lower intensity. This can be probably connected to a lower contamination level associated to this sampling point, which allowed the growth of a higher and diverse microbial population favored by exudes and organic matter supplied by the growing *B.juncea.*

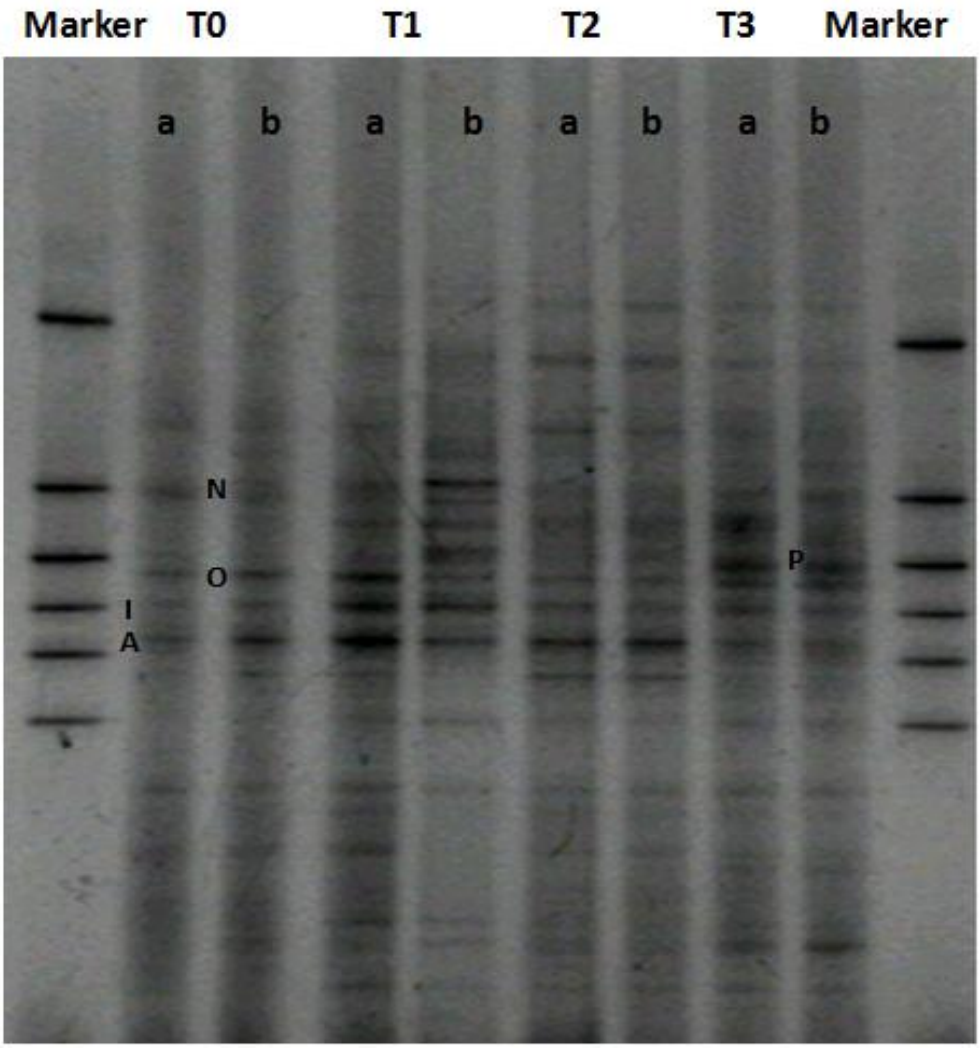



**Fig. 4.22** - DGGE on rhizosphere soil of Pont III *Brassica juncea* mesocosms. A and B refer to the repeated analysis for the same soil samples. T0, T1, T2, T3 refers to sampling times along the trial. Letters refer to sequenced bands.

| Band Point III | Taxonomic Reference ID | Phylogentic group | Homology % |
|---|---|---|---|
| **A** | *Alcaligenes* sp. HQ670710 | β-Proteobacteria | 100% |
| **I** | Uncultered *Xanthomonadacea*e bacterium EU640674 | γ-Proteobacteria | 100% |
| **N** | Alpha proteobacterium FR691422 | α-proteobabacteria | 100% |
| **O** | *Sphingomonas* sp. FM997989 | α-proteobabacteria | 99% |
| **P** | Uncultured bacterium AF257865 | Bacteria | 98% |

**Tab.4.7** Taxonomic characterization of the major DGGE bands identified in point III untreated *B.juncea* mesocosms

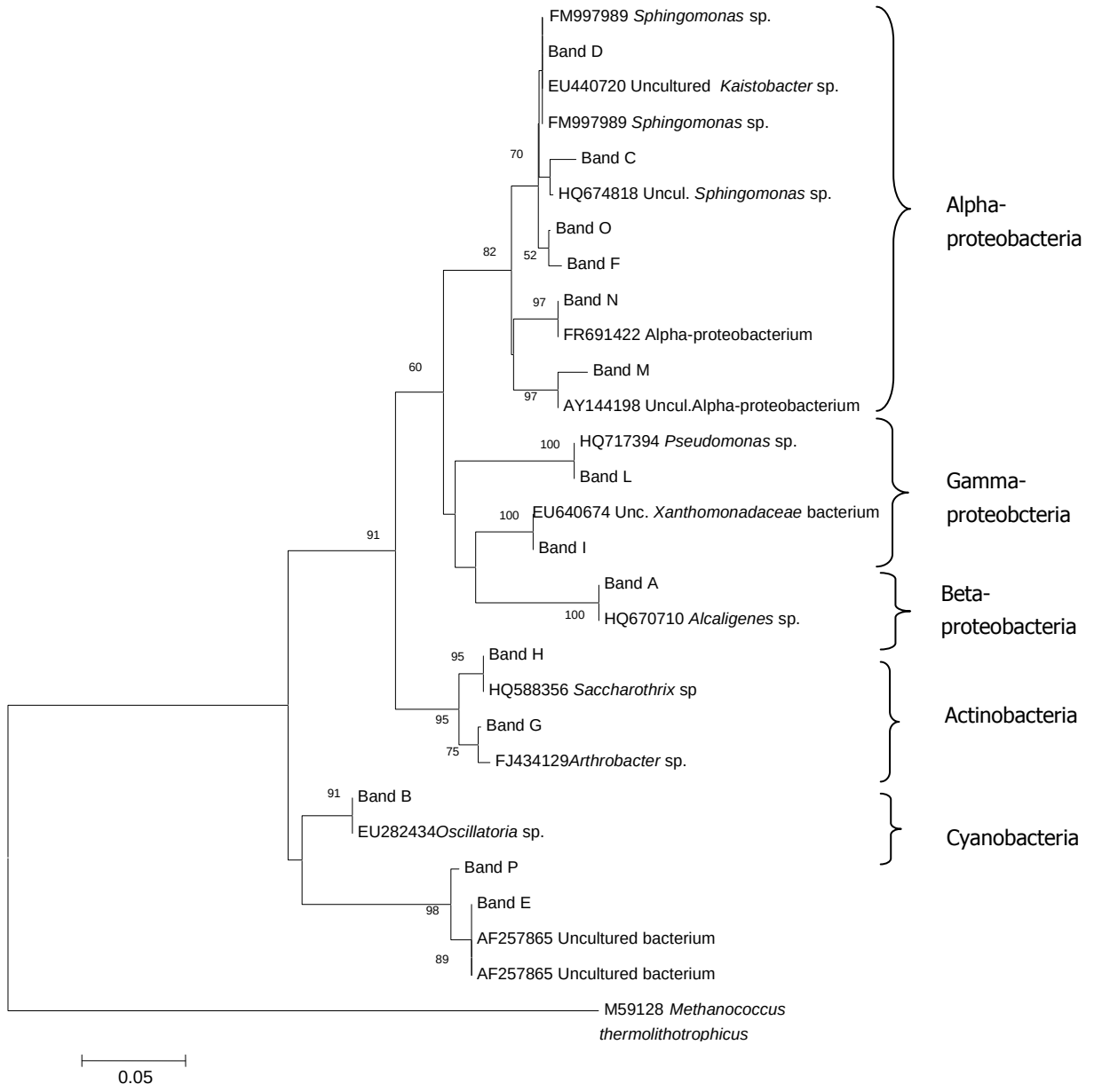



**Fig 4.23**: Phylogenetic neighbour-joining tree of the V3 sequences obtained from excised bands from DGGE analysis of untreated mesocoms and their closest database relatives with Species Name and GenBank accession numbers. Bootstrap values (*n*_1,000) above 50% are indicated at the nodes. The scale bar represents genetic distance (nucleotide substitutions per site).

The molecular analyses of the rhizosphere of *Brassica juncea* indicated therefore the presence along all the experiment of interesting microbes such as heavy metals resistant bacteria and degraders of organic compounds and PAH - co-contaminant in point I - and PGPR, certainly related to the higher biomass production detected in all the untreated mesocosms.

As shown in the phylogenetic tree of the sequenced bands from the DGGE analysis of the untreated mesocoms (Fig 4.23), a higher representation of Proteobacteria is observed at the three analyzed sampling points – accordingly to the dominance of this *phylum* detected in both the culture and molecular study performed for all the sampling points in exame (par 3.1 and 3.2.2). The sequencing results evidenced the class Alpha in particular - as detected in the molecular analysis on the sampling points

under study (par 3.2.2) - along with the detection of few members of the gram-positive *phylum* of Actinobacteria.

These results are in agreement with the predominance of the bacterial communities compared to fungi observed in the rhizosphere of hyperaccumulators grown in heavy metal-contaminated soils, with a high percentage of bacteria belonging to the Alpha-proteobacteria (Idris *et al.*, 2004; Kamaludeen *et* Ramasamy, 2008).

Considering the profiles of sterilized mesocosms, it is interesting to notice that bands at T0, corresponding to dominant indigenous microbial species, disappeared in the next sampling (Fig. 4.24, 4.25, 4.26). This confirmed the sterilization of the autochthonous micro flora previously detected by culture counts (par 4.2.1), followed by the colonization of external microorganisms from the glasshouse environment.

Only major bands detected at T0 and consequently associated to the autochthonous micro flora were excised and sequenced (Tab 4.8). In sterilized mesocoms few bands were detected at T0 probably connected to a degradation of DNA along the thermal treatment (Fig 4.24, 4.25, 4.26).

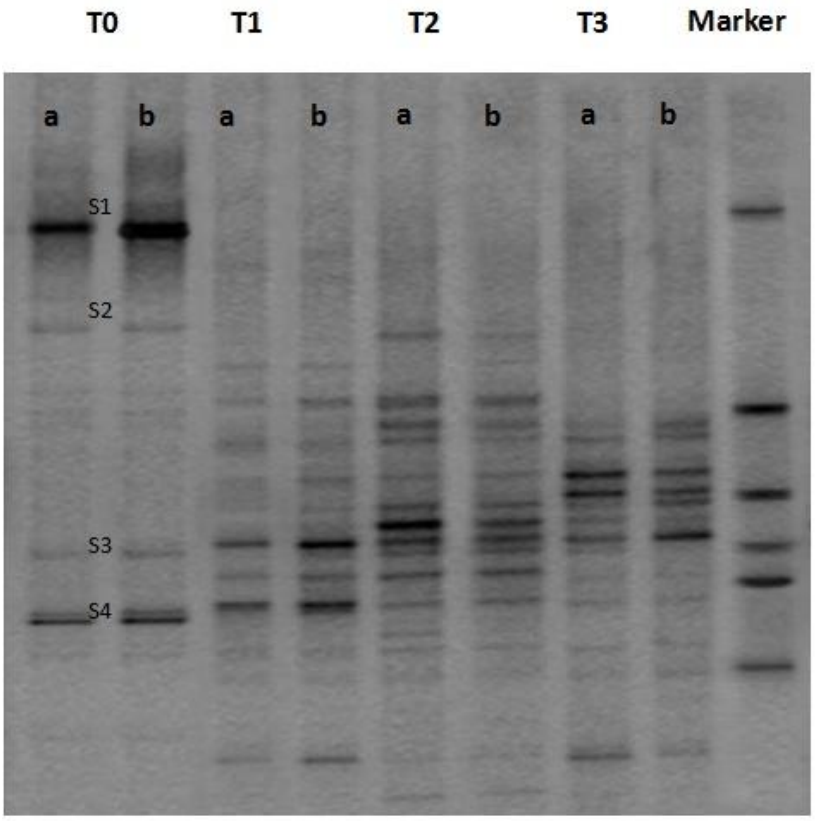



**Fig. 4.24** DGGE on rhizosphere soil of Point I *Brassica juncea* sterilized mesocosms. A and B refer to the repeated analysis for the same soil samples. T0, T1, T2, T3 refer to sampling times along the trial. Alphanumeric codes refer to sequenced bands

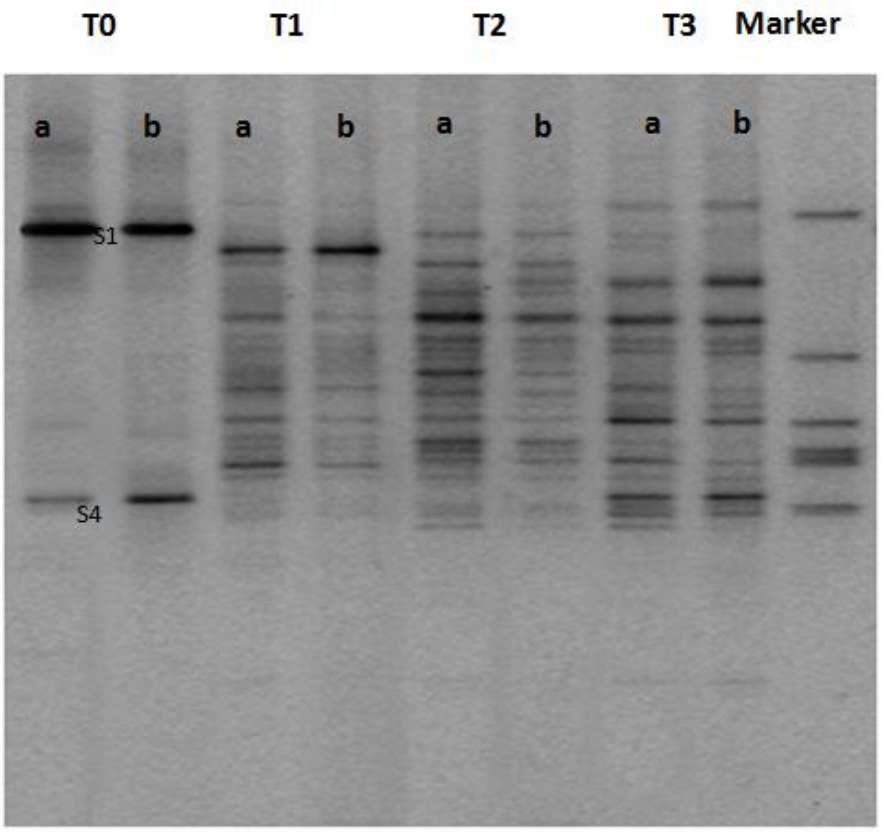



**Fig. 4.25** DGGE on rhizosphere soil of Pont II *Brassica juncea* sterilized mesocosms. A and B refer to the repeated analysis for the same soil samples. T0, T1, T2, T3 refers to sampling times along the trial. Alphanumeric codes refer to sequenced bands

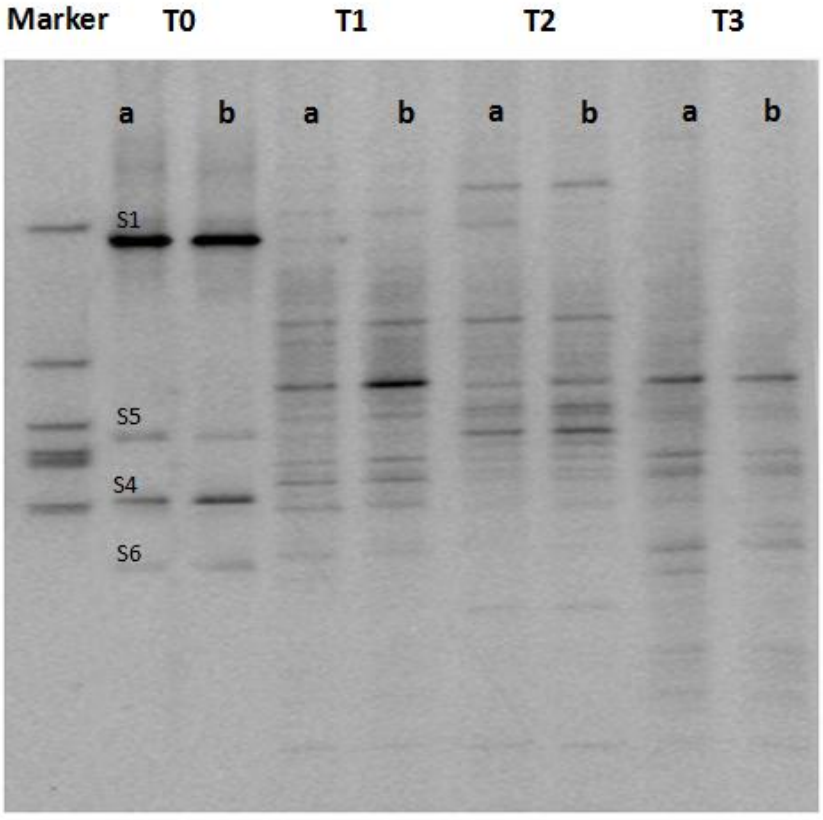


**Fig. 4.26** DGGE on rhizosphere soil of Pont III *Brassica juncea* sterilized mesocosms. A and B refer to the repeated analysis for the same soil samples. T0, T1, T2, T3 refers to sampling times along the trial. Alphanumeric codes refer to sequenced bands

| Band Sterilized Mesocosm | Taxonomic Reference ID | Phylogentic group | Homology % |
|---|---|---|---|
| **S1** | *Alcaligenes* sp. FJ608768 | β-proteobacteria | 100% |
| **S2** | *Bacillus* sp. DQ416782 | Firmicutes | 100% |
| **S3** | *Mesorhizobium mediterraneum* FJ430079 | α-proteobabacteria | 100% |
| **S4** | *Burkholderia sp.* AY177370 | β-proteobacteria | 100% |
| **S5** | *Mesorhizobium* sp. EU722444 | α-proteobabacteria | 98%% |
| **S6** | *Dietzia natronolimnaea* EU373398 | Actinobacteria | 100% |

**Table 4.8** Taxonomic characterization of the major DGGE bands identified at T0 in the sterilized *Brassica juncea* mesocosms

*Alcaligenes* and *Burkolderia* genera of the Beta-proteobacteria class were identified as the most intense bands in T0 of sterilized mesocosm of all the 3 sampling points (S1 and S4 bands). The *Burkholderia* genus is of particular interest in a Phytoremediation approach. Actually it includes strains able to solubilize inorganic phosphate, to increase biomass and tissue Pb concentrations in plants growing in heavy metal-contaminated soils, therefore promoting both biomass production and metal uptake (Jiang *et al.*, 2008). It is interesting to notice at point I (band S2), the detection of the only member of the *phylum* Firmicutes identified in the rhizosphere molecular analysis.

Other bands relate to $N_2$-fixing *Mesorhizobium* genus of Alpha class (band S3 and S5) and to the *Dietzia* of Actinobacteria (band S6), genus including strains with broad PAH degradation ability (Gerbeth *et al.*, 2004; Qureshi *et al.*, 2009).

Considering results obtained for both untreated and sterilized mesocosms, few components of the Actinobacteria *phylum* were identified at all the three sampling points and only one component of *Bacilli* class was detected - in the sterilized mesocosm of point I – accordingly to the lower gram-positive representation observed by culture analysis at the three sampling points (par 3.1). On the other side in literature to Actinobacteria are included phosphate-solubilizing microorganisms of special interest, since these bacteria are able to develop in extremely different soils and produce various substances (anti-fungi, insecticides, phytohormone-like compounds) that could benefit plant growth, pointing out their positive synergistic potential (Hamdali *et al.*, 2007).

Indeed the molecular analysis has allowed the detection along all the experiment of members of the indigenous bacterial community able to positively interact with plants, and also characterized by both resistance and degrading potential in relation to metals and organic compounds. Actually the detection of microorganisms for which are reported plant growth promoting activities, degrading potential and also phytoextraction assistance reflect the observed synergistic role exerted by the autochthonous micro flora in untreated mesocosms on both biomass production and Pb uptake (par 4.2.2. and 4.2.3).

Genera such *Alcaligenes* (Beta-proteobacteria) identified in mesocosm from all the 3 sampling points, *Pseudomonas* (Gamma-proteobacteria), identified at point II mesocosm, and *Sphingomonas* (Alpha-proteobacteria) identified in the mesocosms set-up with Point I soil - also contaminated by hydrocarbons

– include even members involved in degradation of complex organic compounds and hydrocarbons (Seo *et al.*, 2007; Ben Said *et al.*, 2008). They can therefore also play an important role in the solubilization of low soluble metal complexes and in contaminant plant uptake, along with phosphate solubilizing microorganisms such as those belonging to the *Burkholderia* and *Arthrobacter* genera (Banerjee *et al.*, 2010; Jiang *et al.*, 2008).

Besides PGPR can reduce stress symptoms in plants and potentially support heavy metal uptake, increasing both biomass production and Pb accumulation as generally observed in all untreated mesocosms. It is also interesting to notice the detection of the *Cyanobateria phylum* – not identified by the culture study - which also probably plays an important role in stimulating plant growth (Hussain *et* Hasnain, 2011).

### 4.2.7 Role of the soil autochthonous microbial community in the lab-scale trial and comparative considerations on the 2 plants tested

The results of the present lab-scale study showed, for all the three sampling points under study, that cultivating both plants in presence of the indigenous soil micro flora led to a major growth. Moreover in *B.juncea* higher Pb concentrations were also detected in shoot tissues in presence of the examined autochthonous micro flora.

It is therefore worth noting as for both plants higher amounts of Pb in the above-ground parts and consequently higher Phytoremediation efficiency were detected in untreated mesocosms, pointing out the positive effect of the indigenous micro flora, selected by and adapted to Pb contamination at the EX-SLOI area, on both plants growth and on Phytoextraction process.

Actually as reported in literature cultivating plants together with plant growth-promoting bacteria allows plants to germinate to a much greater extent, and then to grow well and rapidly accumulate a large amount of biomass. In addition in PAH removal trials, as a consequence of the treatment of plants with plant growth-promoting bacteria, the plants provide a greater sink for the contaminants, since they were better able to survive and proliferate probably due to an alleviation of a portion of the stress imposed upon the plant by the presence of the contaminant (Glick, 2003).

Actually the results obtained are in agreement with literature evidences on promotion of plant growth and metal uptake in hyperaccumulator or non-hyperaccumulator plants by heavy metal-resistant bacteria (Sheng *et al.*, 2008b). Increased metal uptake in hyperaccumulators is aided by changes in the rhizosphere and rhizobacterial secretions. Actually the chemical condition of the rhizosphere differs from bulk soil as a consequence of various processes induced by plants roots as well as by rhizobacteria like secretion of organic acids followed by reduction in pH, production of siderophores, phytochelains, amino acids and ACC deaminase (Kamaludeen *et* Ramasamy, 2008; Rajkumar *et al.*, 2010).

Soil microorganisms can indeed produce iron chelators and siderophores that ensure iron availability, solubilize metal-phosphates and/or reduce soil pH affecting metal mobility (Abou-Shanab *et al.*, 2008; Rajkumar *et al.*, 2010). Rhizobacteria involved in mobilizing insoluble nutrients by producing various organic acids might desorb Pb as well, which is a challenging metal tightly bound in most soils. Phosphate solubilization and acid production are not the only mechanism adopted by bacteria towards metals in soil; siderophores are also involved in mobilizing metals and their complexes can also be assimilated by the plants (Abou-Shanab *et al.*, 2005; Khan *et al.*, 2009; Cavalca *et al.*, 2010). At the same time the presence of microorganisms with degrading ability of complex organic compounds and hydrocarbons can contribute to the solubilization and contaminant plant uptake, with also a possible role in the degradation of the organometallic compounds (Seo *et al.*, 2007).

As far as the examined plants are concerned, the results so far obtained point out tolerance and resistance of both plants towards this kind of contamination combining inorganic and, even if in much lower levels, organic Pb.

In *B.juncea* the biomass production was in fact lightly affected by the contamination, particularly in presence of the examined autochthonous microbial community in untreated mesocoms. For *A.cannabinum* a higher decrease in biomass production was detected in sterilized mesocosms, while in both untreated and sterilized mesocosms it showed a lower shoot biomass production in comparison to *B.juncea,* despite a much longer time of growth.

Considering Pb concentrations accumulated within plant tissues, both plants reached good Pb concentrations with the higher values detected in *A.cannabinum.* However this plant species reached the highest concentrations within roots, which favors therefore metal immobilization but does not contribute to the accumulation in the harvestable part of the plant, main objective of the phytoextraction. A preferential shoot accumulation can in fact enable phytoremediation of the heavy metal-contaminated soils by only harvesting the aboveground parts of the plants, thus simplifying the agricultural practices (Xiong, 1998; Karami *et* Shamsuddin, 2010).

On the other side considering the global µg Pb phytoextracted/plant for both plants, higher and comparable results were obtained in presence of the examined indigenous micro flora, at all the three sampling points under study. Taking moreover in account the lower time of growth of *B.juncea* trial, results obtained indicate a higher Phytoextraction potential associated to this plant in relation to the examined contamination.

## 4.3 Field-scale Phytoremediation trial

With the 2 plants previously tested in lab-scale mesocosms a scale-up was performed to field-trial. This allowed a better evaluation of the potential of the examined plants and of the system autochthonous micro flora/plant in a Phytoremediation approach related to both inorganic and organic Pb contamination.
Within the contaminated Ex-SLOI area at the location of the three sampling points (I, II and III), parcels of respectively 60, 23 e 45 square meters were set up, depending on the size of accessible and available area. The trial was therefore performed in open field conditions, uncontrolled as those of a real scale process (Fig. 4.26).
For *Brassica juncea* in addition to the cultivar PI173874 (named A) used in lab-scale, a second cultivar PI426308 (named B) has been included in the field trial, as reported to highly accumulate Pb (Kumar *et al.*, 1995). In fact when this cultivar was grown in nutrient solution, more than 1.5% $Pb^{2+}$ was found in its buds tissues (Kumar *et al.*, 1995).
In the case of *Brassica juncea*, plants were collected at three different sampling times: T1 after 6 weeks from the sawing, T2 after 14 weeks and T3 after 18 weeks from the sawing. For *Apocynum cannabinum* as in lab-scale mesocosms a unique sampling was carried out due to its slow growth rate, and performed after 16 months from sawing (T1).
At point II *Brassica juncea* samples were collected only at the final sampling time T3 while for *A.cannabinum* no sampling was performed. Actually at this point only few plants were able to grow. This is in fact the most contaminated point under study and moreover located in a shaded area. Particularly affected was *A.cannabinum,* probably even in connection to its low germination percentage (par. 4.2.2).


**Fig 4.26** Field-scale trial set up with *Brassica juncea* (A) and *A.cannabinum* (B)

### 4.3.1 Enumeration of culturable rhizosphere community

As previously performed in lab-scale mesocosms, at each sampling time microbial counts for rhizosphere soil samples were determined on both Nutrient and R2a media, in order to monitor the dominant eubacteria population along the trial. At each sampling time distinct samples of rhizosphere soils were therefore collected for each point and average value are reported in Fig 4.27 and 4.28.
Counts of the eubacterial population along the whole experiment showed for both *B.juncea* cultivars and for *A.cannabinum* trials $\log_{10}$ values of about 6. Slightly lower values were detected in correspondence of the most contaminated point II - in agreement with the reported data of microbial charge for the 3

analyzed sampling points (par 3.1.1) and of untreated lab-scale mesocosms (par 4.2.1). A slight increase in the microbial charge was associated to *B.juncea* growth, although lower than in the lab-scale trial (par 4.2.1); this probably reflected the lower influence of the plant on a much higher mass of soil per plant under field conditions compared to the mesocosm pots.

In *A.cannabinum* trials slightly lower $\log_{10}$ values were registered at the final sampling after 16 months in both analyzed points I and III, probably also reflecting different environmental, season and soil conditions.

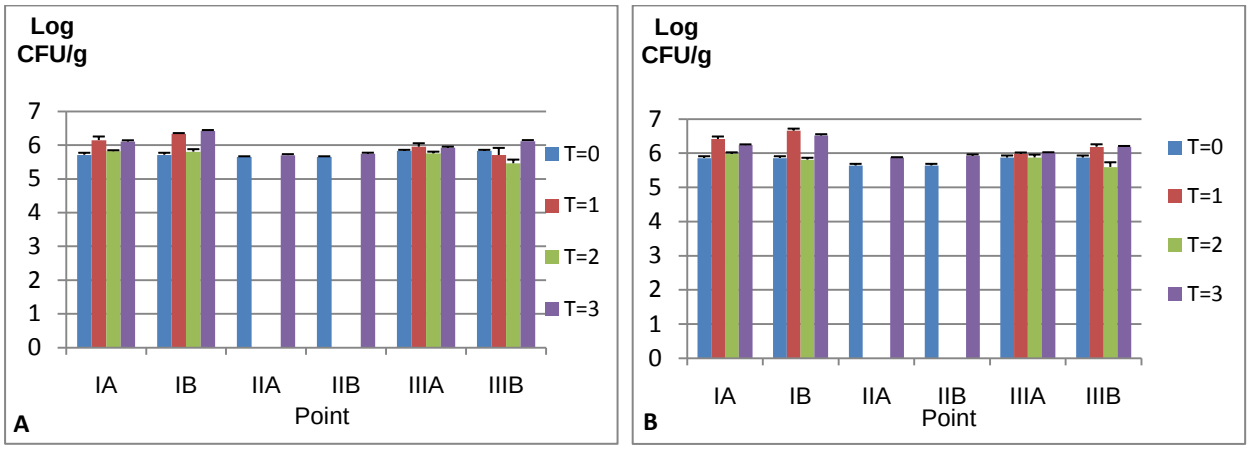



**Fig 4.27** Microbial counts on Nutrient (chart A) and R2a(chart B) media at T0 and at each sampling time for the field-scale *B. juncea* trial with the 2 examined cultivars (A, B) at the 3 sampling points (I,II,III)

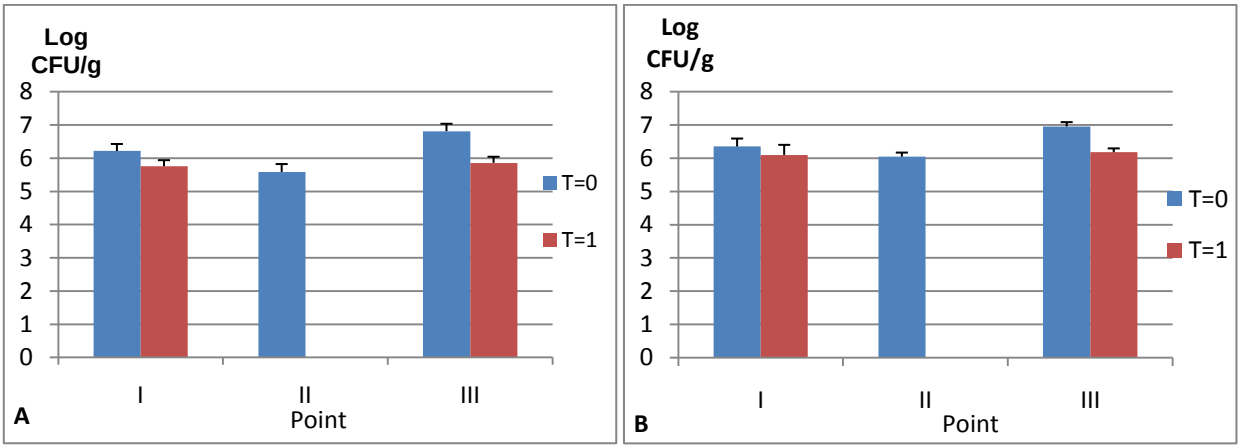



**Fig 4.28:** Microbial counts on Nutrient (chart A) and R2a (chart B) media performed at T0 and at the sampling time T1 for the field-scale *A.cannabinum* trial, performed at the 2 sampling points (I,III)

## 4.3.2 Plant biomass production

At each sampling time dry weight of collected plants was recorded for both shoots and roots of each plant under study (Fig. 4.29-4.32).

Considering *Brassica juncea* trials, plant biomass production of both shoots and roots showed similar values for the 2 tested cultivars (A and B) along all the experiment (fig 4.29, 4.30). A higher biomass production was detected for both cultivars A and B at the point I with values respectively up to 8 and higher than 10 g d.w. in shoots, while unexpectedly a lower biomass was detected in the less contaminated examined point III, in particular at the final sampling time. On the other side this could be related to the numerous variables implied in a field trial such as sun exposition, soil structure and water retention.

As above reported at point II few plants grew, and data were obtained only for the final sampling of *B.juncea* while no sampling was collected for *A.cannabinum*.

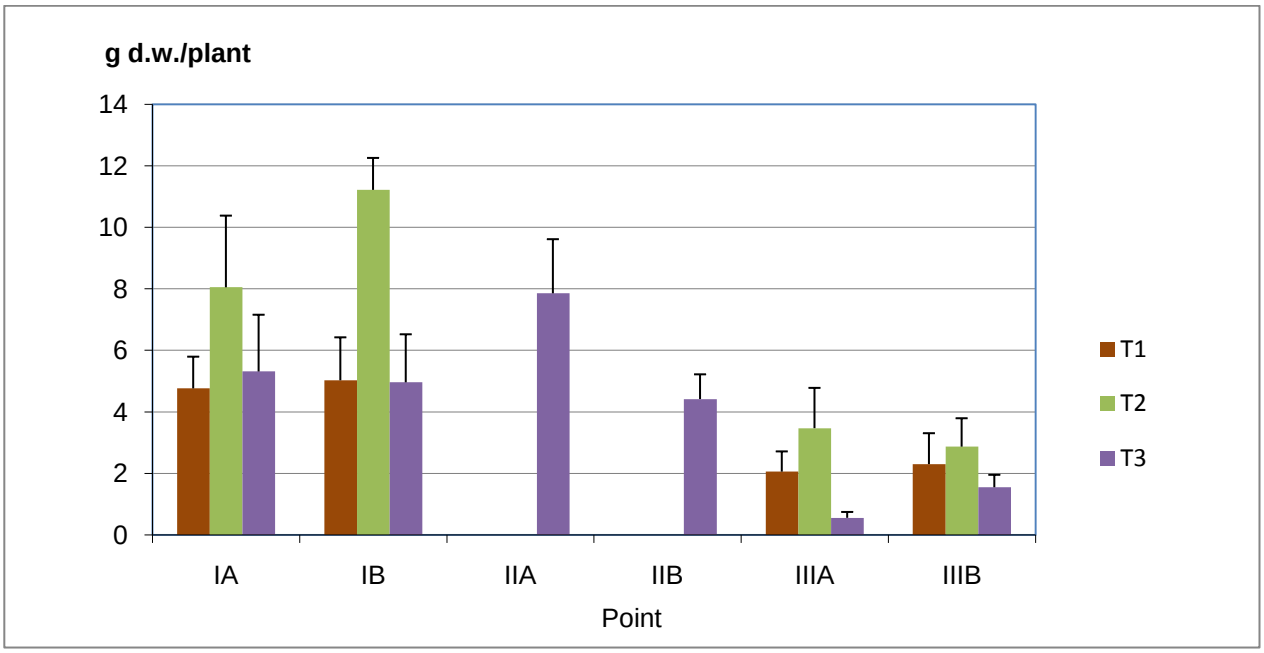



**Fig. 4.29** Shoots biomass production of the *B.juncea* cultivars (A and B) along the field trial at the 3 sampling points (I,II,III)

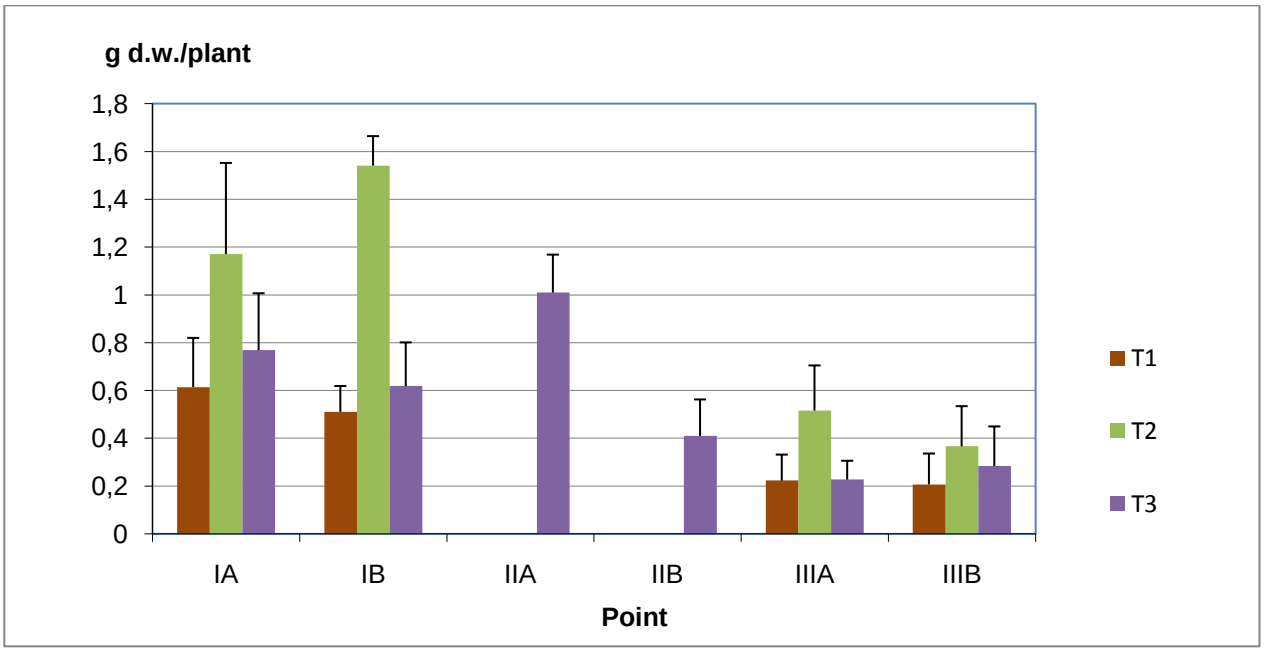



**Fig. 4.30** Roots biomass production of the *B.juncea* cultivars (A and B) along the field trial at the 3 sampling points (I,II,III)

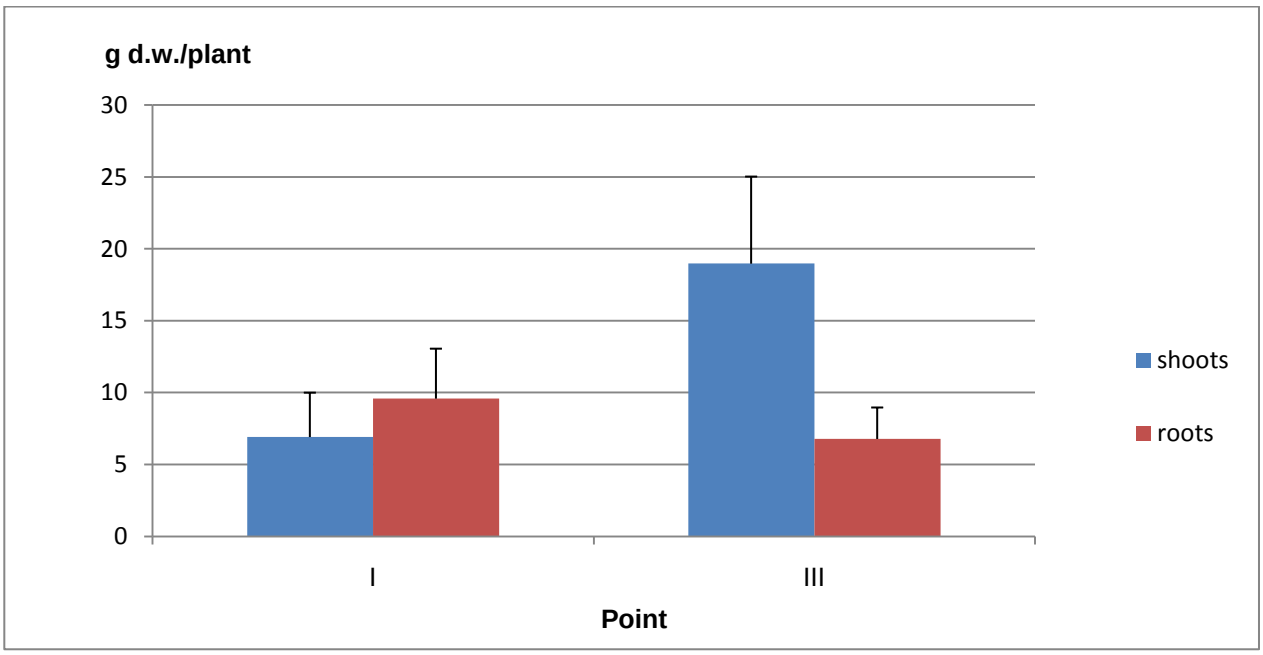



**Fig. 4.31** Biomass production of *A.cannabinum* in field trial at the sampling time T1 at the 2 sampling points (I,III)

Considering *A.cannabinum* plants in field trials (shown in Fig 4.31), a high biomass production was observed ranging from about 5 up to 19 g d.w./plant in shoot tissues, higher than *B.juncea* plants. On the other side a comparison is not feasible as, in comparison to *B.juncea, A.cannabinum* plants were collected after a growth time more than 4 times longer.

In relation to *A.cannabinum* plants, samples collecting was particularly difficult due to the extensive and deep roots system, therefore the root biomass reported underestimate the actual root weight.

It is however worth noting that for both plants a much higher biomass production was observed in comparison to lab-scale trials (par 4.2.2), of 1order of magnitude at least.

Even if for *A.cannabinum* this comparison is also affected by a time-length difference - as the lab-scale trial lasted less than half the time of the field trial - it is anyway indicative of a higher and good growth of the plant in open field condition. Actually for this plant species an increase in shoots biomass up to 2 order of magnitude was observed from lab mesocosm (shown in Fig 4.11 in par 4.2.2) to field trial, probably due not only to the longer time of growth – 16 months compared to 6 months for the lab-scale mesocosms – but also to the much greater amount of soil available for the plant in a scale up from 1kg pot to field conditions.

Conversely to *B.juncea*, in *A.cannabinum* field trial a shoots biomass more than twofold that detected in point I was produced at point III, which is in fact the less contaminated reference point.

As far as *B.juncea* is concerned, the comparison with the lab-scale data of the final sampling - performed at 4 months from sawing and therefore intermediate between T2 and T3 of field trial (Fig. 4.10 of par 4.2.2) - evidenced a much higher biomass production in field condition, of one order of magnitude higher. This data confirmed, even in the examined perturbed condition, the good biomass production reported in literature for this crop plant.

Also for the *A.cannabinum* a good growth was obtained, but in a much longer time of growth.

On the other side it has moreover to be considered that no agronomic practices such as irrigation or use of fertilizers were performed, therefore for both plants higher yield could be obtained by usual agronomic activities.


**Fig. 4.32** *B.juncea* (A) and A.*cannabinum* (B) samples collected at its respective T=1: 6 weeks and 16 months from the sawing


**Fig 4.33** Field-scale trial set up with *Brassica juncea* (A) in point I (B) and III (C)


**Fig 4.34** Field-scale trial set up with *A.cannabinum* (A) at point I (B) and III (C)

### 4.3.3 Lead content in plant tissues

Plant samples of at least 0,5 g d.w.(minimum sample weight for the ICP-OES analysis) were analyzed to determine total lead content in root and shoot tissues of both examined species. Total Pb content determinations were therefore carried out for both *B.juncea* cultivars' samples collected at the three different sampling times (T1:6 weeks from sawing, T2:14 weeks from sawing, T3:18 weeks from sawing and end of the trial) and for *A.cannabinum* plants collected at the only sampling after 16 months from sawing. The results obtained for *B.juncea* cultivars PI173874 (A) and PI426308 (B) are shown in Fig. 4.35 and 4.36.

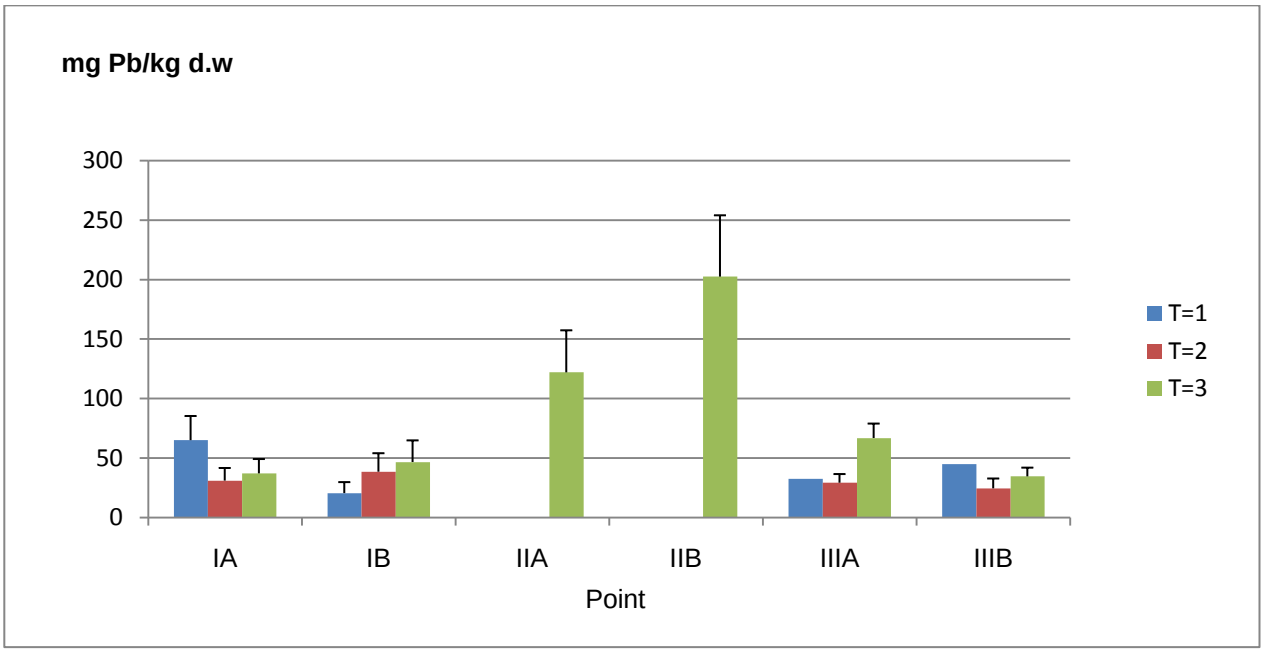



**Fig. 4.35** Pb content in shoots of both B.*juncea* cultivars (A and B) along the trial at the 3 sampling points (I,II,III)

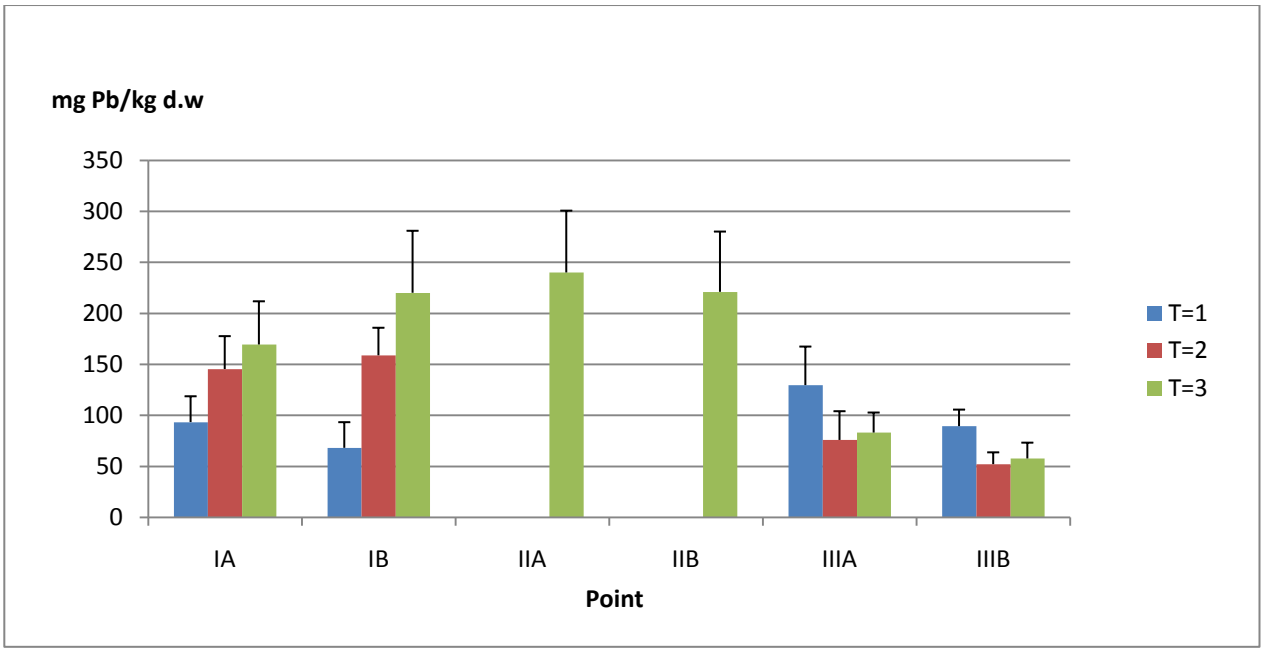



**Fig. 4.36** Pb content in roots of both B.*juncea* cultivars (A and B) along the trial at the 3 sampling points (I,II,III)

Considering Pb concentrations accumulated in both *B.juncea* cultivars (Fig. 4.35, 4.36), similar Pb content were detected in their tissues reaching values > 100 mg Pb/Kg d.w. in shoots and higher values of almost 250 mg Pb/Kg d.w. in roots at the most contaminated reference point II.

Comparing these data with those obtained in the untreated mesocosms of the lab-scale trial, set-up with the cultivar PI173874 (A) (Fig 4.12 reported in par 4.2.3), slightly lower but comparable values were obtained. As in the lab-scale mesocosms the highest Pb concentration were registered in the most contaminated point II, reaching values up to 200 mg/kg, while at the other points I and III in both lab-scale and field trials shoots concentrations lower than 100 mg/kg were reached.

The results for root tissues reported value generally higher than shoots Pb concentrations and, as previously observed in shoots, higher concentration were reached at the most contaminated reference point II. At roots level however a smaller gap was detected between concentrations reached in the most contaminated point II and point I (Fig 4.36), the latter also characterized by a contamination in inorganic Pb of the same order of magnitude of point II.

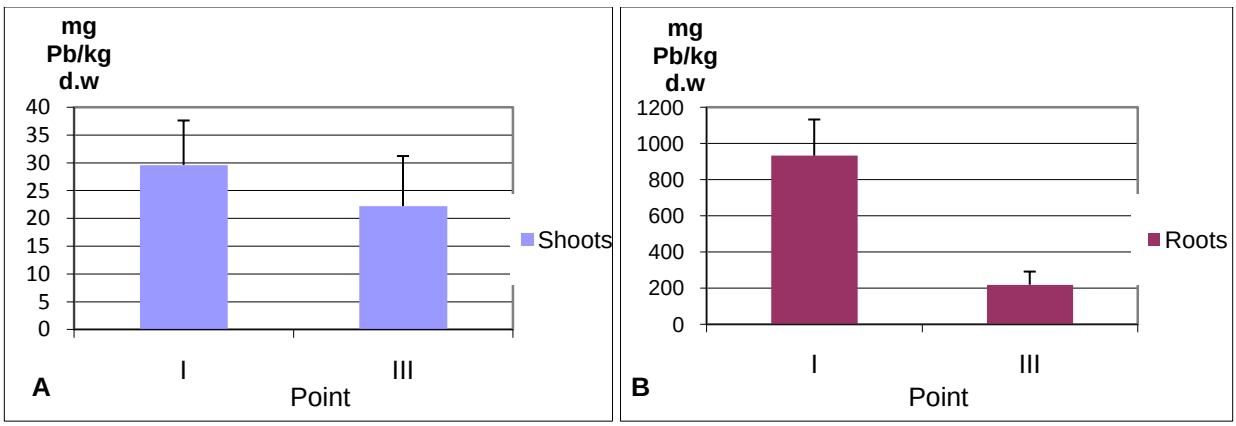



**Fig. 4.37** Pb content in *A.cannabinum* shoots (A) and roots(B) in field trial at the 2 sampling points (I,III)

As far as *A.cannabinum* field trial is concerned, concentrations in the order of hundreds of mg/kg were reached in *A.cannabinum* roots, reaching concentrations up to almost 1000 mg/kg at the point I location, reflecting its higher contamination in comparison to point III (Fig. 4.37).
Moreover, comparing roots Pb concentrations reached in point I to the respective lab-scale mesocosms (fig.4.14 in par 4.2.3), values about 5 times higher were detected, as could be expected due to the longer time of the field trial.
On the other side except for this datum, comparing all results obtained in field trial in both shoots and roots at the sampling point I and III to the respective lab-scale mesocosms (par 4.2.3), Pb concentrations reached in lab conditions were higher, in particular a difference of about one order of magnitude was detected at shoot level. In field conditions Pb uptake in *A.cannabinum* seemed therefore to be negatively affected.
Comparing the concentrations reached in both species, shoot values in the same order of magnitude were detected. However generally lower concentrations were reached in *A.cannabinum* and comparing shoots values of both species at their final samplings, values more than 2 times higher were detected in *B.juncea* in point III, while a lighter difference was detected at point I.
On the other side in comparison to both *B.juncea* cultivars much higher Pb concentrations were reached in *A.cannabinum* roots - more than 4 and 2 times higher respectively in point I and III - indicating for this species a sharp preferential accumulation in root tissues.

### 4.3.4 Evaluation of Phytoextraction efficiency

As previously performed for lab-scale trial (Par 4.2.5), different parameters were monitored to investigate, in relation to this kind of contamination, the actual efficiency of the examined plants grown in the uncontrolled conditions of a real scale Phytoremediation process.
Lead phytoextracted in shoots tissues per plant – defined as µg Pb Accumulated/plant = tissue [Pb] x dried biomass – was therefore calculated at the distinct sampling times along the process of *Brassica juncea* mesocosms and at the unique sampling of *Apocynum cannabinum,* to monitor Pb uptake in the harvestable part of the plant. Moreover Lead phytoextracted in roots tissues was also determined, to evaluate the contribute from the roots to Pb phytoextraction/immobilization.
The Bioconcentration Factor (BF) was not calculated, as soil analysis were not performed. The translocation factor (TF) - defined as $C_{SHOOTS}/C_{ROOTS}$ - was also calculated for both plants and displayed in Table 4.9 and 4.10.

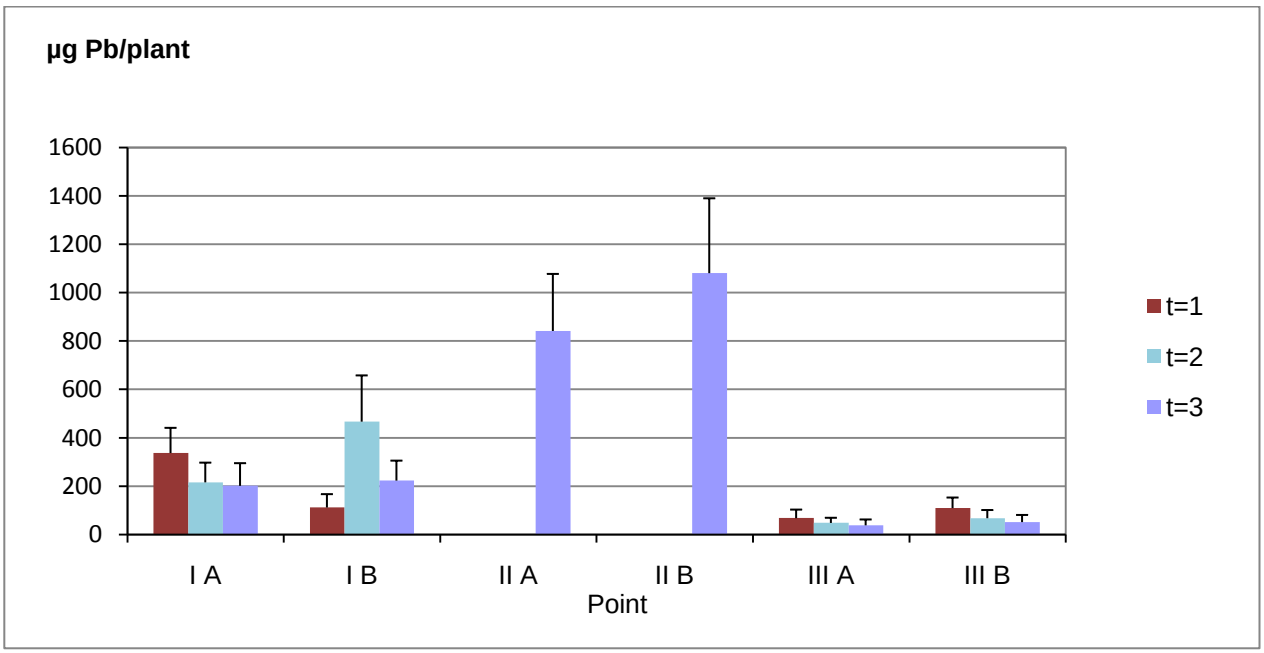



**Fig. 4.38** Pb phtoextracted per plant in shoots of both B.*juncea* cultivars (A and B) in field trial at the 3 sampling points (I,II,III)

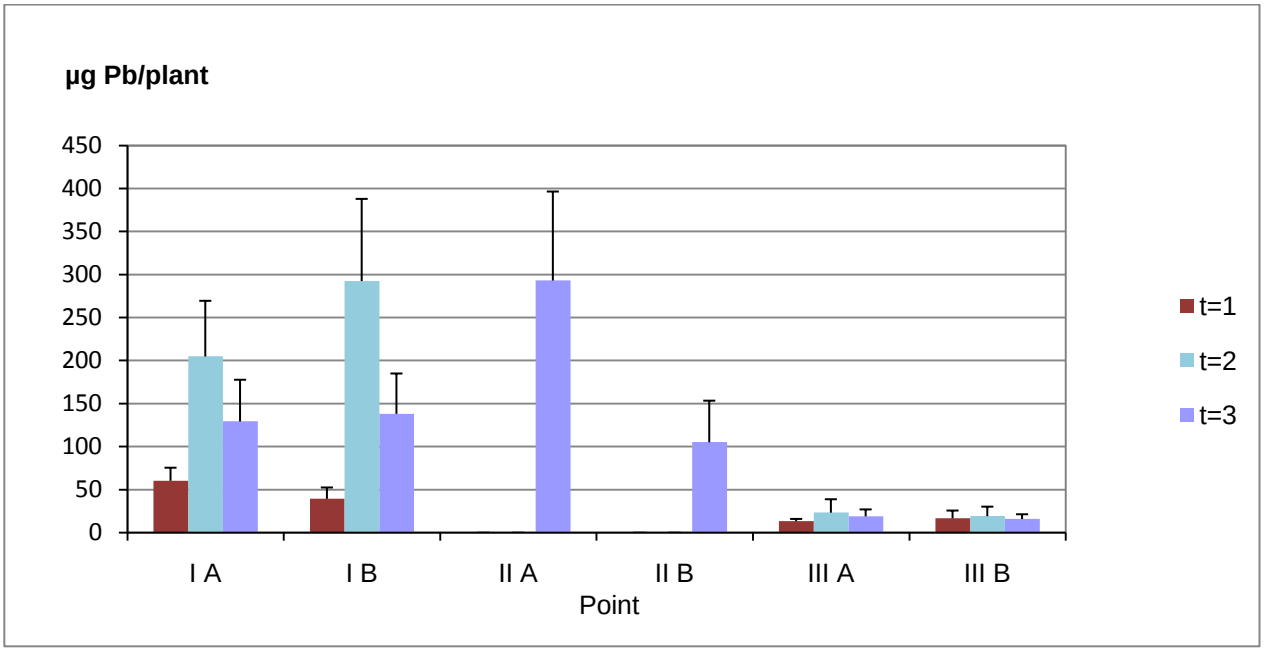



**Fig. 4.39** Pb phtoextracted per plant in roots of both B.*juncea* cultivars (A and B) in field trial at the 3 sampling points (I,II,III)

The evaluation of the total lead phytoextracted in shoots and roots tissues was performed for both *B.juncea* cultivars (fig 4.38, 4.39). The two cultivars showed similar values, as expected due to their similar biomass production and Pb concentration previously detected for their tissues (par 4.3.2 and 4.3.3). Both cultivars showed Pb accumulation per plant 1order of magnitude higher in comparison to lab-scale results (reported in par 4.2.5) - except for the less contaminated point III. This was due to the much higher biomass production of *B.juncea* observed in field conditions at point I and II (par 4.3.3), while in point III an unexpected low biomass was detected in filed trial. Considering Pb accumulated in root tissues (fig 4.39), even if slightly higher concentrations were reached in comparison to shoots, lower value of Pb was accumulated due to the lower root biomass. In general the Pb accumulated per plant in

shoot tissues was about two times higher than those accumulated in roots (fig 4.38, 4.39). In particular in point I both cultivars accumulated in shoots values 50% higher than roots, while in the most contaminated point II shoots of cultivar A and B accumulated respectively 2 and 10 times higher values than roots, and 2 and 3 times higher shoots accumulation in point III.

For both shoots and roots higher values were detected at point I and II, reflecting the higher tissue Pb concentrations and soil contamination associated to this sampling points (fig 4.38, 4.39). A positive correlation between soil contamination and tissues concentrations has in fact been described (Kapourchal *et al.*, 2009).

It is worth noting that no increase in Pb phytoextracted per plant was observed at the last sampling time, this suggest that in possible Phytoremediation processes with *B.juncea* a shorter growth time could be applied, positively affecting the remediation process.

As far as Pb phytoextracted by *A.cannabinum* is concerned, as in lab-scale trial the highest values were detected in roots tissues, in connection to its roots preferential accumulation and greater biomass production, with higher values in correspondence of the more contaminated point I compared to the examined point III (Fig 4.40). As above reported for *B.juncea,* values one order of magnitude higher than lab-scale mesocosms (par 4.2.5) were obtained in *A.cannabinum* tissues. Actually despite the lower Pb concentrations reached in plant of *A.cannabinum* grown in field conditions, in shoot tissue a higher Pb accumulation per plant was obtained due to the higher biomass of field plants.

Moreover comparing roots accumulation of both plants in field conditions (Fig. 4.39, 4.40), an accumulation one order of magnitude higher was detected in *A.cannabinum* due to both high biomass and concentrations reached in its roots. Comparing the root accumulation in point III, a difference of two order of magnitude was observed due to the low growth of *B.juncea* species at this sampling reference point. On the other side *A.cannabinum* roots proved to be of highly difficult harvesting, limiting therefore their phytoextraction contribute.

Considering the values obtained for the harvestable part of the plants (Fig.4.38, 4.40) - the most important in a phytoextraction process - values of about 450 µg Pb/plant were obtained in field plants of *A.cannabinum* at point III, much higher than *B.juncea* in connection to its very low growth detected at this reference point.

On the other side although a comparison of *B.juncea* and *A.cannabinum* for point II was not possible - as *A.cannabinum* growth was too affected - values of 800 and 1000 µg Pb/plant were respectively obtained by the 2 *B.juncea* cultivars A and B.

Besides comparing Pb accumulation of the two species at the most contaminated point I similar values were obtained of the same order of magnitude, however a 4 times shorter time was spent by *B.juncea* trial. These results indicate therefore a higher Phytoextraction accumulation and efficiency in *B.juncea* species, with no significant differences between the two examined cultivars.

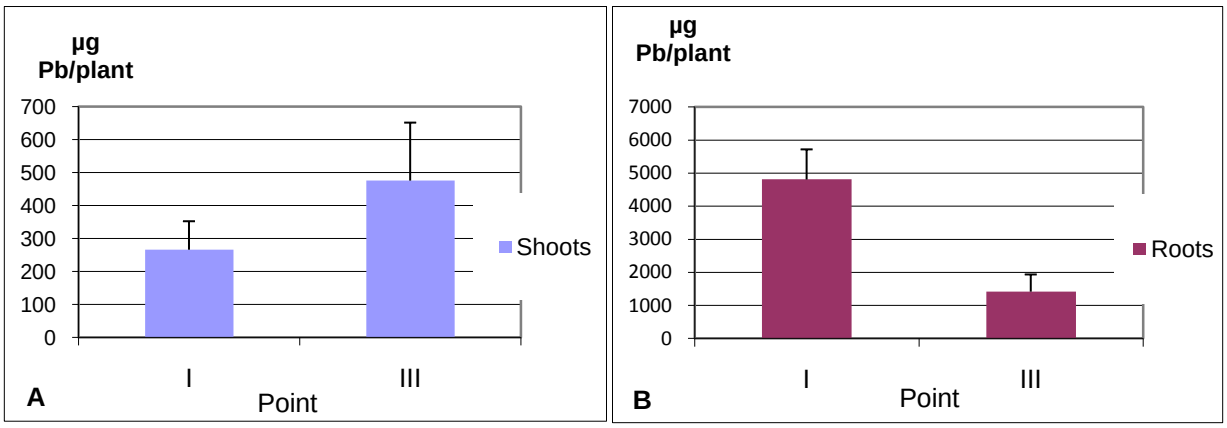



**Fig. 4.40** Pb phtoextracted per plant in shoots(A) and roots(B) of *Apocynum cannabinum* in field trial at the 2 sampling points (I,III)

Considering the Translocation Factor (TF) determined for both *B.juncea* cultivars, values along the trial ranged between 0,2-0,7 for both cultivars and in general lower values were detected in point I (Tab. 4.9). A higher value of 0,94 was detected for the cultivar B (PI426308) at the final sampling at the most contaminated point II. For the *B.juncea* species in fact the highest shoot concentration of about 200 mg Pb/Kg d.w. was reached at this point by cultivar B (par 4.3.3, fig 4.35). Moreover at the final sampling time values of about 0,7 TF were detected for both cultivars in the less contaminated point III.
Considering TF values detected in *A.cannabinum* plants (shown in Tab 4.10) values lower than 0,15 were detected, as expected due to the low shoot concentrations reached in field conditions. Actually the shoot concentrations reached in field by *A.cannabinum* plants were not only lower than those obtained by the same plant in lab-conditions (Fig. 4.13 in par 4.2.3), but even than the concentrations of *B.juncea* obtained both in lab-mesocoms (Fig. 4.12 in par 4.2.3) and in field trial (Fig. 4.38, 4.39). In comparison to both untreated and sterilized lab-scale mesocosms (par 4.2.5, tab 4.2), the TF values calculated in field trial were respectively 1 order of magnitude and 5 times smaller for point I and III. These results indicate a low shoot translocation and phytoextraction efficiency of this plant in the applied open field conditions.

| **T1** | **Cultivar A** | **TF** | **Cultivar B** | **TF** |
|---|---|---|---|---|
| | Point I | 0,69±0,12 | Point I | 0,29±0,02 |
| | Point II | - | Point II | - |
| | Point III | 0,43±0,06 | Point III | 0,46±0,07 |
| **T2** | **Cultivar A** | **TF** | **Cultivar B** | **TF** |
| | Point I | 0,21±0,04 | Point I | 0,23±0,05 |
| | Point II | - | Point II | - |
| | Point III | 0,41±0,08 | Point III | 0,47±0,08 |
| **T3** | **Cultivar A** | **TF** | **Cultivar B** | **TF** |
| | Point I | 0,22±0,07 | Point I | 0,21±0,03 |
| | Point II | 0,51±0,08 | Point II | 0,94±0,10 |
| | Point III | 0,72±0,05 | Point III | 0,69±0,10 |

**Table 4.9** Traslocation Factor (TF) values for *Brassica juncea* in field trial

| *Apocynum cannabinum* | TF |
|---|---|
| Point I | 0,09±0,01 |
| Point III | 0,15±0,01 |

**Table 4.10** Translocation Factor (TF) values for A.*cannabinum* field trial

In relation to TF obtained with *B.juncea* (Tab. 4.9), as previously mentioned lead hyperaccumulating plants, which usually have a higher shoot/root ratio of lead content in plant than the non-hyperaccumulators, are reported values of 0.04 - 0.1 TF (Xiong, 1998; Kumar *et al.*, 1995).
A higher shoot/root ratio of heavy metal content in plant is important in practical phytoremediation of heavy metal-contaminated soils, as it can enable phytoremediation of the heavy metal-contaminated soils by harvesting the aboveground parts of the plants, thus simplifying the agricultural practices.
In the present study, both *B. juncea* cultivars showed 0,2-0,7 TF values, much higher than those reported (0.09) in Literature for the Pb Hyperaccumulator *Brassica pekinensis* and for both *B. juncea* and *B. nigra,* respectively grown in soil and hydroponically after inorganic Pb amendments (Xiong, 1998; Kumar *et al.*, 1995). The presented TF results compared well also with TF of 0,01 and 0,04 reported for *Brassica napus* and *Raphanus sativus* of the *Brassicaceae* family grown in multi-contaminated soil (Marchiol *et al.*, 2004).
Although this ratio is far below the desired level of 1, it demonstrates a great ability to accumulate lead from soil to shoot of this non-hyperaccumulating plant, in particular considering lead low mobility in soils. In fact a comparison of the phytoextraction coefficients of shoots reported for $Cr^{6+}$, Cd, Ni, Zn, Cu, Pb and $Cr^{3+}$ showed that lead is among those which are the most immobile heavy metals from soil to shoot (Kumar *et al.*, 1995), due to tight binding of lead to soils and plant materials (Xiong, 1998). However, as previously mentioned the influence of many variables, such as soil physical/chemical characteristics and Pb biovailability, affect the comparison of distinct experiments.
In field condition the influence and role of the autochthonous micro flora was implied in the results obtained by the examined plants in relation to this kind of contamination. TF values here determined for *B.juncea* were in fact higher than those reported in literature for the same species and even for the same cultivar 426308 ( here named B), reporting a value of about 0,3 TF in hydroponic conditions (Kumar *et al.*, 1995).
PGPR are in fact known to be able to promote plant growth in heavy metal contaminated soils, and in addition a variety of bacteria (mainly PGPR) have been reported as phytoextraction assistant - such as *Pseudomonas* spp. *Bacillus* spp. *Microbacterium* spp. *Rhizobium* spp. *Variovorax* sp. detected within the indigenous micro flora - promoting the phytoextraction process (Koo *et* Kyung-Suk, 2009; Khan *et al.*, 2009).

## 4.3.5 Results and comparative considerations on the 2 plants tested from lab-scale to field-trial

Despite Pb is a challenging metal tightly bound in most soils, data obtained in lab-scale and field trial showed for both examined *Brassica juncea* cultivars quite high Pb concentrations in their shoots tissues up to over 250 mg/kg d.w..
*Apocynum cannabinum* was able to reach the same shoots concentration in lab-conditions, although almost one order of magnitude decrease was observed in field trial. However it reached in its roots tissues the highest concentration detected up to 2000 mg/kg d.w.. A sharp preferential accumulation in roots tissue was in fact detected in *A. cannabinum*, while in *B.juncea* root tissues only slightly higher but similar concentration to shoots were detected.
For the two tested cultivars of *B.juncea* no significant differences were detected in either Pb content or biomass production, reaching almost the same results. On the other side *A.cannabinum* showed a much

slower biomass production. In comparison to the lab-scale trial for both species however a much higher growth was observed in open field conditions, with a biomass production of at least one order of magnitude higher.

The increase in biomass production scaling up from 1kg mesocosms pots to open field was reflected in the Pb phytoextracted - defined as µg Pb/plant and calculated as the product of Pb concentrations reached in plant tissues and biomass produced - which evaluates the actual Phytoremediation efficiency.

Especially for *B.juncea* the results pointed out a positive correlation between the lab-scale experiment performed in the controlled conditions of a glasshouse and the field trial set up along a scale up directly at the Ex-SLOI area. Except for *A.cannabinum* shoots values, the results of Pb content within plant tissues obtained in the field trial were in fact comparable to those of lab-scale mesocosms. Moreover in connection to the higher biomass production detected in the same plant species moved from pot to open field, higher phytoextraction efficiency was obtained for both plants in field-scale experiment.

The data obtained in the experiments performed indicated a tolerance of both plants to the organic and inorganic Pb contamination. However comparing the 2 plant species, *B.juncea* showed a higher efficiency reaching a comparable shoot accumulation both in lab-scale mesocosms and in open field conditions, but in a much shorter time, in particular 4 times shorter in field trial.

It has however to be considered that plant growth was highly affected in the most contaminated sampling point II, stopping from performing the *A.cannabinum* field trial. This confirms the presence of contamination in the area too high for a direct application of a Phytoremediation approach with the examined plants at a global scale. Although *B.juncea* accumulated a considerable amount of Pb after the period of growth on the contaminated soil, it is use could not be considered suitable for planning a realistic and short-term phytoremediation program at the high contamination as those detected at the 3 examined points within the Ex-SLOI.

On the other side in the areas of lower contamination and in a combined approach with physical-chemical techniques a phytoremediation process with *B.juncea* could be interesting.

In literature plant such as radish - reported to accumulate values lower than those registered in this trial of 208 mg/kg in roots and 27.25 mg/kg in shoot - has been proposed to be used to remediate lead-polluted top soils (0–10 cm), as by more crops a high yield per year can be obtained (Kapourchal *et al.*, 2009). These considerations confirm therefore for *B.juncea* a possible applicability in relation to both organic and inorganic Pb contamination in more moderate and shallow contamination levels.

However values obtained indicate, as reported in literature, the need of several crop seasons and of increasing the Phytoremediation performance, whose main drawbacks are the need of time and low efficiency associated to plant limits. In fact among different factors influencing phytoremediation, two important limiting factors are pollutant fixation by soil particles and low absorption and/or transportation by plants (Kapourchal *et al.*, 2009). Therefore growing interest has been drawn by microorganisms characterized by PGP activity and able to increase plant biomass and/or increase metal uptake. Various studies and data have been reported in this context in the last years in relation to phytoremediation enhanced by microorganisms (Karami *et* Shamsuddin, 2010). For phytoremediation to be effective, one more way is to increase the amount of biomass in the contaminated soil by the use of higher plants, such as Hybrid poplar under study (Glick, 2003; Cunningham *et* Berti, 1995; Di Lonardo *et al.*, 2010). In this perspective, the arboreal plant Hybrid poplar characterized by extensive root system and great biomass production has been the object of our study in a biaugmentation protocol – as next described in par 4.3.6 - with strains isolated from the indigenous micro flora selected within the Ex-SLOI area.

Moreover improvements to phytoextraction could also be obtained by simply implementing specific agronomic practices that may have a positive influence on the global efficiency of the process (Quartacci *et al.*, 2006).

### 4.3.6 Selection of a consortium of autochthonous strains for a Phytoremediation study in a biaugmentation protocol

As above mentioned a study on a third plant - Hybrid poplar - has been taking place. This arboreal plant was originally chosen as it is characterized by a huge biomass production and an extensive root system; it presents also the advantage of being generally easy to propagate and to grow fastly. This plant has moreover been reported to be tolerant to organic contaminants and to accumulate metals, and is commonly used as phytoremediation tool (Di Lonardo *et al.*, 2010).

The study has been performed in a biaugmentation 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 heavy 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 even in the perspective of a Phytoremediation approach, increasing biomass production and therefore remediation efficiency through their addiction in biaugmentation protocols. Selecting microorganisms that are both metal-resistant and able to produce plant growth-promoting compounds could in fact prove useful as inocula in phyto-remediation 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 *Variovorax* sp, are able to act as phytoextraction assistant promoting the phytoextraction process (Koo *et* Kyung-Suk, 2009).

On the basis of the study previously performed (par 3.1), OTUs reporting Heavy metals resistances and PGPR traits were chosen among the isolated components of the culturable indigenous micro flora. Therefore a consortium of Gamma and Beta proteobacteria was composed as reported in tab. 4.11. The selected strains are therefore of particular interest as characterized by PGP traits and in the mean time well adapted to this particular soil and contamination, from which they have been isolated.

| OTU | Taxonomic reference ID | Phylogenetic group | Metal resistences | | | PGPR traits | |
|---|---|---|---|---|---|---|---|
| | | | Pb | Hg | Ni | ACC | IAA |
| **A5** | *Delftia* sp. Fj594443 | β-proteobacteria | + | + | - | - | + |
| **A8** | *Variovorax* sp. EU734636 | β-proteobacteria | - | - | - | + | - |
| **A11** | *Pseudomonas* sp. EU375660 | γ-proteobacteria | - | - | - | + | + |
| **A14** | *Alcaligenes* sp. EU37500 | β-proteobacteria | - | - | - | + | - |
| **A16** | *Stenotrophomonas maltophilia* EF620462 | γ-proteobacteria | + | + | + | + | + |

**Tab. 4.11** Resistence determinants and PGP traits detecetd in the OTUs composing the inoculum

The bacterial inoculum thus selected was bioaugmented to the rizosphere of Hybrid poplars plants at an initial concentration of $10^7$ CFU/g of soil (Fig 4.41). The biaugmentation was performed after 5 month

from planting of cuttings in mesocosm of 5 Kg set up in glasshouse with the soil of the sampling point III. In comparison to the other two, the sampling point III has a lower level of contamination, a better soil structure and higher accessibility. Controls were set-up as described in par 2.7.4.2.
In parallel, mesocosms inoculated and not inoculated were amended with the chelating agent EDTA, used in various studies to mobilize metals and increase plant uptake (Komárek *et al.*, 2007). The analysis of Pb content and biomass production at the end of the trial, i.e. after 8 month from planting, will allow evaluating the plant efficiency and the microorganisms' actual role and influence on plant growth and contaminant removal. Besides the molecular DGGE analysis on rhizosphere soil samples will allow monitoring the inoculated microorganisms along the trial. As the analyses are still in progress no data on this experiment are reported in this document.


**Fig 4.41** Lab-scale trial set up with Hybrid Poplar

# 5. Concluding remarks

Nowadays lead is worldwide one of the pollutants of major concern due to its widespread diffusion, persistence and toxicity. Indeed Pb has been widely used since ancient time and its spreading in the environment is connected to industrial, agricultural and urban activities such as manufacturing and smelting of batteries, land application of sewage sludge and use of leaded petrol (Hill, 2004; Thornton *et al.*, 2001; Lu *et al.*, 2005).

Indeed organic lead has been used for almost a century as anti-knocking additive for petrol, leading to an ubiquitous pollution of both organic and inorganic Pb in low concentrations and much more severe contaminations at manufacturing and distributing level, implying the need for remediation (Gallert *et* Winter, 2002; World Health Organization, 2007).

In this context 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 side 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 Phytoremediation 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 *et* Dowling, 2009; Manousaki *et* Nicolas, 2009; Shukla *et al.*, 2010).

This PhD research focused on the autochthonous bacterial community established in a soil exposed for over half century to both organic and inorganic Pb in a former industrial area Ex-SLOI in Trento Nord. The study was performed by complementing both culture-dependent and culture-independent techniques and examined distinct sampling points with different contamination levels chosen in the former industrial area.

The results obtained by the culture analysis indicated the selection within the Ex-SLOI area of a tolerant soil bacterial community, characterized by a heterogenic structure and - for a perturbed soil - rich biodiversity. Actually all the isolated strains - relating respectively to 21 gram-negative and to 7 gram-positive distinct genera - showed high homologies with bacteria diffused within different environmental niches including contaminated soils, capable of degrading recalcitrant pollutants and hydrocarbons and reporting high resistances to heavy metals.

The molecular analysis performed further indicated the selection of dominant members within the autochthonous bacterial community exerted by the long-term contamination. In particular a distinct ribotype was observed in the most polluted Hot spot, i.e. the highest contaminated spot detected within the entire Ex-SLOI area, indicating the selection for a specific microbial community.

Considering the bacterial taxonomic distribution, both the molecular analysis and the culture approach indicated the general predominance of the Proteobacteria *phylum* in the sampling points under study, including the most contaminated Hot spot.

Indeed the gram-negative Proteobacteria included the majority of OTUs directly isolated from the most contaminated examined sampling points and accounted for the totality of strains isolated in the strictest condition of Enrichment culture - performed with TEL as sole carbon source to isolate the strains most resistant and potentially able to degrade organic Pb.

The molecular analysis performed - along with the MIC determination - evidenced the heavy metal resistance within the autochthonous microbial community with strains reporting genes for multiple resistances. A degrading potential towards hydrocarbon was also identified by the metagenomic molecular analysis, targeting the whole autochthonous microbial community.

In particular 8 strains displayed at least one heavy metal resistance determinant, while 5 of them presented the concomitant presence of multiple resistances. It is worth noticing that heavy metal

resistance determinants were exclusively detected within the most represented classes of Beta (*Cupriavidus, Ralstonia* and *Delftia* genera) and Gamma-proteobacteria (*Pseudomonas* and *Stenotrophomonas* genera), confirming the high resistance potential of the gram-negative population within the indigenous community. In particular a strain of *Cupriavidus campinensis* displayed at the same time 4 resistance determinants: for Pb, Hg, Cr and Cd-Zn-Co.

Considering also the microbial ability of promoting plant growth, the high represented class of Gamma-proteobacteria included the 2 genera - *Pseudomonas* and *Stenotrophomonas* - whose members within the autochthonous community demonstrated both examined PGP (Plant growth promoting) traits.

Besides, to the Gamma-proteobacteria genera *Pseudomonas* and *Stenotrophomonas*, and to the *Delftia* genus of the Beta class, belong the 3 OTUs reporting both Heavy metal resistence determinants and PGP traits: namely OTU A5 *Delftia* sp., OTU A10 *Pseudomonas putida* and OTU A16 *Stenotrophomonas maltophilia,* hence of particular interest in the context of a Phytoremediation approach with a biaugmentation protocol. 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).

In the screening for PGP characteristics – performed on the OTUs selected in the strictest conditions imposed either in laboratory by Enrichment culture and *in situ* at Hot spot level - the 68% of examined OTUs possessed 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.

The strain *Stenotrophomonas maltophilia* drew particular attention reporting three heavy metal resistance determinants - to Pb, Hg and Ni - and both analyzed potential PGP traits: IAA production and ACC-deaminase activity.

The study performed indicated therefore the selection imposed by the contamination of a resistant microbial cenosis, with members of particular high resistance potential belonging to the most represented gram-negative Proteobacteria *phylum*, and at the same time characterized by a high PGP potential.

Considering the minor gram-positive population detected within the microbial community, the majority of the gram-positive Firmicutes and Actinobacteria were directly isolated from the less contaminated examined sampling point. Nevertheless few member of the Actinobacteria were also detected in enrichment cultures - set up with the addition of yeast extract as carbon source – and two genera, *Microbacterium* and *Arthrobacter,* were part of the indigenous culturable community selected within the most contaminated Hot spot. Besides, members of these genera also displayed PGP traits. This indicates that also the gram-positive Actinobacteria *phylum* includes community members of high resistance and bioremediation potential.

Examining the interaction of indigenous micro flora with the plants studied in a Phytoremediation approach, the results of the lab-scale study showed in both species a higher growth in presence of the indigenous micro flora. Moreover in *B.juncea* higher Pb concentrations were also detected in shoot tissues in presence of the autochthonous micro flora, pointing out its synergistic effect on both plants growth and on Phytoextraction efficiency.

These results are in agreement with those above reported on the characterization of the soil indigenous micro flora, and with literature evidences on promotion of plant growth and metal uptake in hyperaccumulator or non-hyperaccumulator plants by heavy metal-resistant bacteria (Sheng *et al.*, 2008a).

Moreover, the analyses performed on the speciation of the organic Pb compounds along the trial in soil of *B.juncea* mesocosms pointed out a progressive degradation of organic lead during the phytoremediation

process, suggesting the promotion by the plant-micro flora system of the mineralization of organic Pb, lowering the environmental risk to it associated.

As far as the examined plants are concerned, obtained data also indicated tolerance and resistance of both plants towards this kind of contamination combining inorganic and, even if in lower levels, organic Pb. On the other side comparing the 2 plants, *A. cannabinum* showed a lower shoot biomass production and a much slower growth.

Although in lab-scale *A. cannabinum* reached the highest detected Pb concentration within its tissues, it displayed a sharp preferential accumulation within roots, while accumulation in the harvestable part of the plant is the main objective of the Phytoextraction process.

Despite Pb is a challenging metal tightly bound in most soils, data obtained in lab-scale and field trial showed for *Brassica juncea* quite high Pb concentrations in its shoots tissues up to 270 mg/kg d.w.. On the other side even if *Apocynum cannabinum* was able to reach slightly higher shoots concentration in lab-conditions, almost one order of magnitude decrease was detected in its shoot concentrations reached in field trial.

However in comparison to the lab-scale trial, for both species a much higher growth was observed in open field conditions, with a biomass production of at least one order of magnitude higher.

Especially for *B.juncea*, the results pointed out a positive correlation between the lab-scale experiment performed in the controlled conditions of a glasshouse and the field trial, set up along a scale up directly at the Ex-SLOI area.

Moreover the increase in biomass production scaling up from 1kg mesocosms pots to open field was reflected in the Pb phytoextracted - defined as µg Pb/plant and calculated as the product of Pb concentrations reached in plant tissues and biomass produced - which evaluates the actual Phytoremediation efficiency. Actually, higher phytoextraction efficiency was obtained for both plants in field-scale experiment. In comparison to *A.cannabinum*, *B.juncea* showed a higher efficiency, accumulating a comparable amount of Pb in the harvestable part of the plant, but in about a 4 times shorter time.

In moderate and shallow contamination levels and/or in combined approach with physical-chemical techniques – the latter for higher contaminations as those detected at the examined points within the Ex-SLOI - a phytoremediation process with *B.juncea* could be interesting to remediate and restore soil contaminated by both inorganic and organic Pb.

However values obtained indicated, accordingly to literature, the need of several crop seasons and of increasing the Phytoremediation performance, whose main drawbacks are the need of time and low efficiency, associated to pollutant fixation by soil particles and low absorption and/or transportation by plants (Neugschwandtner *et al.*, 2008; Kapourchal *et al.*, 2009). Therefore growing interest has been drawn by microorganisms characterized by PGP activity, able to increase plant biomass and/or increase metal uptake, and by the use of higher plants such as Hybrid poplar under study (Glick, 2003; Di Lonardo *et al.*, 2010; Karami *et* Shamsuddin, 2010).

Indeed in this perspective a lab-scale trial in a biaugmentation protocol is in progress with the arboreal plant Hybrid poplar - characterized by extensive root system and great biomass production - set up on the Ex-SLOI contaminated soil. Strains amended were chosen from the isolated components of the culturable indigenous micro flora among the Proteobacteria population reporting PGP traits. Results of this trial will allow determining the effect of the Proteobacteria consortium under study on the phytoextraction process and the applicability in a Phytoremediation enhanced by microorganisms applied on a contamination combining organic and inorganic Pb.

On the basis of the results obteined 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 Pb contamination present in the Ex-SLOI 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 predominance of gram-negative Proteobacteria has been detected at the higher contamination levels examined within the area, including strains with multiple Heavy metal resistances and/or PGP traits;
- Among the community members isolated in pure culture, strains of *Deltia* sp., *Pseudomonas putida* and *Stenotrophomonas maltophilia* - reporting both Heavy metal resistance determinants and PGP traits – are of particular interest, even in a Phytoremediation perspective with a biaugmentation protocol.

As far as the study in the context of a Phytoremediation approach with the 2 plants *Brassica juncea* and *Apocynum cannabinum* is concerned, it is interesting to point out the following considerations:

- The examined autochthonous micro flora, selected by and adapted to Pb contamination at the EX-SLOI area, exerted a positive influence on both plant species, improving plant growth and the Phytoremediation efficiency. In *B.juncea* it also positively affected Pb uptake;
- Both plants showed tolerance and resistance towards inorganic and organic Pb, reaching good Pb concentrations in their tissues, despite Pb is a challenging metal which tightly bind to soils particles and plant materials;
- Comparing the 2 plant species, *B.juncea* showed a higher Phytoextraction efficiency reaching a comparable shoot accumulation – most important parameter in a Phytoextraction process – both in lab-scale mesocosms and in open field conditions, but in a much shorter time;
- Despite it is not applicable at the high contaminations as those detected at the examined points within the Ex-SLOI, a Phytoremediation process with *B.juncea* could be interesting at lower/shallow contamination and in a combined approach with physical-chemical techniques;
- Interesting prospective - and trial in progress - is the application of the arboreal plant Hybrid poplar – with extensive root system and great biomass production - in a biaugmentation protocol with strains isolated from the examined indigenous micro flora selected within the Ex-SLOI area.

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# Original papers

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AREA 5 • BIOREMEDIATION • RESEARCH ARTICLE

# Selenite resistant rhizobacteria stimulate $SeO_3^{2-}$ phytoextraction by *Brassica juncea* in bioaugmented water-filtering artificial beds

**Silvia Lampis · Anita Ferrari · A. Cristina F. Cunha-Queda · Paula Alvarenga · Simona Di Gregorio · Giovanni Vallini**

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**Abstract**
*Background, aim, and scope* Selenium is a trace metalloid of global environmental concern. The boundary among its essentiality, deficiency, and toxicity is narrow and mainly depends on the chemical forms and concentrations in which this element occurs. Different plant species—including *Brassica juncea*—have been shown to play a significant role in Se removal from soil as well as water bodies. Furthermore, the interactions between such plants, showing natural capabilities of metal uptake and their rhizospheric microbial communities, might be exploited to increase both Se scavenging and vegetable biomass production in order to improve the whole phytoextraction efficiency. The aim of the present study was to evaluate the capability of selenite removal of *B. juncea* grown in hydroponic conditions on artificially spiked effluents. To optimize phytoextraction efficiency, interactions between *B. juncea* and rhizobacteria were designedly elicited.
*Materials and methods* Firstly, *B. juncea* was grown on water-filtering agriperlite beds in the presence of three different selenite concentrations, namely, 0.2, 1.0, and 2.0 mM. Plant growth was measured after 3 and 6 weeks of incubation in order to establish the selenite concentration at which the best plant biomass production could be obtained. Afterwards, water-filtering agriperlite beds were inoculated either with a selenium-acclimated microbial community deriving from the rhizosphere of *B. juncea* grown, erstwhile, in a selenite-amended soil or with axenic cultures of two bacterial strains, vicelike *Bacillus mycoides* SeITE01 and *Stenotrophomonas maltophilia* SeITE02, previously isolated and described for their high resistance to selenite. These latter were seeded separately or as a dual consortium. Selenite was amended at a final concentration of 1.0 mM. Total Se content in plant tissues (both shoots and roots), plant biomass production, and persistence of bioaugmented microbial inocula during the experimental time were monitored. Moreover, parameters such as bioconcentration factor (BF) and phytoextraction efficiency (PE) were determined at the end of the testing run to evaluate the effects of the different bioaugmentation strategies adopted on selenite phytoextraction efficiency of *B. juncea*.
*Results* A general but significant increase in capacity to extract and transport selenium to the epigeous plant compartments was recorded in *B. juncea* grown in beds augmented with microbial inocula, except for the treatment with *B. mycoides* SeITE01 alone. Nevertheless, a severe decrease in vegetable

S. Lampis · A. Ferrari · G. Vallini (✉)
Department of Biotechnology, Laboratories of Microbial Biotechnology and Environmental Microbiology,
University of Verona,
Strada Le Grazie 15-Ca' Vignal,
I-37134 Verona, Italy
e-mail: giovanni.vallini@univr.it

A. C. F. Cunha-Queda
Departamento de Química Agrícola e Ambiental,
Instituto Superior de Agronomia,
Universidade Técnica de Lisboa (UTL),
Tapada da Ajuda,
P-Lisboa 1349-017, Portugal

P. Alvarenga
Departamento de Ciéncias do Ambiente,
Escola Superior Agrária de Beja, Rua Pedro Soares,
Apartado 158,
P-Beja 7801-902, Portugal

S. Di Gregorio
Department of Biology, University of Pisa,
Via Luca Ghini 5,
I-56126 Pisa, Italy

biomass production was observed after all microbial treatments with the exception of the plants that had received only *S. maltophilia* SeITE02. Actually, an increase in selenium phytoextraction efficiency up to 65% was observed in *B. juncea*, when this bacterial strain was inoculated.

*Discussion* Emendation of *B. juncea* grown in water-filtering beds with a Se(IV)-acclimated microbial community caused a higher Se uptake along with a reduction of plant biomass yield with respect to plants grown without addition of the same bacterial inoculum. The increase of selenium BF in shoots suggests that the Se(IV)-acclimated microbial community not only elicited the plant capacity to absorb selenite, but also did improve the capacity to transport the metalloid to the epigeous compartments. On the other hand, the reduction in plant biomass yield might be related exactly to this improved capability of *B. juncea* to accumulate selenium at concentrations that are actually toxic for plants. Differently, addition of two selenite-resistant bacterial strains, namely, *S. maltophilia* SeITE02 and *B. mycoides* SEITE01, had weaker effects on plant biomass production when compared to those recorded in the presence of the Se(IV)-adapted microbial community. In particular, inoculation of water-filtering beds with the SeITE02 strain alone was the sole strategy resulting in a positive effect on both plant biomass production in stressful conditions and the capacity of shoots to accumulate selenium. In fact, its putative ability of reducing Se(IV) to organo-Se compounds significantly enhanced either selenium absorption by the plants or active metalloid translocation to epigeous parts.

*Conclusions* Bioaugmentation with the bacterial strain *S. malthophila* SeITE02 is suggested to elicit selenite phytoextraction efficiency in *B. juncea*.

*Recommendations* Manipulation of synergistic interactions between plants having phytoextraction capabilities and their associated rhizobacteria may enhance already consolidated treatment processes aimed to detoxify selenite laden wastewater.

**Keywords** *Bacillus mycoides* · Bioaugmentation · *Brassica juncea* · Constructed water-filtering beds · Phytoextraction · Selenite · *Stenotrophomonas maltophilia*

## 1 Background, aim, and scope

Selenium is a naturally occurring metalloid which behaves either as an essential trace element for many living organisms (Birringer et al. 2002) or as a noxious contaminant due to the narrow concentration range between its deficiency and toxicity (Vinceti et al. 2001). Se toxicity, however, is tightly related to the bioavailability of its chemical species, which includes the highly toxic oxy-anions selenite [$SeO_3^{2-}$, Se(IV)] and selenate [$SeO_4^{2-}$, Se(VI)], as well as the insoluble and almost biologically inert elemental selenium ($Se^0$; Barceloux et al. 1999).

In a perspective aimed at exploiting biotechnological tools for selenium decontamination of environmental matrices, constructed wetlands—already proposed for the removal of a wide range of other water-borne contaminants—offer a promising alternative to the traditional treatment methods (Sobolewski 1999; Hansen et al. 1998). The dynamics of Se transformation by means of different mechanisms occurring in this type of vegetated bioreactors has been extensively discussed (Zhang and Moore 1996; Frankenberger and Engberg 1998). Several plant species have been shown to play a significant role in Se removal (Pilon-Smits et al. 1999a; Qian et al. 1999). Unfortunately, adsorption phenomena within the contaminated matrices (Gao et al. 2000) often hinder both recovery and satisfactory disposal of the pollutants of interest (Sobolewski 1999; Hansen et al. 1998). In particular, *Brassica juncea* has revealed a high ability of extracting different heavy metals from contaminated effluents in *ad hoc* tailored hydroponic systems (Dushenkov et al. 1995). Nevertheless, if plant species with marked absorptive characteristics are grown hydroponically (rhizofiltration conditions) or in beds packed with inert/artificial water-filtering matrices, adsorptive entrapment of heavy metals on sedimental particles could be avoided.

In this frame, genetically modified cultivars of *B. juncea* overexpressing ATP sulfurylase have already been proposed—for instance—to increase selenate phytoextraction and accumulation (Pilon-Smits et al. 1999b). However, the recourse to genetically modified plants might be nullified if a comparable efficiency in contaminant removal could be achieved by eliciting plant–microbe interactions at the rhizosphere level (Glick 2003).

The present study was to investigate the effects of inocula with Se-resistant rhizobacteria on selenite phytoextraction capacity of *B. juncea* plants, grown on a water-filtering inert substrate draining an artificially Se(IV) spiked effluent. Filtering beds were thus added either with a suspension of rhizobacteria coming from the rhizosphere of *B. juncea* cultivated on a selenite-amended soil or with axenic cultures of two soil bacterial isolates, namely, *Bacillus mycoides* SeITE01 (Vallini et al. 2005) and *Stenotrophomonas maltophilia* SeITE02 (Di Gregorio et al. 2005; Antonioli et al. 2007). These latter—previously obtained in pure culture and described for their high resistance to Se(IV)—were inoculated either independently as single strains or as a binary consortium of both. Effects of the different bioaugmentation strategies on both selenite bioaccumulation and phytoextraction efficiency of *B. juncea* were evaluated. The persistence of augmented bacterial isolates after addition to the artificial growth substrate was also assessed by means of 16S rDNA DGGE analysis.

## 2 Material and methods

### 2.1 Chemicals and growth media for microbial cultures

Analytical grade chemicals were purchased from Sigma-Aldrich Srl (Milan, Italy), whereas microbiology products were furnished by Oxoid Italia Spa (Garbagnate Milanese, Italy).

### 2.2 Plant growth experiments

Cultivation experiments were carried out in a temperature-controlled glasshouse (24–28°C). Plants were grown in 20 l polyethylene beds filled with 1,330 g of inert material, watered with sterile half-Hoagland solution up to the maximum of its water-holding capacity (WHC=72.5%). The inert material was a silica matrix (agriperlite) containing neither humic acids nor clayey materials, and potentially involved in Se adsorption. Agiperlite was even chosen for its porosity that favored either a good oxygenation of the plant root system or a plentiful vegetative growth. Seeds of *B. juncea* (accession number 173874—USDA, North Central Regional Plant Introduction Station, Iowa State University, Ames, IA, USA) were sown directly in beds. Afterwards, beds were added with different chemical or microbial amendments once the fourth week of plant cultivation had expired (T0).

A first experiment was performed without any microbial inoculum, in the presence of three different selenite concentrations corresponding to 0.2, 1.0, and 2.0 mM, respectively.

A second experiment was then carried out at the sole selenite concentration of 1.0 mM, with the addition of different microbial inocula. Amendments with bacterial cultures were as follows: on one hand, a complex Se(IV)-acclimated microbial community (SeAMC) was used, on the other, inocula of *B. mycoides* SeITE01 (*B*) and *S. maltophilia* SeITE02 (*S*), singularly or mixed together, were added.

The SeAMC was obtained from the rhizosphere of *B. juncea* plants previously grown on soil amended with 0.2 mM selenite. Plant growth had been carried out for 2 months in a temperature-controlled glasshouse (24–28°C). The microbial inoculum was then obtained by the dilution of a proper aliquot of selenite-amended soil in 0.9% (wt $vol^{-1}$) NaCl solution for 2 h at 28°C on an orbital shaker. The suspension was filtered on Whatman paper, added to the Hoagland solution in order to obtain a final inoculum of $2\times10^7$ CFU/g of agriperlite and, lastly, uniformly distributed onto water-filtering beds.

Otherwise, strains SeITE01 and SeITE02 were previously isolated from a selenium-contaminated soil and characterized for their high selenite resistance capacity. The axenic cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml of Nutrient Broth amended with 0.2 mM $Na_2SeO_3$. Flasks were incubated on an orbital shaker at 200 rpm at 28°C for 24 h. As needed, a proper aliquot of each bacterial culture was centrifuged at 8,000×*g* for 10 min at 4°C and re-suspended in 200 ml of half Hoagland solution in order to obtain a final inoculum of $2\times10^7$ CFU/g of agriperlite and then uniformly spread onto hydroponic beds.

Appropriate control beds were arranged. All experiments were carried out for 6 weeks, in duplicate.

### 2.3 Plant and agriperlite sampling

Five plants per bed were collected immediately after selenite amendment (T0) as well as after 3 (T3) and 6 (T6) weeks since this. Plant tissues were separated into roots and shoots, and oven dried at 50°C until constant weight was reached. Dry weight (DW) values were then recorded. Meanwhile, agriperlite samples were collected from plant rhizospheres and stored at –20°C for molecular analyses.

### 2.4 Selenium determination in plant tissues

Se content determinations were performed by relying on both EPA 3052/1996 and EPA 200.8/1994 methods. Analyses were carried out with an Agilent 7500ce ICP–MS (Agilent Technologies, Tokyo, Japan).

BF (i.e., the ratio between selenium concentration in plant tissues and initial selenium concentration in the growth matrix) and plant PE (i.e., the ratio between the amount of selenium present in plant dry biomass and initial selenium content in the growth matrix) were quantified at T6.

### 2.5 Molecular analyses

Total DNA extraction from agriperlite was carried out following the modified cooling–heating method (Chao et al. 1996): 1 g of agriperlite was re-suspended in 5 ml of lysis solution (0.15 M NaCl, 0.1 M Na–EDTA, and 1% CTAB). A volume of 100 μl lysozyme (15 mg $ml^{-1}$) was added, and the mixture was incubated on an orbital shaker at 37°C for 1 h. Successively, 5 ml of sodium dodecyl sulfate (SDS) solution (0.1 M NaCl, 0.5 M Tris–HCl [pH 8.01], and10% SDS) was added to the mixture, then incubated twice at −80°C for 30 min and immediately transferred at 65°C for a further 30 min. The aqueous phase of the solution was extracted twice with chloroform/isoamyl alcohol (25:24:1) in order to recover total DNA, which was successively precipitated by adding 1 volume isopropanol alcohol. After 2 h at –80°C, total DNA was pelleted by centrifugation (12,000×*g* for 30 min at 4°C) and re-suspended in 200 μl of $H_2O$.

Bacterial genomic DNA purification and DGGE analyses were performed in triplicate as described by Zocca et al. (2004). DGGE bands containing DNA to be sequenced were excised and incubated for 4 h in 100 μl of sterile water. Afterwards, polymerase chain reaction (PCR) amplification was carried out according to Zocca et al. (2004), except for the use of non-GC–clamped primers. PCR products were transformed in *E. coli* DH5α using the pGEM-T vector system following the manufacturer's instructions (Promega), sequenced on both strands, and subsequently searched for homology using the BLASTN database (Altschul et al. 1997).

### 2.6 Statistical treatment of data

A one-way analysis of variance (ANOVA) procedure was used to treat the data by means of the variance analysis, and significant differences were taken for $F<0.05\%$.

The interpretation of the DGGE gels with respect to the Similarity index was carried out by relying on the software SPSS 8.0 for the calculation of the Pearson coefficient, while the NTSYS software was used for the dendrogram formation, according to the UPGMA method (Kropf et al. 2004).

## 3 Results

### 3.1 Effects of Se(IV) addition on *B. juncea* plants

Toxic effect on *B. juncea* was exerted by selenite in a concentration-dependent mode, as quantified through comparison of the different plant biomass productions (Table 1). In fact, a decrease in plant biomass yield, both for shoots and roots, was observed in beds amended with the highest selenite concentration. The statistical significance of plant biomass produced both at T3 and T6 was confirmed at the 5% level using a one-way ANOVA model. Moreover, statistical analysis showed that no significant differences in plant biomass production were registered with respect to the control, after 6 weeks of growth, when plants were treated with either 0.2 or 1.0 mM of selenite. The concentration of 1.0 mM selenite was therefore chosen for the setup of the cultivation runs in the presence of different microbial inocula.

### 3.2 Effects of microbial amendments on *B. juncea* plants

As far as Se content in plant tissues is concerned, a preferential accumulation of selenium in roots rather than in shoots was observed (Fig. 1). The statistical significance of Se content in plant tissues, both at T3 and T6, was confirmed at the 5% level using a one-way ANOVA model. As a general rule, Se content in plant tissues increased with the extension of the incubation time except for uninoculated beds in which the highest Se concentration value was observed at T3. The presence of a Se-acclimated microbial community, as well as inocula of either strain SeITE02 alone or strains SeITE02 and SeITE01 mixed together, caused a rising in the Se content in plant tissues if compared with uninoculated plants. This effect was more manifested—especially in shoots—as a consequence of the addition of SeAMC. Interestingly, the influence of the inoculation with *Stenotrophomonas* was similar to that registered with the dual consortium of *S* and *B*. In fact, statistical analysis, carried out using a one-way ANOVA model, indicated no significant differences between Se content values registered at T6 for plant inoculated with *Stenotrophomonas* alone or with the dual consortium of *S* and *B*. On the other hand, a significant difference was observed among SeAMC, *B*, *S* inocula and uninoculated beds. Interestingly, plants bioaugmented with only *B. mycoides* showed the lowest selenium concentration values in both shoots and roots.

With reference to the vegetable production, microbial inocula caused a general depletion of harvestable plant biomass in comparison to that achieved in control beds. This effect was actually detectable at T6. After 3 weeks of

**Table 1** Biomass production as dry weight in plants grown at different selenite concentrations: 0.2, 1.0, and 2.0 mM

| Time sampling | Biomass production as dry weight (g) | | | |
|---|---|---|---|---|
| | CTR | 0.2 mM | 1.0 mM | 2.0 mM |
| Shoots | | | | |
| T0 | 0.148±0.05 | 0.255±0.05 | 0.123±0.03 | 0.052±0.01 |
| T3 | 0.817±0.11 | 0.751±0.08 | 0.332±0.17 | 0.197±0.01 |
| T6 | 1.281±0.28 | 1.534±0.23 | 1.203±0.23 | 0.394±0.10 |
| Roots | | | | |
| T0 | 0.005±0.001 | 0.021±0.008 | 0.006 ±0.004 | 0.015±0.001 |
| T3 | 0.052±0.014 | 0.113±0.055 | 0.047±0.008 | 0.058±0.001 |
| T6 | 0.251±0.074 | 0.292±0.027 | 0.262±0.048 | 0.144±0.035 |

The statistical significance of the results was confirmed at the 5% level using a one-way ANOVA model

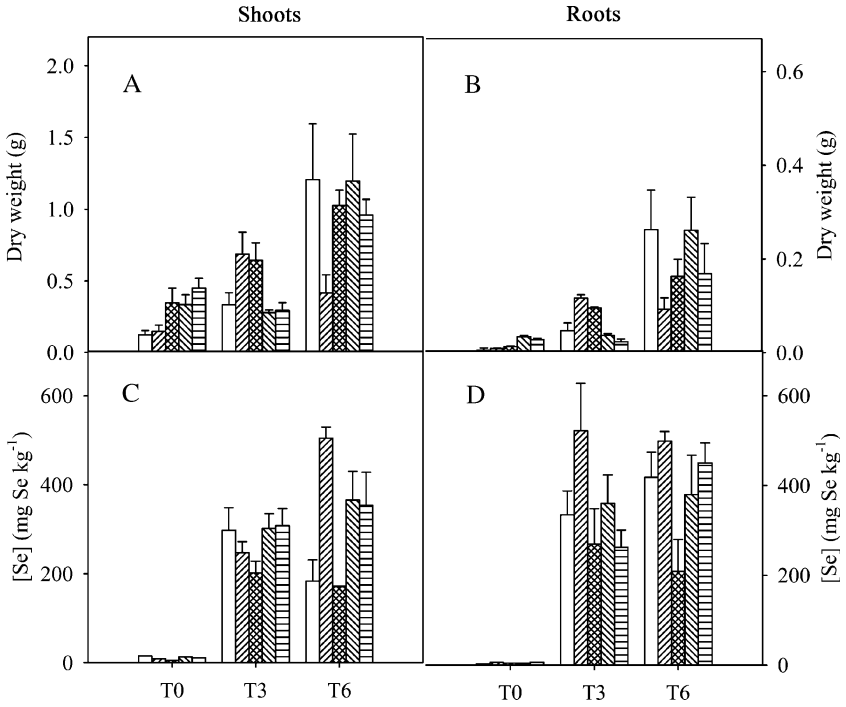


**Fig. 1** Dynamics of shoot and root biomass production (**A** and **B**) and selenium accumulation (**C** and **D**) in compartments of plants grown in beds amended with 1.0 mM Se(IV) either uninoculated (▭) or inoculated with the Se(IV)-acclimated microbial community (▨), SeITE01 (▩), SeITE02 (▧), and SeITE01 and SeITE02 as a dual consortium (▤). The statistical significance of the results was confirmed at the 5% level using a one-way ANOVA model

incubation, in fact, water-filtering beds amended with SeAMC or solely with the *B. mycoides* appeared positively affected in terms of plant biomass production if compared with the control beds. Nevertheless, at the end of the cultivation trial, inoculation with SeAMC caused the severest reduction in vegetable biomass, whereas bioaugmentation with axenic culture of *Stenotrophomonas* resulted in a plant biomass yield similar to that obtained in uninoculated beds, as confirmed by one-way ANOVA analysis.

### 3.3 Evaluation of BF and PE parameters

As mentioned before, selenium BF and plant PE were evaluated at the end of the cultivation trial (T6; Table 2).

**Table 2** Bioconcentration factor (BF) and phytoextraction efficiency (PE) in beds amended with Se(IV) at increasing concentrations

| Bed label | BF | | PE (%)[a] |
|---|---|---|---|
| | Shoot | Root | |
| CTRL | 2.44±0.64 | 5.57±0.53 | 10.58±1.38 |
| SeAMC | 6.73±0.32 | 6.65±0.20 | 8.23±0.38 |
| B | 2.29±0.01 | 2.78±0.67 | 7.47±0.35 |
| S | 4.87±0.61 | 5.06±1.18 | 16.29±1.72 |
| B+S | 4.71±1.00 | 6.00±0.43 | 10.68±2.55 |

*SeAMC* Se(IV)-acclimated microbial community, B *Bacillus mycoides* SeITE01, S *Stenotrophomanas maltophilia* SeITE02;
[a] Selenium content at T0 was 99.75 mg
The statistical significance of the results was confirmed at the 5% level using a one-way ANOVA model

An increase of BF values in shoots from water-filtering beds bioaugmented with SeAMC, *S*, and *B* + *S*, respectively, was detected while comparing untreated beds with plants amended with the different microbial inocula. On the other hand, the addition of solely the *B. mycoides* to agriperlite beds did not affect the BF with respect to the values reached in plants from uninoculated beds. Meanwhile, inoculation with *B* alone caused a marked decrease of BF value in roots with regard to the control. Moreover, in this experimental condition, the lowest values of BF in both shoots and roots were reached.

At the same time, plants treated with SeAMC, *S*, and *B* + *S* inocula did not show significant differences of BF values in roots if compared to uninoculated plants.

Despite the results obtained for BF, PE values obtained with SeAMC inoculation were lower than those registered in the absence of such an amendment. This was related to a high decrease in plant biomass production following treatment with the SeAMC inoculum.

Coming to the effects of the treatments with bacterial axenic cultures on PE values, both the binary consortium of the two isolates and *S. maltophilia* alone caused a rising of phytoextraction efficiency values. On the other hand, inoculation of the sole *B. mycoides* eventually determined a decrease in PE in respect to the values obtained with plants grown either in control beds or in the presence of SeAMC. In particular, the highest PE value was recorded in the presence of SeITE02 (see Table 2). In fact, when this strain was added into the water-filtering beds, Se accumulation in plant tissues was not paralleled by a reduction of

plant biomass yield, contrary to the situation recorded in control beds.

### 3.4 Persistence of selected bacterial inocula within the agriperlite beds

In order to monitor the persistence of the strains SeITE01 and SeITE02 within the water-filtering beds throughout the testing runs, 16S rDNA DGGE analysis was performed on agriperlite samples collected from the rhizosphere of plants at different sampling times (Fig. 2). Amplification products of 16S rDNA of *B. mycoides* and *S. maltophilia* strains were used as molecular markers, corresponding to specific bands on the DGGE profiles.

DGGE profiles shown in Fig. 2 indicated that SeITE01 and SeITE02 strains were still present after 6 weeks, when added singularly. On the other hand, when beds were amended with the binary consortium of both strains, SeITE02 lasted in agriperlite for 6 weeks, whereas SeITE01 progressively disappeared within the time T6. Looking at the DGGE profiles corresponding to the resident bacterial community in agriperlite beds, no taxonomical stabilization was achieved in 6 weeks, as indicated by the few similarity values among the different treatments.

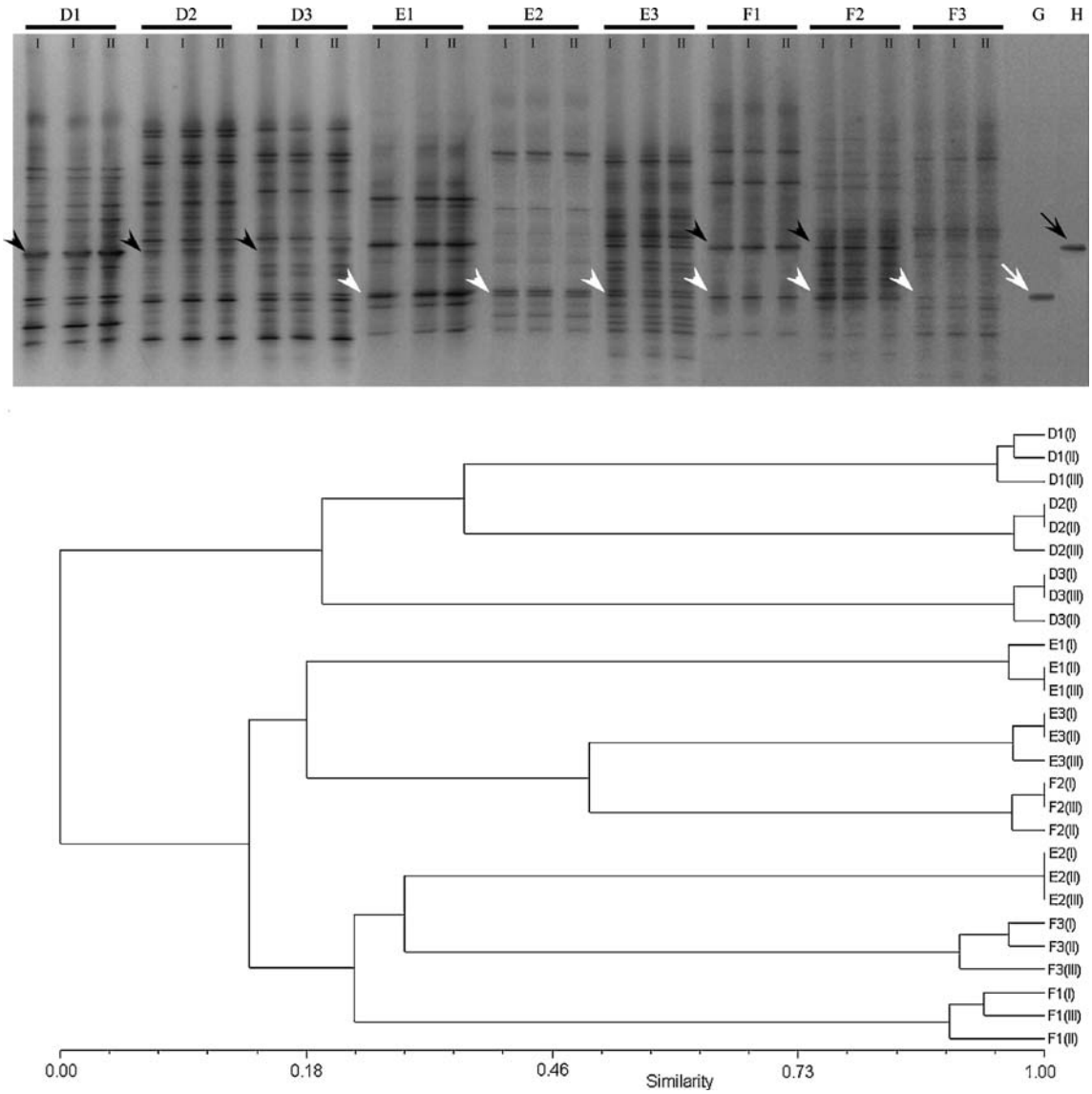


**Fig. 2** DGGE profiles of the rhizosphere of *B. juncea* grown in presence of 1.0 mM Se(IV) and then inoculated with: strain SeITE01 (D1–D3), strain SeITE02 (E1–E3), or the dual consortium of both (F1–F3). Lanes G and H correspond to the V3-16S rDNA PCR products amplified from the genomic DNA of SeITE02 and SeITE01, respectively. Arabic numerals correspond to the sampling time T0 (1), T3 (2), T6 (3), while Roman numerals (I, II, III) to different replicates. The dendrogram indicates the similarity relationships among the different DGGE profiles. *Arrows* indicate bands which were gel-excised and sequenced in order to verify their correspondence to SeITE01 (*black arrows*) and SeITE02 (*white arrows*)

## 4 Discussion

Among the possible different approaches for the decontamination of selenium-laden wastewater, rhizofiltration can be taken into account. Fast growing *B. juncea* cultivars have proved to be suitable for Se remediation because of their high rates of selenium accumulation and volatilization (Banuelos and Meek 1990; Terry et al. 1992). However, plant efficiency should be optimized in order to contrast undesired side effects such as sedimentation phenomena. In this perspective, within the present study, agriperlite-filtering beds have been considered as substrata for growing *B. juncea* plants. Phytoextraction efficiency was tentatively optimized by exploiting the plant interactions with bacterial strains selected for their resistance to selenium. Therefore, agriperlite was first inoculated with a Se(IV)-acclimated microbial population isolated from the rhizosphere of *B. juncea* grown on selenite-amended soil. This specific microbial community was thought to be reasonably capable of a synergistic interaction with *B. juncea* to overcome the toxic effect due to the presence of the metalloid. However, evidence has been gained that the addition of such a microbial inoculum was associated with a reduction of plant biomass yield, throughout the cultivation span, when compared to plants grown without the same bacterial inoculum. This meant a drop in the PE of this plant system. On the other hand, *B. juncea* root absorption capacity for selenium increased. Actually, BF values rose markedly in comparison to those recorded in plants grown in uninoculated beds. At the best of the information so far available in the literature, this is the first report suggesting that plant inoculation with rhizobacteria increases *B. juncea* root capacity to absorb Se(IV). Moreover, the increase of selenium BF in shoots suggests that the Se(IV)-acclimated microbial community not only elicited the plant capacity to absorb selenite, but also did improve the capacity to transport the metalloid to the epigeous compartments. The observed reduction in plant biomass yield might thus be related to an improved capability of *B. juncea* to accumulate selenium at concentrations that are actually toxic for plants (Brown and Shrift 1981).

In a different way, Terry et al. (2000) reported rhizobacteria as capable of increasing the capacity of plants in extracting selenate from Se-laden waters, with no effects on selenite. Synthesis of selenomethionine in the root zone, however, was observed and associated with a general increase in selenium volatilization.

Actually, the capacity of microbes to transform selenium into organo-Se compounds might explain the results achieved in this study. Selenium is generally absorbed by plants as selenite, selenate, and organo-Se compounds. These latter, along with selenate, are actively transported in plant tissues (Terry et al. 2000), whereas selenite is thought to be passively absorbed and metabolized by the root apparatus. Moreover, organo-Se metabolites are certainly important within the selenium assimilation pathway of *B. juncea* (Grant et al. 2004; Kahakachchi et al. 2004). Therefore, it is reasonable to suggest that the Se(IV)-acclimated microbial community probably reduced selenite to organo-Se compounds, increasing the incorporation of metalloid by plants.

Whenever agriperlite beds were bioaugmented with the selenite-resistant bacterial strains *S. maltophilia* SeITE02 and *B. mycoides* SEITE01, these isolates—if inoculated singularly—persisted for 6 weeks in the system as shown by a diachronic reading of the DGGE profiles. Nevertheless, SeITE01 instability was observed when SeITE02 was co-inoculated. Both strains revealed weaker effects on plant biomass production with respect to those observed in the presence of the Se(IV)-adapted microbial community. Moreover, bed inoculation with SeITE02 alone was the sole strategy resulting in a positive effect either on plant biomass production in stressful conditions or on the capacity of shoots to accumulate selenium. This can be held as a probative evidence that SeITE02 is able to boost vegetative growth in the presence of the contaminant. Meanwhile, its putative capacity to reduce Se(IV) to organo-Se compounds significantly increased both selenium absorption by the plants and active metalloid transport to epigeous compartments. On the other hand, the observed decrease in plant biomass production after SeITE01 inoculation could not be ascribed to the concentration of selenium in the plant compartments. In fact, Se concentration in SeITE01-amended plants resulted in significantly lower levels with respect to those reached in the presence of both Se(IV)-acclimated microbial communities and SeITE02. After SeITE01 inoculation, a competition for macronutrients between *B. juncea* and the bacterial strain can be invoked as the cause of the lower plant biomass production. Indeed, the plant response to rhizosphere inoculation with selected bacterial strains—although these were classified as PGPRs—is depending on the general trophic conditions. Therefore, massive microbial inoculations in the rhizospheres may reduce macroelement uptake by plants (Belimov et al. 2002). The interaction between *B. juncea* and the strain SeITE01 might emblematically represent this condition. Moreover, *B. mycoides* SeITE01, by efficiently reducing Se(IV) to $Se^0$ in vitro (Vallini et al. 2005), makes selenium definitely unavailable to plants. This evidence may explain the decrease in plant capacity to absorb the metalloid. Finally, positive effects of the dual consortium on selenium PE in *B. juncea* should be related to the instability of SeITE01 when co-inoculated with SeITE02, which otherwise showed positive effects if added singularly. In fact, the features of the microbial cenosis colonizing agriperlite filtering beds seem to influence selenium speciation favoring—from time to time—metalloid absorption by plants as well as its exclusion.

## 5 Conclusions

In a phytoextraction perspective, selenium-resistant microorganisms can be exploited for a synergistic interaction with accumulating plants. In this study, the inoculation of agriperlite beds with a complex rhizospheric microbial community resistant to selenite was proven to elicit the capacity of plants to either incorporate selenite into the root apparatus or translocate it to the epigeous portion. Nevertheless, this positive effect was associated to a decrease in plant biomass production with a consequent drop of PE with respect to the tests done without any bacterial inoculum. On the other hand, inoculation of water filtering beds with the selected *S. maltophilia* SeITE02 strain stimulated plant uptake of selenite in concomitance to a recovery of plant biomass production, even under stressful conditions. This improved PE for selenite in *B. juncea* up to 65% when compared to uninoculated plants. Meanwhile, bioaugmentation with the selected *B. mycoides* SeITE01 strain determined a sharp reduction of both plant biomass production and selenite uptake. This evidence demonstrates that the exploitation of the interactions between plants and microorganisms can significantly improve already consolidated treatment processes for the detoxification of metal and metalloid-laden wastewater. However, the specificity of possible interactions between different plants and related rhizobacteria plays a pivotal role in determining the whole efficiency of the system chosen.

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