



Centro
Interuniversitario
di Ricerca Per lo
Sviluppo sostenibile



SAPIENZA
UNIVERSITÀ DI ROMA

TOOL BOXES FOR SOIL BIOREMEDIATION ASSESSMENT IN HEAVY METAL POLLUTED AREAS

Laura Emili

Supervisor Prof. Dr. Sara Marinari

Cosupervisor Prof. Dr. Stefano Grego

Coordinator of the Ph.D. Program Prof. Dr. Silvia Macchi

A thesis submitted
for the degree of Doctor of Philosophy
on Sustainable Development and International Cooperation
at the CIRPS (Interuniversity Research Centre for Sustainable Development)
University of Rome “Sapienza”

XXIV Cycle (2008/2011)

Acknowledgements

First and foremost I want to thank my supervisors Prof. Dr. Sara Marinari and Prof. Dr. Stefano Grego. I appreciated all their contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating. I thank my coordinator of the Ph.D. program Prof. Dr. Silvia Macchi, she is very helpful and careful with me and with all Ph.D. students. I thank the scientific group of the Institute of Ecosystem Study (National Research Council-Pisa) in particular Prof. Dr. Brunello Ceccanti, Prof. Dr. Graziana Masciandaro and Dr. Cristina Macci. I had a fantastic collaboration with them, despite the distance. For the “microbiological” part of this thesis, I am particularly thankful to Prof. Dr. Anna Rosa Sprocati, Dr. Chiara Alisi, Dr. Flavia Tasso and Dr. Valentina Pinto of the Department for the Environment, Climate and Sustainable Development of ENEA-Casaccia (Anguillara-Rome). They have been fundamental to elaborate a complete thesis. I would also like to thank Prof. Dr. Erika Köthe and Dr. Götz Haferburg, who supervised my period of research abroad in their Institute of Microbiology and Phytopathology of the Friedrich Schiller Universität in Jena (Germany). My time in Jena was made enjoyable due to many new friends that became a part of my German life. I gratefully acknowledge the funding sources that made my Ph.D. work possible. I was funded by the CIRPS (Interuniversity Research Centre for Sustainable Development-Sapienza University of Rome) for my 3 years of the Ph.D. course. Moreover, I was honored to be granted with a DAAD scholarship for my journey in Germany. I am very grateful of my colleagues of the biochemical soil scientific group of University of Tuscia. This group had been a source of friendship as well as good advice and collaboration. In particular, I am grateful to Alessandra, Daniela, Anita, Renée, Armando, my friends also in my life. This thesis is largely a result of the interaction that I had with my supervisors, colleagues and my dear Ph.D. friends. I thank Clara and all my friends for their support! To each of them I owe a great debt of gratitude for their patience, inspiration and friendship. These three years had been very complicated due to different causes, but now I can say that the Ph.D. course had been an intensive but positive experience. Lastly, I would like to thank my family for all their love and encouragement. My parents Paolo and Letizia, my brother Marco and my sister Silvia had been extremely understanding and supportive of my studies. I feel very lucky to have a family that shares my enthusiasm for academic pursuits. In particular, I'd like to thank my wonderful boyfriend, Fabio, who constantly encourages me so much. I am very indebted with him, he is always precious and fundamental for my life.

Thank you!

Laura Emili

March 2012

Contents

Chapter 1-Introduction.....	5
1.1 Soil quality in regulating ecosystem functions.....	5
1.1.1 Indicators of soil quality.....	7
1.2 Heavy metals contaminated soil.....	9
1.2.1 Diffusion of heavy metals contamination in Europe.....	11
1.2.2 Heavy metals bioavailability: concept and investigation measurements.....	15
1.2.3 Processes and mechanisms involved in heavy metal regulation in soil.....	18
1.2.4 Role of plant-microorganism interactions in heavy metals mobility in soil.....	21
1.3 Sustainable soil reclamation methods: innovative approaches for heavy metals bioremediation.....	24
1.3.1 Phytoremediation: the use of plants.....	25
1.3.1.1 Advantages and disadvantages of phytoremediation.....	27
1.3.2 Microbial assisted phytoremediation: the use of autochthonous plant-growth promoting-bacteria.....	30
Chapter 2- Objectives.....	33
Chapter 3- Set up a tool box of soil quality bioindicators.....	36
3.1 Microbial biomass as bioindicators of soil quality.....	36
3.2 Functional and genetic diversity of soil microbial communities.....	40
3.2.1 Microbial functional diversity.....	41
3.2.2 Microbial genetic diversity.....	46
Chapter 4- Heavy metal bioavailability in soil and bioaccumulation into soil microbial biomass ...	50
4.1 Abstract.....	50
4.2 Introduction.....	51
4.3 Specific aims.....	52
4.4 Materials and methods.....	53
4.5 Results and discussions.....	59
4.6 Conclusions.....	74
Chapter 5- Changes of soil quality in a phytoremediation system of a former dump.....	76
5.1 Abstract.....	76
5.2 Introduction.....	77
5.3 The aim of the study.....	78
5.4 Materials and methods.....	78
5.5 Results and discussions.....	86
5.6 Conclusions.....	100
Chapter 6- Use of Plant Growth-Promoting Bacteria to enhance heavy metals phytoremediation in mining soils: a preliminary study under Umbrella project.....	101
6.1 Abstract.....	101
6.2 Introduction.....	102
6.3 The aim of the study.....	103
6.4 Materials and methods.....	104
6.5 Results and discussions.....	118
6.6 Conclusions.....	130
Chapter 7-General conclusions.....	132
References.....	135

The outline of the thesis

The thesis is divided into seven separate chapters, each of them has a specific purpose. The first chapter is a general introduction. It is based on the accurate bibliographic research and aims to clarify main issues concerning soil and heavy metals (e.g. the concept of soil quality and its indicators, heavy metals bioavailability, the plant-microorganisms interactions in soils, the possible sustainable methods for soil remediation). The general goal of the thesis and the specific aims of the experimental researches are described in chapter 2. The third chapter entitled “Set up a tool box of soil quality bioindicators” describes which “experimental tools” were chosen to assess the soil quality improvement during the bioremediation processes. In the following fourth chapter “Heavy metal bioavailability in soil and bioaccumulation into soil microbial biomass” the methods selected to measure the heavy metal bioavailability and accumulation into soil microbial biomass are described. The fifth and sixth chapters show as the previously selected soil bioindicators respond to two different bioremediation systems. The fifth chapter “Changes of soil quality in a phytoremediation system of a former dump” refers to a phytoremediation system applied to an ex-dump located in “Madonna dell’Acqua”, Municipality of San Giuliano Terme - Pisa (Italy). The discussions of the obtained results are important to evaluate the efficiency of this soil reclamation technology. The sixth chapter, entitled “Use of Plant Growth-Promoting Bacteria to enhance heavy metals phytoremediation in mining soils: a preliminary study under Umbrella project” refers to a pot experiment conducted to test the efficiency of the microbial-assisted phytoremediation based on the soil inoculation of different selected Plant-Growth-Promoting Bacteria. This preliminary test useful for the next pot experiments of the European Umbrella project, was conducted to convey a reflection about the use of PGPB applied to mining soils during a phytoremediation process. Finally, in the general discussion of the seventh chapter, the most important results are summarized and general conclusions are reported, focusing on the aspects of bioremediation to be developed and improved.

Chapter 1-Introduction

1.1 Soil quality in regulating ecosystem functions

Soil is a non-renewable resource essential to civilization (Jenny, 1980). Soils play a key role in completing the cycling of major elements required by biological systems, transforming and recycling sunlight, storing energy and matter through plants and animals, decomposing organic wastes, detoxifying certain hazardous compounds. This resource is a medium for plant growth by supplying physical support, water, essential nutrients, and oxygen for roots, providing human food and fibre needs (Caporali, 2004). The suitability of soil for sustaining plant growth and biological activity is a function of physical properties (porosity, water holding capacity, structure, and tilth) and chemical properties (nutrient supplying ability, pH, salt content, etc.) and depends from the fact that the number of organisms contained in a teaspoon of healthy soil can exceed nine billion, one and one-half times the human population of the Earth (Gregorich, 1996). The resource “soil” is a meaningful crossroads between the different components and processes of terrestrial ecosystems. Soils are necessary to the proper functioning of an ecosystem, contributing to the system’s ability to withstand the adverse effects of such disturbances as drought, pests, pollution, and human exploitation, including agriculture (Gregorich, 1996). In any event, and because the pedosphere, hydrosphere, atmosphere and biosphere are overlapping, intimately associated in the environmental compartments of the ecosystem, whatever occurs in the soil has a profound effect not only on soil quality but also on ecosystem quality (Caporali, 2004). In the past 10 years, research on the soil quality concept has proceeded rapidly, with particular emphasis on understanding the role of the soil resource in maintaining environmental quality (Pickett et al. 2001; Heneghan et al., 2008) and on the application of the soil quality concept to restoration and management of nonagricultural lands (Karlen et al. 2001). Much like air or water, the quality of soil has a profound effect on the health and productivity of a given ecosystem and the environments related to it and its maintenance is critical for ensuring the sustainability of the environment and the biosphere (Arshad and Martin, 2002). However, when referring to air or water, the term “quality” usually involves the analysis of specific contaminants which are known to have well defined threshold values, and there is no need to specify dynamic, chemical, physical, biological and even ecological properties that would define an ideal state for what there is an almost limitless number of environmental scenarios (Sojka and Upchurch, 1999), but the concept of quality applied to soil gives rise to more controversy. It is ascertained that soil quality requires a holistic approach, and it is not possible to consider one element of quality in isolation from the other ecosystem components (Moody, 1996; Trasar-Cepeda

et al., 1998). A variety of definitions have been proposed for the term soil quality, ranging from a purely agricultural point of view to a more environmental perspective (Carter, 1996). Anderson and Gregorich (1984) proposed that soil quality be defined as *"the sustained capability of a soil to accept, store and recycle water, nutrients and energy"*. Then a holistic approach considered soil as part of a much broader ecological system, which interacts with, and affects other various parts of the system and so the expanded concept of soil quality has been evident in the work of Larson and Pierce (1991) defined soil quality *"as the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation"*. This definition also recognises that soil serves other functions both within and beyond ecosystems. Another successive and detailed definition has been developed by the Soil Science Society of America (1995) as follows: *"Soil quality is the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation"*. This definition is similar to that following of Doran and Parkin, (1996), used commonly in the last years in the scientific articles, where soil quality is the :*"the continued capacity of a specific kind of soil to function as a vital living system, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, to maintain and enhance the quality of air and water environments, and to support human health and habitation"* (Doran and Parkin, 1996; Palma et al.,2000; Epelde,2010). However, to manage and maintain soils in an acceptable state for future generations, soil quality must be defined and quantified in a standardize way, and the definition must be broad enough to encompass the many functions of soil. Much research into soil science is attempting to make objective an area that is subjective by nature and difficult to quantify the quality of a soil (Sojka and Upchurch, 1999; Bastida et al., 2008). The quality of life on Earth is inextricably linked to the quality of soils, which determine agricultural sustainability, environmental quality and, as a consequence of both, plant, animal, and human health. Within a community, a strong link can be found between soil quality, food quantity and quality, and the health, well-being, and prosperity of its citizens. In this respect, history has repeatedly shown that mismanagement of the soil resource base can lead to poverty, malnutrition and economic disaster (Bezdicsek et al. 1996; Epelde, 2010) but nevertheless this invaluable resource has often been degraded in the name of progress; as a means to the end of meeting the increasing 'needs' of humanity and so its resiliency and its natural processes to maintain global balances of energy and matter are under threat (Gregorich, 1996).

1.1.1 Indicators of soil quality

The growing awareness of soil degradation has increased the demand for quick and reproducible indicators useful to obtain information related to the impact of soil management practices or pollution on soil quality (Palma et al., 2000). The challenge is in quantitatively defining the state of soil quality by the standardized indicators, which can be defined as measurable properties that influence the capacity of a soil to carry out a given function (Acton et Padbury, 1993; Epelde et al., 2009). The importance of soil quality indicators is that they: 1) provide reference material to measure trends and patterns; 2) relate soil quality to other components of the system; 3) where trigger or precautionary levels are set the quality may be assessed (Nortcliff, 2002; Bastida et al., 2008). The soil quality depends on a large number of physical, chemical, biological and biochemical soil properties, and thus its characterization requires the definition of the properties most sensitive to changes (De la Paz Jimenez et al., 2002), but in many cases the specific property of soil may be difficult to measure directly, so an indicator (an associative property; i.e. surrogate or proxy) can be used to serve as an indirect and practical measure of the attribute. Moreover, the indicator can represent a single attribute or may also represent a set of attributes or properties (Carter, 1996). Various studies have attempted to identify sets of attributes which can characterize a soil process or processes in regard to a specific soil function (Doran and Parkin, 1994; Gregorich, 1996). Attributes of intrinsic soil quality, such as mineralogy and particle size distribution, soil porosity, nutrient retention, and both physical and chemical rooting conditions (e.g. physical and chemical barriers to root growth, total organic matter) are mainly viewed as almost static and usually show little change over time. Attributes of dynamic soil quality (e.g. microbial biomass and populations, soil respiration, nutrient mineralisation rates, macroporosity, pH and labile organic matter fractions) are subject to change over relatively short time periods (Nortcliff et al., 1996). In the elaboration of suitable indicator, it would be obvious to consider parameters where the biotic–abiotic interlinkages would find their expression. Moreover, it is generally acknowledged that indicators should be easily measured, have some sensitivity to variations in soil management (but not overly sensitive), and have a relatively low sampling error (Carter, 1996). Given the complex nature of the soil and the exceptionally large number of soil properties that may be determined, it is important to be able to select indicators that are appropriate to the task (Nortcliff, 2002). Many biological parameters have been proposed as bioindicators of soil quality, such as microbial biomass, basal and substrate-induced respiration, mineralizable nitrogen, soil enzyme activities, abundance of soil microflora and fauna, root pathogens, structural and functional biodiversity, food-web structure, plant growth and diversity, and so on (Bastida et al., 2008). There has been

widespread agreement on the importance of measuring these soil biochemical and microbiological properties in order to evaluate soil quality, being more sensitive to changes in the soil, able to respond to disturbance over shorter time scales than other soil properties, as well as to their capacity to provide information that integrates many environmental factors (De la Paz Jimenez et al., 2002; Alkorta et al., 2003; Izquierdo et al., 2005). Another current challenge for soil science is the definition of a satisfactory index that is a set of aggregated or weighted parameters or indicators (OECD, 1993). The use of different indices based on a combination of different soil properties would be a more appropriate approach to assess soil quality (Trasar-Cepeda et al., 1998). Any soil quality index should be composed of a minimum data set, including several biological and biochemical variables so to reflect better the complex processes affecting soil quality and to compensate for the wide variations occurring in individual properties (Doran and Parkin, 1994). Indeed, many studies have analyzed soil quality using different indicators but only a few have used the obtained results to establish a soil quality index. The most important problem is that in spite of soil quality is a concept widely defined, there is no a universally applicable formula to measure it. The setting of the standards for the soil quality raises many problems, both for the soil scientists and for the legislators, in part these problems are generated because of the soil is such a diverse material consisting of varying proportions of mineral material, organic material, water and air (Shepherd et al., 1992). The interactions between these materials are complex and the nature of these interactions must be considered in the development of reference values (Nortcliff, 1996). Most handicaps concerning the application of soil quality indicators and indices are related with the poor standardization of the methods, much better resolved for physical and chemical measurements, and with problems related with the spatial scale where they can be applied (Bastida et al., 2008). In this latter case identifying and using some important site-specific factors for the study area, such as climate parameters and vegetation type and density can solve problems related to spatial scale. Many biological techniques are relatively new, and inherently difficult to standardise because we are dealing with living organisms. For example, precise biological standard materials hardly exist, whereas for chemical measurements, international certified standards are often readily available (Bastida et al., 2008). Addressing the problem of contaminated land has provided much of the focus for recent developments in the standardisation of methods for soil analysis to assess the soil quality and the setting of soil quality reference or indicator values (Epelde et al., 2009). During a bioremediation process, different appropriate indicators and indices might be valid monitoring tools to assess the effects on the soil biochemical and microbial properties and so it could express a reflection about the efficiency of soil reclamation technology to clean up the soil by heavy metals and so to restore the soil quality (Epelde, 2010).

1.2 Heavy metals contaminated soil

Currently, the quantity and quality of the soil's ecosystem services and functions are nowadays being diminished at an alarming rate, 47% of the world's land is moderately to very severely degraded, and 22% of all cropland, pasture, forest, and woodland have been degraded only since 1950 (GLASOD, 2004; Filser et al., 2008) making soil degradation an environmental issue that demands immediate attention and response. Soils are presently being degraded through salinization, erosion, sealing, pollution, loss of organic matter and biodiversity, reduction of soil fertility etc., leading to the deterioration of the soil's physical, chemical and biological properties worldwide. In particular, the release of contaminants into soils by human activities has increased enormously over the past several decades, overwhelming the self-cleaning capacity of the soil ecosystem and, as a consequence, resulting in the accumulation of dangerous toxic substances (Epelde et al., 2009). Contaminated soils represent an important issue with clear consequences on the three dimensions of sustainability, the environmental, the economic and the social one (Fernández et al., 2006). The environmental perspective covers not only terrestrial communities, but also aquatic ecosystems exposed to drainage and run-off processes. Regarding the economic point of view, in addition to the cost of prevention, control and remediation, the historically contaminated sites represent a burden from the past, which affects the possibilities for current development. And finally, considering the social dimension the regulation of soils has a main difference with other environmental compartments, as it has to be taken into account that most soils are privately owned (Fernández et al., 2006). In particular, the contamination of soils by heavy metals originating from agricultural (e.g., fertilizers and sewage sludge) or industrial activities (e.g., metal mining and smelting) is one of the major environmental problems in many parts of the world, representing a permanent threat to soil ecosystems. Metal pollution in soils has become one of the most serious environmental problems of worldwide concern, because of their widespread use and distribution, and particularly their toxicity to human beings and the biosphere (Alkorta et al., 2004b). Accumulation of metal ions and metalloids in different compartments of the biosphere, and their possible mobilization under environmentally changing conditions induce a perturbation of both the structure and function of the ecosystem and might cause adverse health effects to biota (Fedotov and Mirò, 2008). Heavy metals and metalloids enter in the ecosystem through both natural and anthropogenic processes.

In spite of some soils have been found to have a high background of some trace elements, toxic to plants and wild life, due to extremely high concentrations of these elements in the parent materials (Violante et al., 2010), the heavy metals are present in soils above all as the result of human activities, such as the burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, agricultural activities means of fertilizers, pesticides, agrochemicals, long-term application

of urban sewage sludges, industrial activities such as waste disposal, waste incineration and vehicle exhausts (Garbisu and Alkorta, 2001; Alkorta et al., 2004c). All these human sources accelerate greatly the biogeochemical cycle of metals and cause their accumulation in soils, so that their concentrations found in contaminated areas exceed those required as nutrients for living organisms (Alloway, 1990; Epelde et al., 2009). These increasing levels pose a threat to food safety and a potential health risks due to soil-to plant transfer of metals and therefore a danger for the food chain (Collins and Stotzky, 1989). Specifically, metal contamination is linked to birth defects, cancer, skin lesions, mental and physical retardation, learning disability, liver and kidney damage and a host of other maladies (Giller et al., 1998). Metals in soils have frequently been reported to have a negative effect on soil biological parameters (soil microbial biomass, composition and diversity of the biomass, mineralisation of organic matter, different microbial processes, soil enzyme activities) with concomitant negative effects on soil fertility and functioning (Leita et al., 1995; Kelly and Tate, 1998; Giller et al., 1998). This is because heavy metals in high concentration can cause the protein denaturation, react with nucleic acids and phospholipids, destroy the integrity of cell membranes, heavy metal ions form unspecific complex compounds in the cell and thus arrest cellular proliferation, affecting the growth, morphology and metabolism of organisms (Giller et al., 1998; McGrath et al., 2001). Metals have traditionally been classified into categories such as light, heavy, semimetal (e.i. metalloids), toxic and trace depending on several chemical and physical criteria such as density, weight, atomic number and degree of toxicity (Alloway, 1990). The term “heavy metals” refers to elements with densities greater than 5.0 g cm^{-3} thus the transition elements from V (but not Sc and Ti) to the half-metal As, from Zr (but not Y) to Sb, from La to Po, the lanthanides and the actinides can be referred to as heavy metals. The heavy metals constitute a group of about 40 elements, of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals and the remaining 53 (with As included) are heavy metals (Weast 1984). Some heavy metal cations, e.g. Hg^{2+} , Cd^{2+} and Ag^+ , form strong toxic complexes, which make them too dangerous for any physiological function (Nies, 1999). Thus, the intracellular concentration of heavy-metal ions has to be tightly controlled, and heavy metal resistance is just a specific case of the general demand of every living cell for some heavy-metal homeostasis system (Nies, 1999). Heavy metals persist in soils for very long periods of time with residence times in the order of thousands of years (Brookes, 1995; Garbisu and Alkorta, 2001; Dai et al., 2004) due to their immutable nature. Indeed, the metals are unique in that they do not undergo either chemically or biologically induced degradation that could reduce their toxicity, but rather are transformed from one oxidation state or organic complex to another (Alkorta et al., 2004b; Epelde et al., 2009).

Some typical examples of the heavy metals polluted soils could be represented by the ex-mining areas or the dumps, where the heavy metals concentration is high and toxic to many organisms and plants, as shown by the absence of vegetation over most of the basin surface (Cao et al., 2009). Migration of contaminants from these areas into non-contaminated sites as dust or leachate through the soil, the spreading of sewage sludge contributes towards contamination of ecosystems. As a result, large areas of agricultural land can be contaminated. Hence, the remediation of such soils is important to avoid the diffusion of contamination and to recover these large areas that are rendered unsuitable for agricultural and other human use (Khan, 2005). Indeed, it is generally accepted that the risk factor associated with this kind of pollution is related to their bioavailability rather than their total concentrations. The bioavailability is available elemental pools for organisms and determines the diffusion and accumulation of these inorganic pollutants in the ecosystems through the food chain (Alkorta et al., 2004b). Thus, it is necessary to plan some different adequate and sustainable projects to remediate and reevaluate the heavy metals polluted areas, choosing adequate treatments necessary to modify their bioavailability and avoid the heavy metals dissemination in the environment.

1.2.1 Diffusion of heavy metals contamination in Europe

The largest and probably most heavily contaminated areas are found near industrialized regions in northwestern Europe, but many contaminated areas exist around most major European cities (EEA, 2003). There could be between 300.000 and 1.5 million of these sites in the EU (EC, 2002), the uncertainty in this estimate is due to the lack of common definitions and a scarcity of accurate data on the size and the level of contamination of affected sites. Potentially polluting activities are estimated at nearly 3 million sites across the EU, many of which need further investigation to establish the damage and whether soil remediation (clean up) is required. Although considerable efforts have been made in some countries, it will take decades to clean up the legacy of soil contamination. Over the last 30 years approximately 80 000 sites have been cleaned up in the countries where data on remediation is available (EEA, 2009) (Figure 1.1).

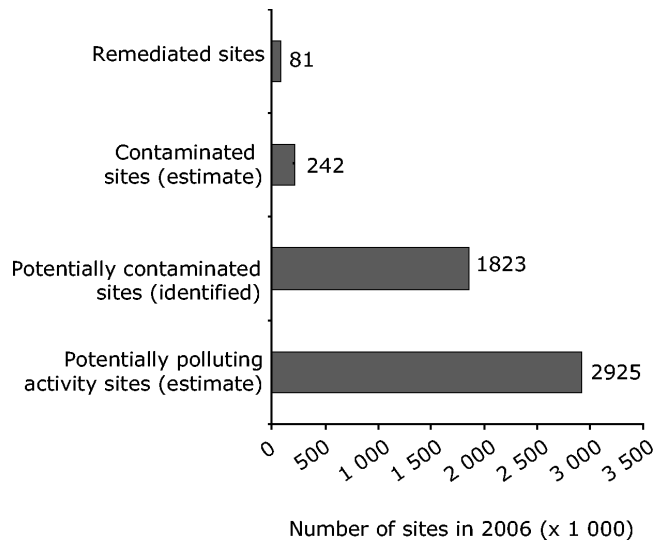


Figure 1.1 Overview of progress in the management of contaminated sites in Europe (EEA, 2009)

All countries have been affected by heavy metals pollution though the area and severity of pollution vary enormously (Lone et al., 2008). Reports from countries across Europe indicate that heavy metals and mineral oil are the most frequent soil contaminants at investigated sites (37.3% and 33.7%, respectively), while mineral oil and chlorinated hydrocarbons are the most frequent contaminants found in groundwater (Fig.1.2). These estimates are based on the frequency of a contaminant is reported at the investigated site. Other contaminants include polycyclic aromatic hydrocarbons (PAH), aromatic hydrocarbons (BTEX), phenols and chlorinated hydrocarbons (CHC) (EEA, 2009).

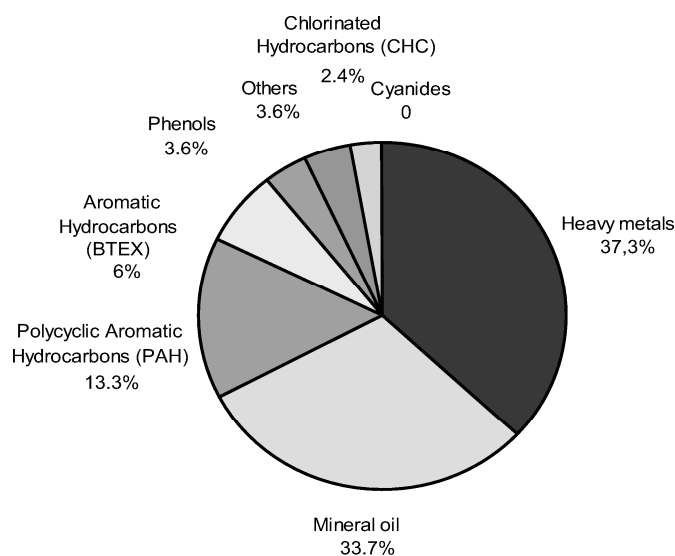


Figure 1.2 Overview of contaminants affecting soil and groundwater in Europe (EEA, 2009)

Based on information from national inventories, the progress in the management of contaminated sites varies significantly across Europe, depending on the different national management approaches and legal requirements. In most of the countries for which data are available, site identification activities are generally advanced. As a result of preliminary surveys, just over 60% of the sites have already been confirmed as potentially contaminated and need to be submitted for detailed investigations. Detailed investigations and remediation activities are generally progressing slowly. More is being learned on the size of the problem but the speed of the clean-up is slow. In the future, the implementation of the EU and national legislative and regulatory frameworks already in place (e.g. Landfill Directive, Integrated Pollution Prevention and Control Directive, Water Framework Directive, Environmental Liability Directive) should result in the more efficient prevention of releases of contaminants into the environment, and into soil in particular. As a consequence, most of the efforts for remediation are expected to be concentrated on historical contamination. Inventories or registers of contaminated sites represent an important tool for the effective management of soil contamination from local sources. Soil remediation projects need to incorporate environmental, technical, legislative, and economic factors, all of which are site specific. In particular, remediation of soils with heavy metal contamination resulting from mining activities represents a strategic target for European policies. The land influenced by mining activities in the European Union has been estimated to be up to 0.6%, compared to a worldwide average of 0.2%. Exploitation of ores and minerals often results in the degradation of the surrounding environment, exposing and liberating metals and sulphides, creating acid mine drainage which remains a long-term problem with high costs for the local communities. The European Environment Agency has estimated the total costs for the clean up of contaminated sites in Europe to be between € 59 and 109 billion. Several techniques are available for the reduction of the risks caused by soil contamination. In the reporting countries, there is a balance in the application of innovative in situ (on-site) and ex situ (off-site) techniques (Fig. 1.4). A significant high percentage of the most-frequently applied techniques can be defined as traditional such as the so-called “dig and dump” techniques and the containment of the contaminated area. This reflects the fact that contaminated soil is frequently treated as waste to be disposed of rather than a valuable resource to be cleaned and reused.

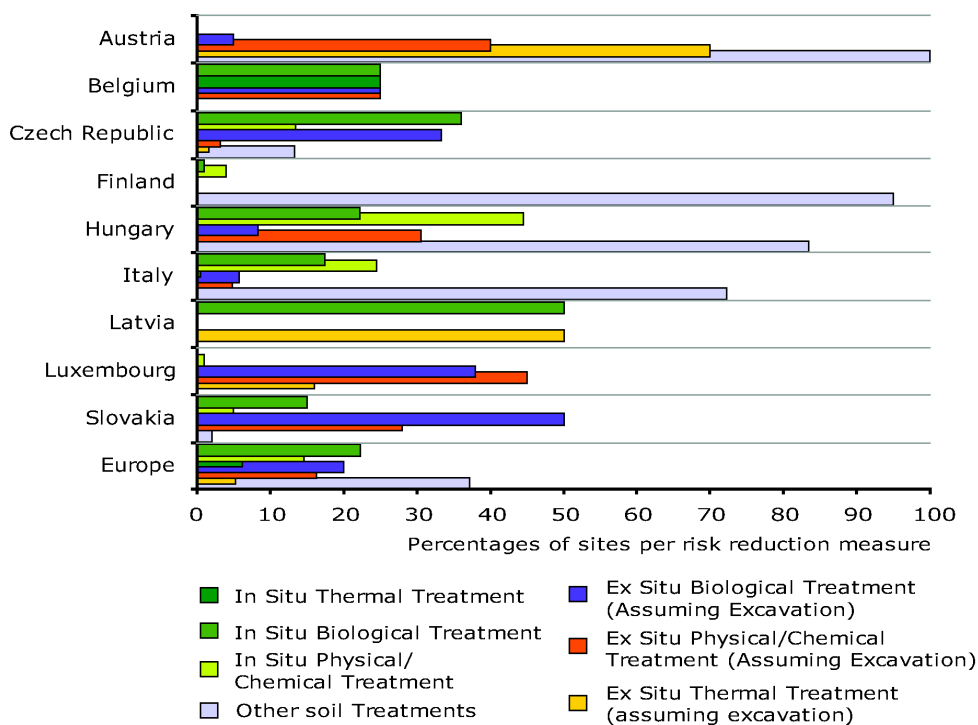


Figure 1.3 Remediation technologies. The graph shows remediation technologies applied in the surveyed countries as percentages of number of sites per type of treatment (data source: EIONET, 2007)

Costs are highly related to the different remediation targets and local site conditions. An analysis of the costs per site covering seven countries and three regions shows that: the average unitary costs for the investigation of a (potentially) contaminated site range between 500 and 50000 €. Due to economic and logistical reasons, the management of contaminated sites follows a tiered approach with increased efforts and expenses at each step. Furthermore, current methods of soil remediation do not really solve the problem. In Germany, for instance, only 30% of soils from contaminated sites are cleaned up in soil remediation facilities (SRU, 2004); the remaining soil must be stored in waste disposal facilities. This does not solve the problem, it merely transfers it to future generations. Obviously, there is an urgent need for alternative, cheap and efficient methods to clean up heavily contaminated industrial areas.

1.2.2 Heavy metals bioavailability: concept and investigation measurements

The knowledge of the heavy metals bioavailability is very important to understand the behavior and the destiny of these inorganic pollutants in the food chain (Kalis, 2006). When soils are polluted with heavy metals, the question always arises how large the risks for organisms living on or in that soil will be. Bioavailability is a concept for which no simple generic definition can be formulated, although a large number of definitions can be found in literature. According to the definition given in ISO 11074 (ISO, 2005) the bioavailability is “*the degree to which chemicals present in the soil may be absorbed or metabolised by human or ecological receptors or are available for interaction with biological systems*”. The bioavailability, depending on a specific target organism and specific contaminants, includes also the following aspects: exposure time, transfer of contaminants from soil to organisms, their accumulation in the target organisms and the subsequent effects (Violante et al., 2010). The most of the existing European legislations for clean-up, target values for remediation and values for the reuse of contaminated soils, are based on a risk assessment procedure that takes into account only the total concentration of the heavy metals (Cattani et al., 2006). This latter, that includes all fractions of a metal, from the readily available to the highly unavailable, does not give valuable information on the ability of the elements to be absorbed by plants and organisms, nor does it predict the transfer of toxic elements in the food chain (Morel, 1997). Biological effects are not related to the total concentration of a contaminant in the soil and soil-like materials, the organisms respond only to the fraction that is biologically available, the bioavailable one, ready to be adsorbed by roots and microorganisms. Therefore, total concentrations of metals are a poor indicator of metal toxicity since metals exist in different solid-phase forms that can vary greatly in terms of their bioavailability (Krishnamurti et al., 1997; Krishnamurti and Naidu, 2002; Huang and Gobran, 2005; Violante et al., 2010). Consequently, the determination of total soil metal content alone is not a good measure of bioavailability and not a very useful tool to quantify contamination and potential environmental and human health risks (Harmsen, 2007). The free ion concentration in soil solution and the labile amount in the solid phase represent the real potentially toxic amount (Sauvé et al., 1996; Cattani et al., 2006), the potentially bioavailable concentration of metals. However, in the European Soil Strategy is recognized the importance to evaluate the heavy metal bioavailability in soil: “*Most member states take conservative assumptions to take care of bioavailability (i.e., effective toxicity) of soil-bound contaminants. Guidelines are needed to account for research results. This should be harmonized, but gaps in knowledge should be taken into account*”. The European Soil Strategy also states, “*If bioavailability in some form or another is to*

be implemented in EU policy, there should be some agreement amongst scientists what is meant by bioavailability” (Van Camp et al., 2004; Harmsen, 2007).

Factors like pH, cation exchange, clays and hydrous oxides and specific adsorption, oxidation and reduction reactions, organic matter content, electrical conductivity, plant species, especially soil-plant interactions, the rate of soil evolution should be considered, because they can modify the bioavailability. These soil characteristics, among others, influence the speciation of the metal, which leads to a higher or lower available fraction, and to a long or short residence time of heavy metals in soils (Kalis, 2006; Feng et al., 2005; Epelde et al., 2009). The speciation is fundamental to the chemical and physical reactions that heavy metals undergo within a soil and consequently establishes the mobility and transport between different soil compartments and other environmental systems, their potential toxicity, the nature of their association with other soluble species. Moreover, also the soil ability to release the metal from the solid phase to replenish that removed from soil solution by the plants depends on their speciation (D’Amore et al., 2005). Consequently, speciation determines the bioavailability of the heavy metals and their pathways in terms of transport and uptake in different food chains (Keepax et al., 1999). Biota, especially bacteria and fungi, and plants can also alter the mobility and bioavailability of heavy metals particularly in the rhizosphere by modifying pH or by releasing oxygen and soluble organic compounds to complex heavy metals (Lombi et al., 2001; Soriano-Disla et al., 2010). All these parameters which determine the speciation of the metal constitute also uncertainties when estimating bioavailability (Krishnamurti and Naidu, 2002; Krishnamurti et al., 2007; Violante et al., 2010). Although considerable effort has been made during the last few decades to estimate the bioavailability of heavy metals and thereby predict their impact on the soil ecosystem, no single method is recognized universally (Soriano-Disla et al., 2010). In general, there are two complementary ways to assess it: (1) by chemical methods (e.g., extraction methods), which quantify a defined available fraction of a well defined class of contaminants assumed to be available for specific receptors (e.g., macro- and meso-fauna living in soil); (2) by biological methods, which expose organisms to soil or soil eluates to predict the amount of contaminants taken up by the organisms and to monitor effects (Harmsen, 2007). The chemical methods are based on the special sequences of extracting reagents aimed to the fractionation of heavy metals identifying the species that are more available for plants and microorganisms (Violante et al., 2010). Fractionation of heavy metal into operationally defined forms under the sequential action of given reagents with increasing aggressiveness is a common approach for distinguishing various species of trace elements according to their physicochemical mobility and potential bioavailability (exchangeable, carbonates, oxides, organic and residual) (Tessier et al., 1979; Martínez-Sánchez et al., 2008; Fedotov and Mirò, 2008) and there are some

different extraction methods for bioavailability evaluation. Through demineralized water (1:5 soil/water) is possible to obtain the water-soluble species made up of free ions and ions complexed with soluble organic matter and other constituents. It constitutes the most mobile and potentially the most available metal and metalloid species, but usually negligible (Chaignon et al., 2003). Through the use of diethylenetriaminepentaacetic acid (DTPA) (0.005 M) (Quevauviller, 1998; Brun et al., 2001), metal is released after dissolution of a soil fraction at pH close to 5 (Gleyzes et al., 2002), this fraction, useful for studies of metal mobility, soil-plant transfers, study of physic-chemical processes, gives a good indication of the amount of metals available to plant roots. Salts solutions of replaceable cations such as 0.1 M NaNO₃, 1 M NH₄NO₃, 0.01 M CaCl₂ and 1 M KCl are used for the exchangeable fraction which includes weakly adsorbed metals retained on the solid surface by relatively weak electrostatic interaction, metals that can be released by ion-exchange processes and metals that can be coprecipitated with carbonates present in many types of sediment (Hlavay et al., 2004). Nitrate salts are advantageous over chloride and acetate salts, since no metal complexing takes place due to the nitrate anion, and as a result, cation exchange is the only operating mechanism, moreover ammonium salts of strong acids, such as NH₄Cl or NH₄NO₃ can lower the pH and encourage the hydrolysis of clays (Filgueiras et al., 2002). Total reactive metal content of the soil measured in a HNO₃ or *aqua regia* extraction is only interesting for toxicity potential of the soils during hundreds or even thousands of years, but they say nothing about bioavailability (Ernst, 1997; Maiz et al., 1997; Kalis, 2006). The common features of those extraction procedures are focused on metal fractions associated with certain soil geochemical phases, but all have ignored biological reactions in soil, especially soil-plant interactions in the rhizosphere zone (Feng et al., 2005). These reactions determine the metal speciation, transformation, uptake by plants and accumulation in plants, thus determining overall metal bioavailability (Feng et al., 2005). The sequential extraction procedures should be described in International Standards, and the standardization process should result in a limited set of established methods for the measurement of bioavailability (Harmsen, 2007). All this work should lead to the application of bioavailability in risk assessment of contaminated sites within the common regulations. Then, adequate and standardized methods to assess the bioavailability of heavy metals in soils are essential for the determination of the soil quality, the potential risk derived from the presence of heavy metals and for the development of satisfactory remediation strategies (Soriano-Disla et al., 2010).

1.2.3 Processes and mechanisms involved in heavy metal regulation in soil

Soil is a major reservoir for contaminants as it possesses an ability to bind various chemicals. In particular, once deposited in soils, heavy metals interact with the soil minerals and organic constituents and different forces could keep them bound to soil particles (Dube et. al., 2001). The heavy metals can exist in various forms in soil and their pathways and availability is dependent on both soil properties (e.g. pH, clay and organic substances, aqueous and gaseous components, variations in moisture content, redox potential conditions) and environmental factors (e.g. climate changes) (Alloway, 1990; Adriano et al., 2002). It is of principal importance to study the interactions soil-metals which affect the speciation (form) of the metal and in turn control their solubility, mobility, bioavailability and toxicity (Dube et. al., 2001). In soils, metals are associated with several fractions: in soil solution as free metal ions and soluble metal complexes; adsorbed to inorganic soil constituents at ion exchange sites; bound to soil organic matter; precipitated such as oxides, hydroxides and carbonates; embedded in structure of the silicate minerals. These fractions are all in dynamic equilibrium with each other (Norvell, 1991). The release of contaminants from the soil matrix depends on (local) environmental parameters, particularly pH, and can be subdivided into different amounts (i) the dissolved amount at ambient conditions; (ii) the potentially available amount (i.e., the maximum amount that can be released under predefined worst-case conditions [environmental availability]); and (iii) the non-available fraction that is not released during the predefined conditions. Both soil properties and soil solution composition (the aqueous liquid phase of the soil and its solutes) determine the equilibrium of metals between solution and solid phases (Maiz et al., 1997). Metal ions may enter the soil solution and be subjected to numerous pathways, all of which can potentially overlap. The soil solution can contain metals as free ions or complexed to inorganic or organic ligands. Both the free ions and the metal-ligand complexes can be: taken up by plants; retained on mineral surfaces, natural organic matter and microbes; transported through the soil profile into groundwater via leaching or by colloid-facilitated transport; precipitated as solid phase; diffused in porous media such as soils. Root exudates and microbes affect the transport and solubility of metals (Diels et al., 2002; Al Chami Z., 2006). However, while the soluble metal in the soil solution is directly available for plant uptake other soil metal pools are less available (Davis and Leckie, 1978). Soil texture, cation exchange capacity (CEC), organic matter content but above all the pH and the redox potential, are the factors controlling the solubility and bioavailability of heavy metals (Ross, 1994; Harder and Naidu, 1995). In general, at an acid pH metals exist as free ionic cations, but at an alkaline pH the ionic cations precipitate as insoluble hydroxides or oxides and the most of heavy metal hydroxides are insoluble.

The pH at which precipitation occurs varies among different metals and among oxidation states of the same element. Some metals, for example, copper, have more than one valence state and the oxidized state is favored by high pH. The hydroxides of these oxidized states are less soluble than those of reduced states and precipitate at low pH values. Thus low pH generally increases the availability of metal ions, whereas high pH decreases availability. This has been illustrated in soils, where in very acid conditions toxicity due to an abundance of iron, manganese, copper, and zinc can be removed by adding lime which raises the pH (Hodgson, 1963). Besides the pH has a direct influence on metal uptake itself, it might have a positive effect as well as a negative effect on metal uptake by plants (Weng et al., 2003). On the one hand a lower pH will increase the metal transfer from the solid phase towards the solution phase, which increases the bioavailability of metals (Hatch et al., 1988; Plette et al., 1999; Kalis, 2006). Another important speciation parameter is the redox state of a heavy metal in solution, because it can drastically affect its toxicity, adsorptive behavior and metal transport (Mertz and Cornazer, 1971). Redox reactions can mobilize or immobilize metals, depending on the particular metal species and microenvironments. The reductive transformation of some heavy metals may proceed chemically, for example Cu(II) reduction to Cu(I) by Fe^{2+} or H_2S and reduction of Cu(II), Ag(II), and Hg(II) to elemental forms by Fe(II)-bearing green-rust (Borch et al., 2010). Sulfate reduction in contaminated soils may mobilize Cu, Pb, and Cd through the formation of Cu-rich sulphide colloids. Redox reactions also control the transformation and Fe- reactivity and Mn-oxides in soils, which are the major sinks for heavy metals and metalloids. Redox reactive metals often do have different degrees of toxicity depending on the specific metal oxidation state. For example, chromate is toxic to plants, animals and humans and is a suspected carcinogen, mobile in soils and readily available, whereas Cr(III) is not toxic to plants and is necessary in animal nutrition, so that reactions that reduce Cr (VI) to Cr (III) are of great importance. Organic material, sulfides, and ferrous species appear to be the dominant reductants. Moreover, many chemical and biochemical processes such as precipitation–dissolution, sorption–desorption, complexation–dissociation, and oxidation–reduction control the mobility and availability of the heavy metals. Not all these processes are equally important for each element, but all of them are affected by soil pH and biological processes. Therefore, it is crucial to understand some major reactions control the release of a specific trace element in the soil and environment in order to overcome problems related to deficiency and contamination of these elements (Violante et al., 2010). Change in the concentration of metal in the matrix of soil minerals is slow relative to exchange and desorption reactions between clays, hydrous oxides, organic matter and the soil solution (Shuman, 1991). Soil has the ability to immobilise introduced chemicals like heavy metal ions. The immobilisation of xenobiotics is mainly due to sorption properties which are

determined by physicochemical properties of the soil such as: soil humic substances, amount of clay, phyllosilicates, carbonates and variable charge minerals, pH, water content, temperature of the soil and properties of the particular metal ion (Weber, 1991). The inorganic colloidal fraction of soil is the most responsible for sorption by its mineral particles and the total amount of clay minerals in soil bulk. Clay particles, usually negatively charged, can also bind metal cations, and some metals such as zinc may enter the crystal lattice and become unavailable to organisms (Hodgson, 1963). Soil organic matter (SOM) is the second main component of the soil solid fraction and the adsorption of the metals cations on organic substances is mainly due to the general negative charge of these colloidal substances. Humic acids are especially important and it has been stated that practically every aspect of the chemistry of heavy metals in soils, sediments, and natural waters is related in some way to the formation of complexes with humic substances (Benes et al., 1976; Dube et al., 2001). Moreover, trace metals form both inorganic and organic complexes with a range of solutes in soils, and complexation reactions are important regulators of heavy metal ion speciation in water. As regard the precipitation appears to be the predominant process of metal immobilization in alkaline soils in the presence of anions such as sulphate, carbonate, hydroxide and phosphate, especially when the concentration of metal is high (Adriano et al., 2002). For example, precipitation as metal phosphates is considered to be one of the mechanisms for the phosphate-induced immobilization of heavy metals, especially in substrates containing high concentration of metals. As well as adsorption, metals may also leave the soil solution via precipitation, as metal hydroxides, removing OH^- ions from solution (Violante et al., 2010). The complexity of the soil matrix makes difficult to selectively choose interactions, which mostly contribute to the speciation and so to the bioavailability of a specific metal. This problem contributes more difficulty in the process of formulating meaningful soil models for the prediction of metal transport. It is imperative to fully understand the metal binding properties of the soil, develop and validate procedures for metal speciation in soils and carefully choose appropriate models, to understand the adsorption and migration of heavy metals in the soil matrices (Dube et al., 2001).

1.2.4 Role of plant-microorganism interactions in heavy metals mobility in soil

Potential for bioremediation applied to the heavy metals polluted soils depends upon the interactions among soil, heavy metals, bacteria and plants. These complex interactions are affected by a variety of factors, such as characteristics and activity of plant and rhizobacteria, climatic conditions, soil properties, etc. Moreover, soil conditions influence the specificity of the plant-bacteria interactions, which can alter contaminant bioavailability, root exudates composition and nutrient levels (Fig.1.4) (Jing et al., 2007).

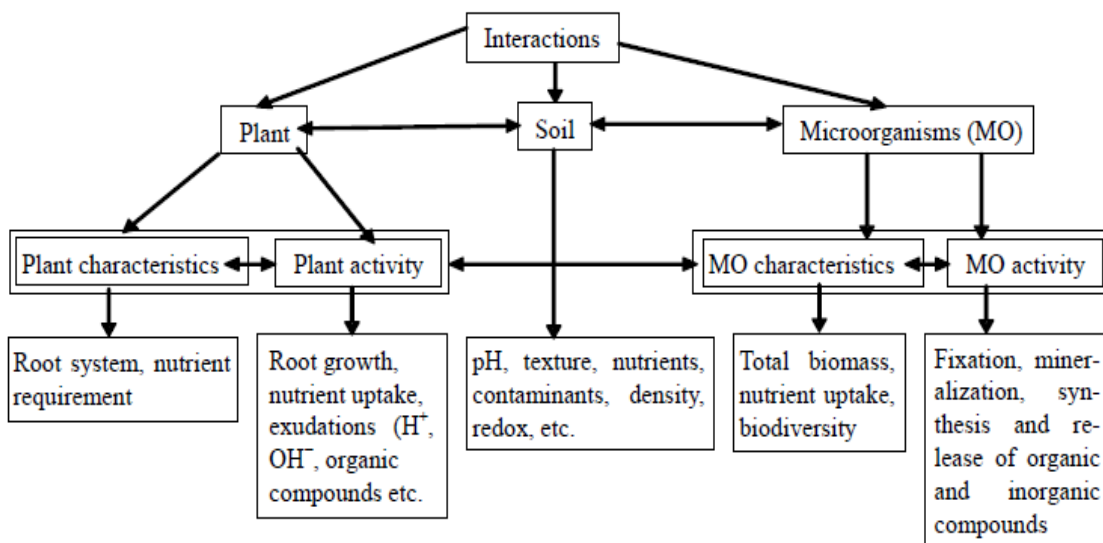


Figure 1.4 Plant-soil-microbial interactions in the rhizosphere (Jing et al., 2007)

Plants affect constantly the metal concentration and metal speciation in contaminated soils: by the uptake of metal ions and the simultaneous exudation of protons due to an antiport uptake system they acidify the rhizosphere and enhance metal availability in weakly buffered soils; by the exudation of simple phenolics and other organic acids (Kuiters and Mulder, 1993). Plant uptake of trace elements by roots to shoots proceeds through the solution phase of the soil and is controlled by plant physiology (Violante et al., 2010). Between the root cells on the outside of the roots there is a free space, and in this free space metal concentrations may accumulate the metals due to a Donnan potential created by the charged binding sites of the cell walls (Soriano-Disla et al., 2010). Plant roots can decrease the bioavailability of cationic metals/metalloids via uptake mechanisms, properties of their root system and root activities via adsorption onto root surfaces or root-derived biomolecules (Hinsinger and Courchesne, 2008). They may change metal speciation through acidification/alkalinisation, modify the redox potential, potentially increase metal/metalloid

solubility through exudation of metal chelants and organic ligands, like mucilage, polysaccharides and ectoenzymes, in particular releasing low molecular mass compounds like carbohydrates, organic acids, amino acids, peptides, phenolics and phytosiderophores, that compete with anionic species (e.g. arsenate) for binding sites (Ernst, 1996; McGrath et al., 2001; Fitz and Wenzel, 2002; Wenzel, 2009). In particular, the phytosiderophores are very specific organic compounds, i.e. not only affect the speciation of iron but also that of other heavy metals (Treeby et al., 1989). These organic acids can influence the metal speciation by complexing metals and therefore decrease the free metal ion concentration but also increasing the metal desorption from the solid phase of the soil. Similar to the other organic acids can bring protons into the rhizosphere which may induce a local lowering of the pH, which leads to a higher free metal ion concentration (Violante et al., 2010). The impact of these plant-borne organic compounds on the decontamination efficiency in the bioremediation process applied to heavy metal contaminated soils is great. The rhizosphere as an important interface of soil and plant plays a significant role in bioremediation. The rhizosphere processes, products and their proper management may be crucial for the success of phytostabilisation and/or phytoextraction of heavy metals in the soils. Moreover, plant-rhizobacteria interactions could stimulate the production of compounds that could alter soil chemical properties in rhizosphere and enhance heavy metals accumulation in plants. The uptake of metals by the plants can reduce consequently the bioavailability of these inorganic pollutants to microorganisms (Hinsinger, 2001; Wenzel, 2009). Metals are toxic to all biological systems from microbial to plant and animal, with microorganisms affected more so than other systems, due, in part, to their small size and direct involvement with their environment (Giller et al., 1999). Nevertheless, high concentrations of metals can harmfully effect the soil microbial activity and functioning, researchers have shown that many soil microorganisms survive to heavy metal pollution thanks to different life-support systems and moreover they play important roles in mobilization or immobilization of metals, because able to carry out various metal transformations (Turpeinen, 2002). The phenomenon of microbial resistance to metals, not only specific to microbes growing in metal-contaminated environments, is of some fundamental importance and is particularly relevant to microbial ecology, especially in connection with the roles of microbes in polluted ecosystems and in the reclamation of metal-contaminated natural habitats (Turpeinen, 2002). Frequently, microbial transformations of metals as complexation, precipitation, solubilization by releasing of chelating agents, acidification, phosphate solubilization and redox changes are the result of their resistance mechanisms (Nies, 1999; Roane and Pepper, 2000; Hietala and Roane, 2009). These microbial transformations affect heavy metal mobility and availability to the plant and therefore have potential to enhance bioremediation processes (McGrath et al., 2001; Lasat, 2002; Jing et al.,

2007). Generally, microbial transformations of metals can be divided into two broad categories: redox conversions of inorganic forms; and conversions from inorganic to organic form and *vice versa*, typically methylation and demethylation. In particular, through oxidations of metals, microbes can obtain energy (i.e. iron, sulfur, manganese and arsenic) (Tebo et al., 1997) and through reductions they may utilize metals as a terminal electron acceptor for anaerobic respiration. Microbial reduction of certain metals to a lower redox state may result in reduced metal mobility and toxicity, and one of the most widely studied forms for metal bioremediation is the reduction of Cr^{6+} to Cr^{3+} (Lovley, 1995; Wang and Shen, 1995; Jing et al., 2007). Similar to plants, microorganisms produce a number of extracellular metabolites including polysaccharides, pigments, organic acids and siderophores, that can change metal speciation, lower pH and so increasing solubility of metals and making them available for recovery (Hietala et al., 2009). For example, citric and oxalic acids can form soluble metal complexes with Fe, Al, and Zn (Strasser et al., 1994). Additionally, siderophores produced by microbes may favour attraction to (immobilisation) or repulsion from (mobilisation) charged soil minerals of metals, depending on the differential surface charge of metal–siderophore complexes and below metal-specific pH values (Neubauer et al., 2002; Wenzel, 2009). Soil rhizobacteria, with their activity and high surface area-to-volume ratio because of their small size and therefore providing a large contact area, may have the potential to act as microbial chelates associated with phytoremediation (Anderson et al., 1993; Sitaula et al., 1999; Jing et al., 2007). Microorganisms could take up the metals and accumulate them in their biomass via intracellular sequestration or precipitation, or adsorb them onto cell walls (Fein et al., 2001; Gadd, 2004; Zaidi and Musarrat, 2004) due to the release of exopolymers into their surroundings, consequently they immobilise metals. In summary, microbiological processes can either solubilize metals, thereby increasing their bioavailability and potential toxicity, or immobilize them, and thereby reduce the bioavailability of metals. These biotransformations are important components of biogeochemical cycles of metals and may also be exploited in bioremediation of metal contaminated soils (Lovley and Coates, 1997; Gadd, 2000; Barkay and Schaefer, 2001; Lloyd and Lovley, 2001). Therefore, bacteria play an important role determining the speciation and so the bioavailability of heavy metals in soil (Wenzel, 2009). The overall result of plant–rhizosphere microbe interactions is a higher microbial density and metabolic activity which in turn stimulate plant growth even in metal contaminated soils (van der Lelie, 1998; Kamnev et al., 2000; Khan, 2005). As concluded by Anderson et al. (1993) “further understanding of critical factors influencing the plant–microbe–toxicant interactions in soils will permit more rapid realization of plant-assisted bioremediation”.

1.3 Sustainable soil reclamation methods: innovative approaches for heavy metals bioremediation

The clean-up of soils contaminated with heavy metals is one of the most difficult tasks for environmental engineering, presenting a different set of problems, due to the dynamic nature of metal interactions in soils (Hietala et al., 2009). Compared to organic pollutants, the toxic metals cannot be mineralized/degraded and then their residence time in soil is of the order of thousands of years. Consequently, it is necessary to plan some suitable projects for soil remediation, often aimed to the reduction of metal toxicity versus removal, choosing satisfactory treatments necessary to avoid the heavy metals dissemination in the environment and/or the food chain (McGrath et al., 2001). The application of the traditional remediation technologies such as excavation and land fill, thermal treatment and electroreclamation is inappropriate due to the high costs, to the potential impacts to the environment, with particular regard to the destruction of landscape and soil structure, and to the extent of many areas subjected to heavy metals contamination. Traditional physicochemical methods for the remediation of metal-polluted soils have stimulated the development of innovative biological technologies to economically and sustainably reclaim these soils. Bioremediation is an emerging technology and is currently viewed as the ecologically responsible alternative to the environmentally destructive physicochemical remediation methods (Meagher et al., 2005). It is defined as the elimination, attenuation or transformation of polluting or contaminating substances by the use of living organisms with their biological processes and products for the reclamation of soil quality of polluted soils. Soil bioremediation aims to overcome constraints on ecosystem recovery through natural processes to produce resilient ecosystems that are resistant to invasion, capture and use resources efficiently, contain biological complexity needed to function effectively, and provide human-valued services. In general, the term bioremediation defines “a managed treatment process that uses microorganisms to degrade and transform chemicals in contaminated soil, aquifer material, sludges and residues” (Dasappa and Loehr, 1991) and the phytoremediation “the use of green plants to remove pollutants from the environment or to render them harmless” (Cunningham and Berti, 1993). Mixing the use of resistant plants and the application of microorganisms with their beneficial effects to plants and to soil could represent a valid tool for soil remediation (Wenzel et al., 2009). It should never be forgotten that the ultimate goal of any soil remediation process must be not only to remove the contaminants from the polluted soil but, most importantly, to restore the continued capacity of the soil to perform or function according to its potential (i.e. to recover soil quality) (Epelde et al., 2009).

1.3.1 Phytoremediation: the use of plants

Phytoremediation is commonly defined as the use of green or higher terrestrial plants to extract, sequester, and/or detoxify pollutants through physical, chemical, and biological processes (Cunningham and Ow, 1996). The efficiency of phytoremediation relies on the establishment of vital plants with sufficient shoot and root biomass growth and activities that can support a flourishing microbial consortium assisting phytoremediation in the rhizosphere. To make phytoremediation a viable and efficient technology, it is necessary to find fast growing and metal tolerant and/or hyperaccumulating plants with extensive root system and abundant production of aerial biomass. Baker (1981) suggested that plants could be classified into three categories: (1) excluders: those that grow in metal-contaminated soil and maintain shoot concentration at low level up to a critical soil value above which relatively unrestricted root-to-shoot transport result, (2) accumulators: those that concentrate metals in the aerial part, and (3) indicators: the uptake and transport of metals to the shoot is regulated so that internal concentration reflects external levels, at least until toxicity occurs. A number of biochemical reactions occur in plants stressed by heavy metal/metalloids. Most of these reactions are produced by the displacement of protein cationic centres or the increase of reactive oxygen species. Those plants with better ability to adjust to toxicity effects are able to survive in heavy metal impacted sites and are better candidates for phytoremediation purposes (Violante et al., 2010). The phytoremediation process can be categorized under five major subgroups, essentially depending on the plants used for the remediation aims: (i) Phytoextraction refers to processes in which plants are used to concentrate metals from the soil into the roots and shoots of the plant; (ii) Phytodegradation/rhizodegradation refer to the use of metabolic capabilities of plants and rhizosphere microorganisms to degrade organic pollutants; (iii) Rhizofiltration is the use of plant roots to absorb, concentrate or precipitate metals from effluents; (iv) Phytostabilisation (and immobilisation) is a containment process using plants and their associated microbes to mechanically stabilize the site and reduce the mobility of heavy metals through absorption and precipitation by plants, thus reducing their bioavailability and pollutant transfer to other ecosystem compartments and the food chain; (v) Phytovolatilisation/rhizovolatilisation are removal processes employing metabolic capabilities of plants and associated rhizosphere microorganisms to transform pollutants into volatile materials such as mercury- or arsenic-containing compounds that are released to the atmosphere (Chaudhry et al., 1998; Khan, 2005). With regard to soil metal remediation, two of these categories are most relevant: phytoextraction and phytostabilization (Garbisu et al., 2002; Epelde, 2010).

The choice of metal phytoremediation strategy (phytoextraction vs phytostabilization) will depend on a variety of factors: type of metal(s) present in the soil, level of metal pollution, future use of the site etc. Interestingly, the combination of both strategies, so that the limitations of one strategy might be overcome by the advantages of the other strategy, appears a most promising approach (Epelde et al., 2009). Phytoextraction, or the utilization of hyperaccumulator plants that have the capacity to transport and concentrate metals from the soil into the harvestable parts of roots and aboveground shoots, appears a promising, cost-effective technology for the remediation of metal polluted soils (Salt et al., 1995; Baker et al., 2000). The ideal plant for phytoextraction should grow rapidly, produce a high amount of biomass, and be able to tolerate and accumulate high concentrations of metals in shoots. To date, about 400 plant species have been identified as metal hyperaccumulators, representing <0.2% of all angiosperms (Brooks, 1998; Baker et al., 2000). Most of the commonly known heavy metal accumulators belong to the *Brassicaceae* family (Kumar et al., 1995). One of the key features that distinguish metal hyperaccumulators from non-hyperaccumulators is the extremely efficient translocation of metals from roots to shoots. This may be partly explained by a smaller sequestration of metals in the root vacuoles of hyperaccumulators than non-hyperaccumulators. It is also possible that hyperaccumulators have a more efficient xylem loading (McGrath et al., 2001). Brooks et al. (1977) introduced the term 'hyperaccumulators' to describe plants capable of accumulating more than $1000 \mu\text{g Ni g}^{-1}$ on a dry leaf basis in their natural habitats. This criterion is also applied to other metals including Co, Cu and Pb, whereas for Cd and Zn the respective threshold is 100 and $10\,000 \mu\text{g g}^{-1}$ dry leaves (Brooks, 1998; Baker et al., 2000). Compared to non-hyperaccumulator plants, metal concentrations in hyperaccumulator plants are 1–3 orders of magnitudes higher. Apart from these rather arbitrary criteria, hyperaccumulator plants usually have a shoot to root metal concentration ratio of >1 , whereas non-hyperaccumulator plants generally have higher metal concentrations in roots than in shoots (Shen et al., 1997; McGrath et al., 2001). Although hyperaccumulator plants have exceptionally high metal accumulating capacity, most of these have a slow growth rate and often produce limited amounts of biomass when the concentration of available metal in the contaminated soil is very high and lack any established cultivation, pest management or harvesting practices (Wenzel et al., 1999). An alternative is to use species with a lower metal accumulating capacity but higher growth rates (Glick, 2003; Jing et al., 2007). As regards, the aim of the phytostabilisation is not to extract the metals from soil, but to immobilize them. This is useful in situations where phytoextraction is not possible (McGrath et al., 2000). In this case, metal tolerant (non accumulator) plants are established, which decrease transport of metals in the environment (McGrath et al., 2001).

Phytostabilization refers to the immobilization of a contaminant in the rhizosphere through absorption and accumulation by roots, adsorption onto roots, or precipitation within the root zone of plants, so that contaminant migration via wind and water erosion, leaching and soil dispersion are prevented (EPA, 2000). Efficient phytostabilization in longterm stabilized heavy metal polluted soils may have beneficial effects on soil functionality and complexity of microbial communities due to reduction of labile elemental pools (Kumpiene et al., 2009). Thus, although metals are not removed from the soil, their adverse environmental effects are reduced. Ideally, plants for phytostabilization should develop an extensive root system and produce a large amount of biomass in the presence of high concentrations of metals, while keeping root-to-shoot metal translocation as small as possible (Wong, 2003). The success of phytoremediation will depend on three factors: (1) the degree of metal contamination of the soil; (2) the degree of metal bioavailability (its chemical and physical aspects); (3) the capacity of the higher plants to accumulate the metals in the shoots or to immobilize them in soils.

1.3.1.1 Advantages and disadvantages of phytoremediation

Phytoremediation appears attractive because in contrast to most other remediation technologies, it is not invasive and, in principle, delivers intact, biologically active soil. The technical aspects of phytoremediation, together with the advantages and limitations of this technology have been extensively reviewed (Chaney et al., 1997; Raskin et al., 1997; Alkorta and Garbisu, 2001; Garbisu et al., 2002; McGrath et al., 2002; Garbisu and Alkorta, 2003; Alkorta et al., 2004; Pilon-Smits, 2005; Epelde, 2010). It is generally considered as an environmentally friendly, effective, in situ, non-intrusive, low-cost, aesthetically pleasing, socially accepted technology which uses solar-driven biological processes to remediate polluted soils (Alkorta and Garbisu, 2001; Garbisu et al., 2002; Wenzel, 2009). It also improves the chemical characteristics of the contaminated soil by increasing organic matter content, nutrient levels, cation exchange capacity, helps to prevent landscape destruction and enhances activity and diversity of soil microorganisms to maintain healthy and self-sustaining ecosystems, prevents water and wind erosion as well as runoff and leaching of metals by rhizosphere-induced adsorption and precipitation processes (Cunningham and Berti, 1993; Cunningham et al., 1995; Salt et al., 1995; Cunningham and Ow, 1996; Garbisu et al., 2002). Despite these advantages, the application of phytoremediation for pollution control has several limitations that require further intensive research on plants and site-specific soil conditions (Sarma, 2011).

Phytoremediation must be considered as a long-term strategy, it usually takes many years and most probably decades (i.e. between 10 and 20 years) to reduce metal contents in soil to a safe and acceptable level representing an important limit for its diffusion (Cunningham et al., 1995). Another concern associated with the application of phytotechnology is handling and disposal of contaminated plant waste. The need to harvest contaminated biomass, and possibly dispose of it as hazardous waste creates an added cost and represents a potential drawback to the technology. The success of phytoremediation can be ensured when naturally selected and highly adapted plant species can be used economically to overcome this Achilles heel. It has been suggested that this phytotechnology should be combined with a profit-making operation such as forestry or bioenergy production (Khan, 2005), circumventing the recent food versus biofuel debate, since the polluted soils, not suitable for food crops, are exploited for energy biomass production. Obtaining products with economic value from plants used in the cleanup of polluted soils would be an additional benefit to phytoremediation, which could indeed help sustain its long-term use. Environmental conditions also determine the efficiency of phytoremediation as the survival and growth of plants are adversely affected by extreme environmental conditions, toxicity and the general conditions of soil in contaminated lands (Conesa et al., 2011). Phytoremediation is a cost-effective alternative to the conventional methods (e.g. soil excavation, landfilling, soil washing). Cost estimates associated with the use of several technologies for the cleanup of metal-contaminated soil are shown in Table 1.1. Cleaning of metal-contaminated soils via conventional engineering methods can be prohibitively expensive. Phytoremediation costs are still far below those of traditional remediation methods, such as stripping the contaminants from the soil using physical, chemical or thermal processes.

Table 1.1 Cost and time of soil treatment (Schnoor, 1997; Glass, 1999)

Treatment	Cost (\$/ton)	Additional factors/expenses	Time required (months)
Vitrification	75-425	Long-term monitoring	6-9
Landfilling	100-500	Transport/excavation/monitoring	6-9
Chemical treatment	100-500	Recycling of contaminants	6-9
Electrokinetics	20-200	Monitoring	6-9
Phytoextraction	5-40	Monitoring/ "Time consuming"	18-60

However, the cost of soil remediation is highly variable and depends on the contaminants of concern, soil properties, and site conditions. The presentation of phytoremediation as novel low cost remediation technology may not be borne out if the time when the land is out of production is taken into consideration. In a real cost evaluation, “time consuming” is assumed to be an additional cost, and this makes the cost of phytoremediation uncertain because it is difficult to evaluate (Conesa et al., 2011). Large remediation operations usually come in association with big projects (urban, commercial, industrial). Most commercial soil remediation occurs in relation to the growth of urban areas, where low levels of contaminations must be reached, change of soil use or toxic spills, where urgent solutions are needed to maintain sociopolitical acceptance. In these cases, conventional remediation options are often the best option due to their rapidity, despite their high initial cost. This makes it difficult for phytotechnologies to compete. Therefore, they are relegated to projects with low economic value and the following profile: (a) long term period is possible; (b) current use of soil does not imply risks for people/ecosystems. These kinds of projects are usually restricted to marginal areas without short term economic value, such as former mining areas, landfills, abandoned shooting ranges, or postindustrial sites (Conesa et al., 2011). Therefore, to make phytotechnologies more commercially attractive, they should be linked to the production of valuable biomass products (Lewandowski et al., 2006; Conesa et al., 2011). Despite some limitations, this phytotechnology is using worldwide and various research laboratories are at present engaged to overcome these limitations.

1.3.2 Microbial assisted phytoremediation: the use of autochthonous plant-growth promoting-bacteria

The potential of microorganisms to enhance phytoremediation processes and the exact mechanisms by which bacteria could enhance heavy metal accumulation in plants have recently received some attention (Khan, 2005). Soil microbial communities are responsible for important physiological and metabolic processes of a paramount interest on soil quality (Jing et al., 2007) and as well the microbe-plant interactions are major determinants of the extent of phytoremediation (Glick, 1995; de Souza et al., 1999; Jing et al., 2007). Especially, plant growth-promoting bacteria (PGPB) and among these in particular, rhizosphere microorganisms, termed plant growth promoting rhizobacteria (PGPR), which include a diverse group of free-living soil bacteria closely associated with roots (Glick, 1995), may exert many beneficial effects on plant growth, nutrition and development in heavy metal contaminated soils by mitigating toxic effects of heavy metals on the plants (Belimov et al., 2004; Jing et al., 2007). These soil microorganisms are essentially stimulated by plant root exudates including a wide range of organic molecules which are used by microorganisms as nutrients (especially small molecules such as amino acids, sugars and organic acids) (Glick, 2003). Plant growth-promoting bacteria can positively influence plant growth and development in two different ways: indirectly or directly (Glick et al., 1999). The indirect promotion of plant growth occurs when these bacteria decrease or prevent some of the deleterious effects of phytopathogenic organisms, inducing resistance in plants against fungal, bacterial and viral diseases, insect and nematode pests and also by increasing the overall fertility of the contaminated soil (Sikora and Hoffmann-Hergarten, 1992; Zehnder et al., 1997). PGPB can directly promote plant growth by catabolizing certain organics and or intermediate partly oxidized biodegradation products, providing the plant with a compound that is synthesized by the bacterium or by facilitating the uptake of nutrients from the environment by the plant (Kamnev et al., 2000). Moreover, they may fix atmospheric nitrogen and supply it to plants; synthesize siderophores which can solubilize and sequester iron from the soil and provide it to plant cells; support plant protection from diseases by producing anti-biotic and other pathogen-depressing substances (Kloepper et al., 1989; Glick, 1995; Kamnev and van der Lelie, 2000), have mechanisms for the solubilization of minerals such as phosphorus which then become more readily available for plant growth (Davison, 1988; Glick et al., 1998), favour specific enzymatic activities, synthesize several different phytohormones including auxins, cytokinins, gibberellins and lowering of plant ethylene levels, modifying the hormonal balance within the affected plant (Patten and Glick, 1996; Glick et al., 1999; Jing et al., 2007).

A particular bacterium may affect plant growth and development using any one, or more, of these mechanisms and a bacterium may utilize different mechanisms under different conditions. For example, bacteria do not fix nitrogen when sufficient fixed nitrogen is available (Glick, 2003), similarly, bacterial siderophore synthesis is likely to be induced only in soils that do not contain sufficient levels of iron. The siderophores produced by plant growth-promoting bacteria, having the effect of scavenging Fe^{3+} , significantly enhance the bioavailability of soil bound iron and may help plants to obtain sufficient iron (Bar-Ness et al., 1992; Wallace et al., 1992; Burd et al., 2000). They have a key-role, due to plants that are grown in the presence of high levels of heavy metals generally suffer for low iron content, becoming chlorotic, since iron deficiency inhibits both chloroplast development and chlorophyll biosynthesis. Also IAA, indole-3-acetic acid, phytohormone of the auxin series is important for plant growth favouring an improved uptake of iron, zinc, magnesium, calcium, potassium and phosphorus by crop plants (Lippmann et al., 1995; Kamnev et al., 2000). It should be mentioned that 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 (Khan, 2005). Therefore, microbial secretions and processes may play a major role among mechanisms of phytoremediation assisted by plant growth-promoting bacteria and the understanding metal-microbe relationships has led to advances in soil bioremediation (Malik, 2004; Bruins et al., 2000; Hietala et al., 2009). The field of microbial metal remediation is a continuing research and is making great strides towards applicable technologies. There are many new and exciting proposed uses of microorganisms and their products in metal polluted soils. For example, the addition of specific microbial populations, defined microbial bioaugmentation, aims to enlarge the biological potential of a system that is to increase the metabolic capabilities of the microbiota present in the soil. In that respect, bioaugmentation corresponds to an increase in the gene pool and, thus, the enlargement of the genetic diversity and capacity of that site. This technology is essential to increase the biological activity, especially for mineral soils with a low content of organic matter (Priha et al., 1999; Kamnev et al., 2000). The bioaugmentation can facilitate the proliferation and growth of various plants that even if are relatively tolerant of various environmental contaminants often remain small in the presence of the contaminant, making phytoremediation in the presence of plant growth-promoting bacteria a much faster and more efficient process (Glick, 2003; Violante et al., 2010). Controversies that concern the merits of bioaugmentation as a viable methodology are indicative of the fragmented knowledge that still exists in the field of bioremediation science and technology.

However, on balance, the recent studies are encouraging and thus bioaugmentation might well be used as a rational methodology for site remediation, subject to a thorough understanding of the site's ecology and of the local physicochemical constraints (Khan et al., 2004; Lebeau et al., 2008). The relationship of the inoculated microorganism with the new biotic and abiotic environments, in terms of survival, activity and migration, can be decisive in the outcome of any bioaugmentation strategy. It is important to choose PGPB which can survive and succeed when used in phytoremediation practices, once released into the target habitat (Kuiper et al., 2004). The selection of strains should be taken on the basis of some understanding of the kind of microbial communities present in the source habitat, and preferably with some knowledge of the type of organisms that are common in the target habitat, that is fundamental to overcome the ecological barriers. This suggests that such dominant populations at polluted sites can be good candidates as hosts for desired catabolic activities that are to be exogenously introduced for bioremediation in situ (Thompson I.P. et al., 2005). It seems that the better choice for the bioaugmentation purpose is the application of different and pre-selected bacterial strains autochthonous of the site to be remediated. Further research needs to be carried out to find suitable combination of plant, PGPR, and soil type in order to investigate their potential in increasing metal uptake by hyperaccumulator plants and improving the process of phytoextraction or the phytostabilization one of soil heavy metals (Khan, 2005). Although the role of PGPB is potentially important in the phytoremediation strategies, research in this area, as pointed out by Lucy et al. (2004), is very limited and requires field studies to support greenhouse or growth chamber results.

Chapter 2- Objectives

Soil is a non-renewable resource, which performs a number of key environmental, social and economic functions vital for life. Nevertheless, it is increasingly threatened by a range of human activities, which cause soil degradation and consequently a more rapid land desertification. One of the most dangerous and permanent threat for soil is represented by the heavy metals, a widespread environmental problem in large proportions of industrialized countries, which can cause an irreversible loss of soil quality. For future generations, new challenges in the field of soil protection, conservation and remediation are to be faced. Soil remediation is defined by Allen (1988) as the return of soil to a condition of ecological stability together with the establishment of plant and microorganisms communities it supports or supported to conditions prior to disturbance. In recent years, scientists and engineers plan cost effective and environmentally friendly technologies that include use of microorganisms or live plants for soil remediation, since current methods are expensive and environmentally destructive. Phytoremediation, based on the use of plants, is an emerging technology for cleaning up contaminated sites, which is cost effective, and has aesthetic advantages and long term applicability. The phytoremediation processes can be improved and more rapid combined with the bioaugmentation, which corresponds to the inoculation of appropriate bacterial species in soil. Several studies have indicated that microorganisms can be important for metal accumulation and for their potential use to clean up the metal-polluted soil. Changes in microorganisms' activity or in biochemical process rates may be used to assess the effects on soil quality of these two bioremediation strategies, phytoremediation and bioaugmentation (Parkin et al., 1996; Palma et al., 2000). Indeed, many soil scientific researchers reported that soil biological and biochemical properties can be the most important and appropriate bioindicators of soil quality (Trasar-Cepeda et al., 1998). Generally, the greatest problems posed by the use of microbiological and biochemical properties as indicators include the lack of reference values, the contradictory behavior shown by these properties when a soil is degraded, and the regional variations in expression levels. This implies that the standardization of the methods and the construction of databases of soil biological and biochemical properties have to be carried out (Gil-Sotres et al., 2005).

Currently, two main questions need to be clarified about soil pollution and remediation: (i) are the phytoremediation and bioaugmentation efficient to remediate the heavy metals polluted soils in a medium-term period? (ii) Which threshold values of heavy metals should be used to classify soil as polluted?

It is well accepted that the analysis of the risk assessment procedures about soil contamination can no longer be based only on total metal contents, but especially on the assessment of the bioavailable fractions of metals to estimate their potential environmental and human health risks. However, no single method has been recognized as a universal approach for prediction of heavy metal bioavailability (Sauvé et al., 2000).

In this context, the present thesis had two principal objectives:

- (i) to set up (a) a tool box of bioindicators, able to assess soil quality improvement due to bioremediation processes and (b) methods able to evaluate the heavy metal bioavailability or accumulation into soil microbial biomass;
- (ii) to use the preselected bioindicators to define the efficiency of different bioremediation systems including a phytoremediation and a bioaugmentation treatments on soil quality conservation or improvement.

In order to accomplish the first aim, a systematic bibliographic research and different preliminary tests were carried out. For the second aim, the preselected soil quality bioindicators were applied in two experimental trials: (i) a field study was conducted in a contaminated area of a former dump to assess the efficiency of a phytoremediation process; (ii) a pot experiment under control conditions of a greenhouse was established to evaluate the effects of bioaugmentation on soil microbial communities.

The specific objectives of the experimental researches were the following:

- to assess the mobility and the bioaccumulation of heavy metals using different methodological approaches, such as the leaching test, the content of different heavy metal extractable fractions. Moreover, the fumigation-extraction method was applied to quantify the CHCl_3 -labile metals, as a tool to investigate the accumulation of heavy metals into soil microbial biomass. A microbiological test was also carried out to assess if the bacterial consortium used for the bioaugmentation purpose was able to accumulate zinc (Chapter 4);

- to monitor soil quality changes in a phytoremediation system applied to a illegal former dump using three Mediterranean species (*Populus nigra*, var. *italica* L., *Paulownia tomentosa* (Thunb) Sieb. & Zucc. ex Steud, and *Cytisus scoparius* L.). Soil chemical and biochemical properties were determined during two years of field experiment to assess the effects of the phytoremediation system on soil quality (Chapter 5);
- to evaluate the effects of the bioaugmentation, performed with the Plant Growth-Promoting Bacteria (PGPB), on soil quality improvement in terms of microbial biomass content, activity and diversity (Chapter 6).

Chapter 3- Set up a tool box of soil quality bioindicators

The choice of soil quality indicators is related mainly to their utility in defining ecosystem processes and integrating physical, chemical, and biological properties, to their sensitivity to management and climatic variations, to their accessibility and utility to agricultural specialists, producers, conservationists, and policy makers (Doran and Parkin, 1996; Doran and Zeiss, 2000). There is an increasing evidence that biochemical properties related to the biocycles of the elements (C, N, P and S) and measurements of size and activity of soil microbial community hold considerable potential as early indicators of soil quality degradation or improvement (Anderson, 2003). The physical and chemical parameters are of little use as they alter only when the soil undergo a really drastic change (Filip, 2002). On the contrary, biochemical parameters are sensitive to the slight modifications that the soil can undergo in the presence of any degrading agent (Klein et al., 1985; Nannipieri et al., 1990; Yakovchenko et al., 1996). Considering the wide number of biochemical properties involved in the functioning of the soil, Visser and Parkinson (1992) point out the need to consider different levels of study which entails the use of specific groups of properties. One level is that of the biotic community, which implies the use of properties that are related to the structure of the microbial population and classically used to verify the composition and distribution of different functional groups of microorganisms of soil. A second level involves population studies, which considers the dynamics of specific organisms or communities of organisms (biological indicators). A third level, ecosystem level, considers the use of properties that are related to the cycles of bio-elements (C, N, P and S), especially when related to the transformation of the organic matter in the soil; in other words, properties related to the size, diversity and activity of microbial biomass as well as to the activity of the soil hydrolytic enzymes (Gil-Sotres et al., 2005).

3.1 Microbial biomass as bioindicators of soil quality

Since soil functioning, development, preservation of soil fertility is governed largely by the activities of the microorganisms, there is particularly a need for indicators of soil quality based soil microbial and biochemical characteristics. Soil microorganisms perform a wide range of ecologically significant functions, which are essential for a normal and healthy soil. They play a key role in the energy flows, nutrient transformations and element cycles in the environment. Activities of the soil microorganisms are irreplaceable in the organic matter transformations, and the microbial biomass itself is the essential source and sink of nutrients for the whole terrestrial ecosystem, supporting the soil fertility (Hofman et al., 2003).

Elevated concentrations of heavy metals can be generally toxic to many biological processes including those catalyzed by soil microorganisms negatively impacts all cellular processes, influencing metabolism and reducing their growth, in particular, affecting their enzyme activities with the exception of metal-tolerant species (Kamnev and van der Lelie, 2000). Significant reductions in microbial biomass (Fliessbach et al., 1994) and soil respiration (Bååth et al., 1991; Moscatelli et al., 2005) have been found in metal contaminated soils compared to uncontaminated soils. Loss of microbial populations in metal-contaminated soils impacts elemental cycling, organic remediation efforts, plant growth, and soil structure (Turpeinen, 2002). Studies of soil microbial properties are very often conducted at biomass level that is the soil microbial community is rated as the microbial biomass. It means that the quantity of microorganisms is expressed as a mass of carbon immobilized in microbial cells (C_{mic}), and overall activities such as respiration are measured without specifications of microbial groups or diversity. The soil microbial biomass has been proposed as a more sensitive indicator of changes in soil than organic C. The term microbial activity comprehends a vast range of activities and microbial respiration rates, N mineralization, enzymatic activities, substrates utilization rates and microbial functional diversity (Community Level Physiological Profiles-CLPP) together with the content of the microbial pool, expressed as the content of microbial carbon (C_{mic}), are some of the most used indicators of soil quality and nutrients cycling (Lagomarsino and Marinari, 2008). A merit of biomass level approach is in methods that are cheap, simple, consume little time, and are well standardized (Hofman et al., 2003). The term soil microbial biomass refers to dormant and metabolically active organisms living in soil that are generally smaller than approximately active organisms living in soil that are generally smaller than approximately $5 \times 10^3 \text{ mm}^3$ and include mainly bacteria and fungi (Lagomarsino and Marinari, 2008), it is one of the few fractions of soil organic matter that is biologically meaningful, sensitive to management or pollution and measurable. Variations in this “live” fraction of C may reflect changes in soil due to management practices, ploughing, amendment, contamination or even climate change, which are perhaps more related with the concept of the environmental quality of a soil. However, it should be noted that differences might exist in microbial biomass between different soil types without existing differences in quality, for which reason it is necessary to evaluate the quality of soils by integrating a variety of indicators (Hofman et al., 2003). The use of several indicators and the combination of soil microbial estimates (e.g. in index or quotients) can be of greater relevance to evaluate soil quality and can give a more comprehensive perception of changes in the soil biota (Lagomarsino and Marinari, 2008). Literature exhibit a great number of soil quality indicators for both agro-ecosystems and natural or contaminated soils and the microbial parameters, particularly those related to the size, activity and

biodiversity of the soil microbial communities are most relevant as bioindicators of soil quality. After all, microbial mediated processes are central to the functions that soil performs and, what's more, microorganisms are responsible for 70–85% of the soil biological activity (Masciandaro et al., 1998). Among the bioindicators of soil quality related to size and activity of the microbial pool, the most diffuse are *Cmic:Corg ratio* (“microbial quotient” percent portion of microbial biomass to total soil organic carbon) and qCO_2 (“metabolic coefficient” basal respiration of microbial biomass unit) which can be used currently for the bioindication of adverse processes in soils (Insam and Domsch, 1988; Anderson and Domsch, 1990, 1993; Brookes, 1995). The other microbial indices often found in some articles are: the qM (mineralization quotient) expresses the fraction of total organic carbon mineralized throughout the incubation time (Pinzari et al., 1999); the qC (microbial biomass change rate quotient) expresses the daily enrichment or loss of soil microbial C and is calculated based on qD (the C-loss quotient based on microbial-C loss) as reported by Anderson and Domsch (1990). A necessary research demand lies in the determination of natural deviations of such parameters. However, a strong deviation from a site-specific baseline value would be indicative of a changing environment and the establishment of a new soil community.

Cmic:Corg ratio

Comparative investigations on agricultural and forest plots have demonstrated the very close quantitative relationship between microbial carbon and the total soil carbon. Indeed, each metabolic activity of organisms is dependent on available carbon sources. The microbial biomass C to total organic C ratio (the so-called *Cmic:Corg ratio*), which reflects the contribution of microbial biomass to soil organic carbon (Anderson and Domsch, 1990), indicates also the substrate availability to the soil microflora or, in reverse, the fraction of recalcitrant organic matter in the soil; in fact this ratio declines as the concentration of available organic matter decreases (Brookes, 1995). This ratio has been proposed as a more sensitive index of soil changes than total organic C, since the microbial biomass of a soil responds more rapidly to changes than organic matter (Powlson and Jenkinson, 1981). If a soil is in a degradation process, this degradation could be primarily detected by microbial changes whereas changes in organic matter would not be detected at an early degradation state. Killham (1985) suggested that soil microorganisms under stress divert energy from growth to cell maintenance functions which may be a plausible explanation for lower *Cmic* values. The *Cmic:Corg ratio* of agricultural and forest soils at neutral pH is very similar and in the range between 2.0 and 4.4% *Cmic* of total *Corg*, depending on nutrient status and soil management. Values below 2.0 for the *Cmic:Corg ratio* could be considered as critical for soils with a neutral soil pH.

The content of soil microbial biomass was quantified as microbial biomass carbon (Cmic) estimated following the Fumigation Extraction (FE) method and total organic carbon (Corg) was estimated following the method reported by Springer and Klee (1954). After the measurement of the content of Cmic was calculated the microbial quotient (Cmic:Corg), which gives the important information in quantitative terms about the physiological condition of soil microorganisms (Kunito et al., 1999; Anderson and Domsch, 1989):

$$\text{Cmic:Corg} = \mu\text{g of Cmic} / \mu\text{g total organic carbon}^{-1}$$

Metabolic quotient (qCO₂)

Other widely used eco-physiological index for estimating soil quality in addition to the Cmic:Corg ratio, is the metabolic quotient qCO₂, that is the specific respiration on the total microbial biomass per unit time. The microbial respiration, which is an important microbial activity correlated to ecosystem productivity, soil fertility and carbon cycles, indicates the oxidative capacity of soil microorganisms, depends on both by the energy sources that there are in the soil and the number of microorganisms and may vary with several factors, such as climate, soil management practices, amendment, contamination, etc. (Epelde et al., 2009). The metabolic quotient (qCO₂) is the CO₂-C produced for unit of microbial carbon and hour (Leita et al., 1999). It gives information about the activity and the energetic needs of microbial pool. This microbial quotient was calculated as follows:

$$q\text{CO}_2 = \mu\text{g CO}_2\text{-C g}^{-1} \text{ h}^{-1} / \mu\text{g C-mic g}^{-1} \text{ (Dilly and Munch, 1998)}$$

The qCO₂, which describes physiologically the substrate mineralized per unit of microbial biomass carbon, ranges between 0.5 and 2.0 mgCO₂-C g⁻¹ Cmic h⁻¹ in neutral soils. Values above 2.0 could be considered as critical for soils with a neutral soil pH. Increasing qCO₂ has been attributed to soil microorganisms under stress, when they divert energy from growth to cell maintenance function increasing in the maintenance energy requirement and lowering the efficiency of substrate utilization for growth (Killham, 1985; Epelde et al., 2009). Decreasing qCO₂ indicated an amelioration of microbial habitat and reduced microbial stress in the treated soils (Brookes, 1995; Kumpiene et al., 2009).

The metabolic quotient has been widely used as a good indicator of the alterations that take place in soil due to heavy metal contamination (Brookes, 1995; Liao and Xiao, 2007), deforestation (Bastida et al., 2008), temperature (Joergensen et al., 1990) or changes in soil management practices (Dilly et al., 2003). In many studies, values of $q\text{CO}_2$ have been shown to increase along with the increased levels of heavy metals with a reduction of the ratio of microbial biomass C to soil organic C as a result of metal pollution. Nevertheless, this quotient has also been criticized for its insensitivity to certain disturbances and to the ecosystem's development (Wardle and Ghani, 1995).

3.2 Functional and genetic diversity of soil microbial communities

The microbial community is recognized as the essential living component of soil and its size, activity and diversity are seen as an indicator of soil quality. The characterization of soil microbial diversity is receiving much attention due to interest in the soil microorganisms as a reservoir of genes with hitherto unknown functions, which can be exploited in biotechnological industries, as important players in soil function and quality, as supporters of plant growth, and players in degradation of xenobiotic compounds (Winding and Hendriksen, 2007). Isolating members of microbial communities in soil and linking them with their functions has long been a goal of microbial ecologists, yet this task has proven to be difficult for a variety of reasons. First, soil microbial communities are phylogenetically diverse (Torsvik and Ovreas, 2002; Fierer et al., 2007) making it difficult to accurately survey and document changes in microbial community composition. Second, since most soil microbial taxa, including dominant taxa, cannot be readily isolated and cultivated in the laboratory, the metabolic capabilities of many taxa are not well known (Torsvik and Ovreas, 2002). Third, microbes exhibit a broad array of 'functions' in soil and, for certain microbial processes there is likely to be a high degree of functional redundancy among bacterial taxa, with phylogenetically dissimilar taxa carrying out similar processes (Allison and Martiny, 2008). This functional redundancy within communities (and functional similarity between different soil communities) can often obscure linkages between microbial taxonomy and functional traits (Schimel, 1995; Allison and Martiny, 2008) particularly when examining more broadly-defined biogeochemical processes (e.g. metabolism of root exudates compounds) where many taxa may be responsible (directly or indirectly) for the same biogeochemical function (Schimel, 1995; Eilers et al., 2010). Therefore, even with recent developments in molecular techniques, the vast genetic and taxonomic diversity of soil microorganisms makes the analysis of microbial community structure and function problematic (Pankhurst, 1997).

However, there are different and innovative methods to characterize microbial populations, which can be divided into molecular and physiological approaches (Stevenson et al., 2004). Analysis of microbial community composition using molecular methods generally involves the extraction of deoxyribonucleic acid (DNA) which is present in all organisms and holds phylogenetic information. Assessments of genetic structure of soil communities, however, provide little information about which organisms are active and the functioning of the whole community. A different approach is to assess the functional diversity by enzyme activity and the community-level physiological profiles (CLPPs) of whole communities through measurement of carbon substrate use, either as substrate-induced respiration in soil (Leckie, 2005).

3.2.1 Microbial functional diversity

Functional diversity, defined as the potential activity of a microbial community in contrast to microbial community function which is defined as actual catabolic activity, is central to understanding ecosystem-level processes such as decomposition and nutrient cycling and has been suggested as a way of assessing the quality of soils (Preston-Mafham et al. 2002; Winding and Hendriksen, 2007). Indeed, any loss in ability of the microbial biomass to maintain its wide range of functions (e.g. changes in catabolic evenness, defined as the uniformity of substrate use, Degens et al., 2001) is seen as a warning sign of decreased soil quality. Therefore, investigating the diversity of microbial heterotrophic functions in soil may provide more relevant information about the roles of microorganisms in ecosystem function (Zak et al., 1994). While molecular (genetic) or biochemical (phenotypic) diversity measurements have their place and generally measure the diversity of the numerically dominant members of the community (Loisel et al., 2006), functional diversity is popular in that it relates to the activity of the soil and gives information on the functioning of those members of the soil microflora involved in carbon cycling (Chapman et al., 2007). Soil functional diversity is generally simpler to assess without the recourse to the specialized laboratory requirements, e.g., DNA or PLFA (Phospholipid fatty acid) analysis (Chapman et al., 2007). It can be determined through the enzyme activities and the utilization of community level physiological profiles (CLPPs) which reflect the potential of the culturable portion of the microbial community to respond to carbon substrates (Bending et al., 2004; Epelde et al., 2009). In this thesis the functional diversity of analyzed soils was evaluated through the enzyme activities and CLPPs in order to monitor the effects of metal bioremediation process on soil microbial communities.

Enzyme activities

Kandeler et al. (1996) have indicated that the composition of the microbial community determines the potential of that community for enzyme synthesis, and thus any modification of microbial community due to environmental factors should be reflected on the level of soil enzymatic activities. Moreover, they are very sensitive to changes due to soil management practices, climatic variations, contamination, etc. For these reasons they have been reported to provide a unique integrative biochemical assessment of soil function and condition (Dick, 1997; Epelde et al., 2009). Consequently, enzyme activities also have been used early as sensitive indicators for reflecting the degree of quality reached by a soil in the bioremediation process applied to heavy metal polluted areas (Izquierdo et al., 2005). Published findings on the influence of heavy metals on soil enzyme activities indicate that metals are toxic to living organisms primarily due to their protein-binding capacity and hence ability to inhibit enzymes. Kandeler et al. (1996) found that C-acquiring enzymes (cellulase, xylanase, and β -glucosidase) were the least affected by soil pollution, phosphatase and sulfatase the most affected and N-acquiring enzymes (urease) had an intermediate response. The behaviour of dehydrogenase activity, which is only present in viable cells and may therefore be considered as a direct measure of soil microbial activity is very variable (Trasar-Cepeda et al., 2000). However, the use of a single enzyme as indicators has been criticised by several authors (Skujins, 1978; Nannipieri, 1994), mainly because enzyme activities catalyse specific reactions and they are substrate specific. The best candidates for bioindicators are those enzymes participating in soil organic matter synthesis (oxidative enzymes) and those that are involved in crop residue decomposition (hydrolytic enzymes), the products of which are relevant to plant nutrition (Palma et al., 2000; Nannipieri et al., 2002). Enzyme assays are based on the quantitative evaluation of the product(s) released or of the substrate(s) consumed and thus they do not distinguish the contribution of the different enzyme categories to the overall enzyme activity (Burns, 1982). Consequently, they give the maximum potential activity of enzymes occurring in sediment rather than the actual enzyme activity due to experimental conditions adopted in the determination (Alef and Nannipieri, 1995). This obviously restricts the usefulness of enzymatic activity data and limits their interpretation. Another problem of these enzyme assays is the lack of standardisation because different protocols are used to determine an enzyme activity in soil, as indicated by Burns (1978) (Gianfreda e Ruggero, 2006). However, enzymatic measurements are considered very helpful methodologies because they are short-term laboratory procedures, without expensive, sophisticated instruments (Dick, 1997), usually performed under standardised environmental conditions (use of sieved samples, optimal temperature and pH, and standardized water content), which prevent or minimise changes in the composition of soil biota and allow

comparison of data from different soils obtained in different laboratories. The most frequently used soil enzyme assays are based on the use of artificial colorimetric (Tabatabai, 1994) and fluorometric substrates (Marx et al., 2001). The colorimetric one uses different specific *p*-nitrophenyl substrates (*p*-nitrophenyl-sulphate, *p*-nitrophenyl- glucoside, and *p*-nitrophenyl-phosphate) and buffers with a pH close to the natural soil pH in the determination of several soil enzymes (acid and alkaline phosphatases, glucosidases, galactosidases, and arylsulfatases). This enzyme assay exploits the hydrolysis of the *p*-nitrophenyl substrate and its release to *p*-nitrophenol (pNP) (Tabatabai, 1994). For the present thesis the fluorimetric one was chosen. It is based on the use of substrate derivatives, conjugates of the highly fluorescent compounds 4-methylumbelliferone (MUB) with enzymatic production of fluorescent end products, has been proposed as a valid alternative to increase the sensitivity of the enzymatic assays and to avoid some of the problems cited above (Marx et al. 2001). This microplate assay with multi-well plate reader technology allows a large number of soil samples and/or enzymes to be analysed in a short time and inexpensive development of large data sets (Marx et al., 2001; Caldwell, 2005). The main advantage of using fluorimetrically-labelled substrates is that product formation can be measured directly in the microplate without previous extraction and purification of the product.

Community Level physiological profiles (CLPPs)

Additionally to the enzyme activities, the community level physiological profiles (CLPPs) are used to determine the functional diversity in soil (Chapman, 2007). In recent years, different rapid methods of fingerprinting for the metabolic potentials of microbial communities have supplanted traditional methodology. These methods are based on assessing the ability of soil microbial communities to metabolize a range of organic substrates that vary in structural complexity (Garland and Mills, 1991; Degens and Harris, 1997; Campbell et al., 2003). For the present thesis, two approaches were chosen to assess the CLPPs of the analyzed soils: Biolog® EcoPlates and the MicroResp™. A comparison of these two principal methods for CLPP determination reveals that each has its advantages and drawbacks. The most important difference between the Biolog® and MicroResp™ (Table 3.1) is that the former uses a soil extract and the latter the fresh soil. Since the Biolog method essentially only targets the small fraction of the microbial community that can grow within the microtitre plate wells. Moreover, the Biolog plates need to be incubated over 1-3 days, or even longer, while MicroResp™ give results within a few hours. All are designed to be non-limiting in terms of water activity but different mixing ratios of soil/water will also give different gas transfer rates and may favour some organisms over others. In both cases, the number and type of carbon substrates used in the standard descriptions differ but these can be readily varied in each

of the applications to whatever is desired or considered to give a useful CLPP. The choice of which of these two methods to use for CLPP determination may depend upon the particular hypotheses or questions in mind, as well as on the facilities available within individual laboratories. However, the MicroResp™ technique combines both relevance and convenience, being a 'whole soil' method in a flexible microtitre plate format that is fairly simple to execute. The user has a wide choice of permutations of number and type of substrates, number of replicates and number of soils to assay at any one time (Chapman, 2007).

Table 3.1 The main characteristics of Biolog® and MicroResp™ (Chapman, 2007)

Biolog	MicroResp
Assesses only cultivable organisms	Assesses whole population
Uses diluted soil extract	Uses ca.350 mg fresh soil
Incubated 24-72 h	Incubated 6 h (colorimetric detection)
Typically 95 carbon substrates	Typically 15 carbon substrates
Microtitre plate format (96-well)	Microtitre plate format (96-well)
Radiolabelled substrates not possible	Radiolabelled substrates possible

Biolog®

The Biolog microtiter plate consists of 96-well that contain a range of different carbon (C) substrates plus a no C control (Garland and Mills, 1991). During the incubation time (24-72h) in each well of the plate, which contains also a tetrazolium dye, there is the growth of the microbial population, utilization of the carbon substrate and reduction of the dye by the presence of CO₂ of microbial respiration to produce a red-violet coloration, which is then read on a standard laboratory microplate reader. The main phases of the Biolog® are reported in Fig. 3.1. Several variants of Biolog plates are commercially available containing different sets and numbers of carbon sources, e.g. Biolog® EcoPlates that contain 31 of the most useful carbon source for soil community analysis (Insam, 1997). The kinetic analysis from the results of Biolog® was performed using AWCD (Average Well Colour Development). The AWCD measure gives a general indication of the metabolic capacity of the community, it enables to capture an integral picture of differences in carbon sources utilisation of soil. AWCD was calculated as the arithmetic mean of the OD values of all of the wells in the plate per reading time (Sprocati et al., 2006).

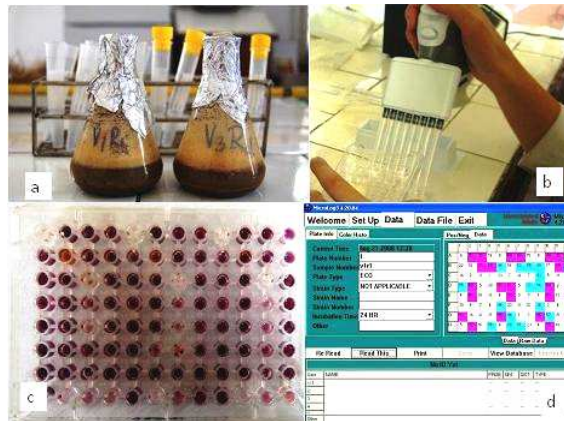


Figure 3.1 The main phases of the Biolog®

*MicroResp*TM

A valid alternative to Biolog method is represented by the *MicroResp*TM, which offers a convenient, rapid and sensitive method for the determination of microbial community functional diversity. Its application in a number of case studies has demonstrated its utility in a wide range of soils and land-cover types (Campbell et al., 2003). *MicroResp*TM was designed to be a 'whole soil' technique while still maintaining the convenience of the 96-well microtitre plate format that Biolog has. Essentially, the soil is placed within the wells of a microtitre deep-well plate and the carbon substrate is added in solution. The plate is then closed, face-to-face, with a second microtitre plate, the detection plate, such that each well of the deep-well plate communicates with one well of the detection plate. The two plates are hermetically sealed together with a suitable gasket (Fig. 3.2). The detection plate contains a gel-based bicarbonate buffer with indicator dye that responds to the pH change within the gel resulting from carbon dioxide evolved from the soil. The colour change is read after 6 h incubation on a standard laboratory microplate reader (Campbell et al., 2003).

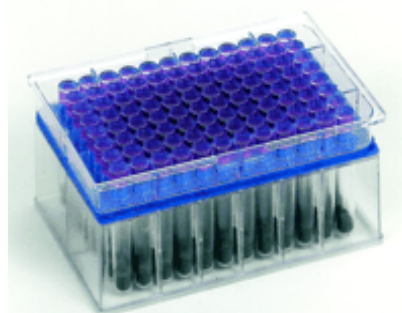


Figure 3.2 The components of the *MicroResp*TM (96-well Deepwell plate with soil, 96-well microplate with the detection gel and *MicroResp*TM Seal - which connects the two plates together (Campbell et al., 2003).

Microbial functional diversity indices

From the results of MicroResp™ and enzyme activities, the index used to describe the functional microbial diversity was the catabolic evenness (or Simpson-Yule index). Catabolic evenness, a component of microbial functional diversity defined as the uniformity of substrate use, can be easily calculated.

Catabolic evenness (CE) by MicroResp™ was calculated using the Substrate-Induced Respiration (SIR) data of all substrates as described in equation (e), where p_i is summed for all substrates and $p_i = r_i / \sum r_i$ (defined as the respiration response of each substrate (r_i) as a proportion of total respiration responses summed over all substrates ($\sum r_i$)).

$$CE = 1 / \sum p_i^2 \text{ (Magurran, 1988)} \quad (e)$$

The catabolic evenness was also computed from enzymatic rates as a measure of functional diversity where p_i is the ratio of the activity of a particular enzyme to the sum of all enzymatic activities (Degens et al., 2000).

3.2.2 Microbial genetic diversity

The molecular methods have led to the discovery of unique and previously unrecognized microorganisms as well as complex microbial diversity in contaminated soil which shows an exciting opportunity for the development of suitable bioremediation strategies for environment clean up (Malik et al., 2008), for understanding the key drivers of processes related to soil quality and to global microbial ecology (Schmeisser et al., 2007). The application of molecular techniques, which rely on the characterization of cellular constituents such as nucleic acids, proteins, fatty acids and other taxa-specific compounds (Rossello-Mora and Amann, 2001) represent an important complement to conventional methods that require cultivation or that measure microbial activities. Nevertheless, these molecular techniques need costly equipment and specialized staff and thus their usefulness as a routine diagnostic tool is limited. A possible solution is the development of diagnostic ‘kits’ this will become less of a problem. Microbial identification and diversity characterization has been enhanced by utilising the highly conserved gene, 16S rRNA which is ubiquitous in all microorganisms (Woese, 1987; Watanabe, 2001). 16S rRNA gene sequences are conserved enough to enable the design of PCR primers which target different taxonomic groups (from kingdom to genus), but have enough variability to provide phylogenetic comparisons of microbial communities (Woese, 1987).

A convincing approach to the study of microorganisms in their habitat is offered by the nucleic acid extraction and their subsequent amplification by polymerase chain reaction (PCR), in combination with denaturing gradient gel electrophoresis (DGGE) (Fig. 3.3). This combination has been proved extremely useful in assessing the changes in microbial community structure by several microbial community profiling techniques (Malik et al., 2008). DGGE separates amplified rDNA fragments of the same length but with different base pair compositions. The separation of bands in DGGE is dependent on the decreased electrophoretic mobility of partially melted double stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (Muyzer and Smalla, 1998). The PCR-amplified DNA fragments are generally limited in size to 500 bp and are separated on the basis of sequence differences, not variation in length. The number of bands produced during DGGE is proportional to the number of dominant species in the sample. For environmental or contaminated source samples where microbial diversity is largely unknown (Amann et al., 1995), DGGE technique provides the opportunity for the identification of the microbial population through the excision and sequencing of bands (Malik et al., 2008). The main advantages of DGGE are that it enables the monitoring of the spatial/temporal changes in microbial community structure and provides a simple view of the dominant microbial species within a sample. The limitations of DGGE in microbial community studies include: sequence information derived from microbial populations is limited to 500 bp fragments of 16S rRNA sequences which may lack the specificity required for the phylogenetic identification of some organisms; due to the existence of multiple copies of rRNA in an organism, multiple bands for a single species may occur; and different 16S rRNA sequences may have identical mobilities (Nubel et al., 1997). Band intensity may not truly reflect the abundance of microbial population (strong band may just mean more copies) and perceived community diversity may be underestimated (Malik et al., 2008).



Figure 3.3 The DGGE (denaturing gradient gel electrophoresis) equipments

Microbial genetic diversity indices

Different ecological indices were determined from the results of DGGE analysis: Simpson's diversity index (1-D), Simpson's evenness index (Ed), range-weighted richness (Rr) and functional organization (Fo), according to Simpson (1949) and Marzorati et al. (2008). The Simpson's diversity index (1-D) was developed for the description of species diversity within an ecological habitat and is based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups. This index is given by the following equation:

$$1-D = 1 - \sum \left(\frac{n}{N} \right)^2$$

where n is the band intensity for individual bands and N is the sum of intensities of bands in a lane. The Simpson's diversity index ranges between 0 and 1, it is greater, the microbial diversity is higher. The Simpson's evenness index (Ed) is a measure of how similar the abundances of different species are. When there are similar proportions of all subspecies then evenness is one, but when the abundances are very dissimilar (some rare and some common species) then the value increases. It is given by the following equation:

$$Ed = \frac{1 - D}{1 - \frac{1}{M}}$$

where 1 - D = Simpson's index diversity and M is the total number of bands in a lane.

Range-weighted richness (Rr) is a measure of the total number of different organisms present. This is the simplest of all the measures of subspecies diversity, it is the total number of bands multiplied by the percentage of denaturing gradient needed to describe the total diversity of the sample analysed, according to the following formula:

$$Rr = (N^2 \times Dg)$$

where N represents the total number of bands in the pattern, and Dg the denaturing gradient comprised between the first and the last band of the pattern.

A third parameter, which is recommended in complementation with the three previous ones to score bacterial communities based on DGGE analysis, is the functional organization. This organization is the result of the action of the microorganisms that are most fitting to the ongoing environmental-microbiological interactions. For this reason, they tend to become dominant within the structure of the microbial community. In order to graphically represent the structure of a bacterial community (species distribution), Pareto–Lorenz (PL) evenness curves (Lorenz, 1905) can be constructed based on the DGGE profiles as previously described (Mertens et al., 2005) (Fig. 3.4). The more the PL curve deviates from the 45° diagonal (the theoretical perfect evenness line), the less evenness can be observed in the structure of the studied community. The latter means that a smaller fraction of the different species is present in dominant numbers (Marzorati et al., 2008).

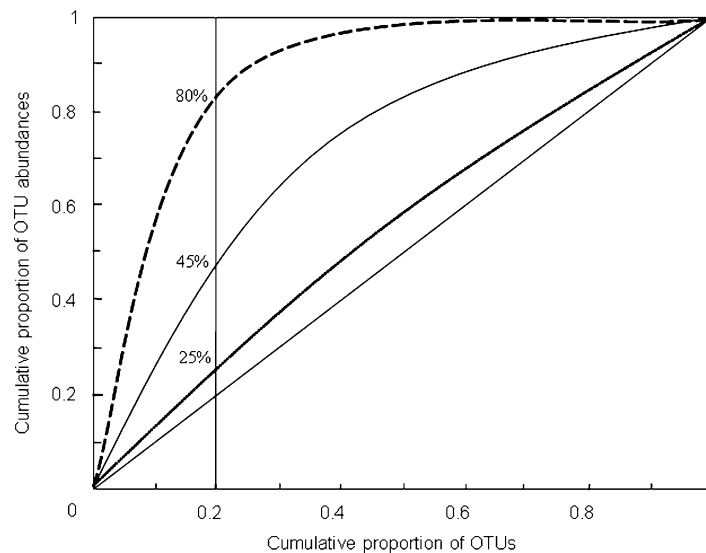


Figure 3.4 Pareto–Lorenz curves derived from three hypothetical DGGE patterns. The 25%, the 45% and the 80% curves refer to a low, medium and high functional organization respectively. The 45° diagonal represents the perfect evenness of a community (Marzorati et al., 2008).

Chapter 4- Heavy metal bioavailability in soil and bioaccumulation into soil microbial biomass

4.1 Abstract

Several chemical and biological methods have been developed in the last decade to evaluate heavy metals bioavailability in contaminated soils. The bioavailability is a complex issue that determines whether or not adverse effects are to be expected when soil organisms or plants are exposed to heavy metals. As regards, it is important improving knowledge on soil microorganisms and heavy metals relationship, with particular attention to the bioaccumulation, because the soil microorganisms could be used in the bioremediation process of the polluted soils. The present study aimed to investigate the mobility of heavy metals and the accumulation of these inorganic pollutants into soil microbial pool. Therefore three methods, fumigation extraction method (FE), leaching test and a metal uptake test were used to evaluate heavy metals availability, mobility and accumulation into soil microbial biomass. The results showed that the CHCl_3 -labile metals may give information on the heavy metals stored into soil microbial biomass, but further research is needed to test the reliability of this indicator to quantify the heavy metals bioaccumulation. As well, more knowledge is required on the contribution of bacteria to the CHCl_3 -labile fraction and on their capacity to accumulate the heavy metals. The leaching test demonstrated to be a reliable tool for a rapid evaluation of soil mobile fractions of heavy metals. The results concerning the microbiological uptake test showed a clear negative effect of Zn on the bacterial consortium, but it was not possible to quantify exactly its active accumulation inside the bacterial cells. It was supposed rather than a passive accumulation mechanism of Zn on the cell membranes. In conclusion, without an interdisciplinary approach, the bioavailability will stay a pure scientific tool, and it will not fulfill its role in the environmental risk assessment and in a sustainable approach to remediate the heavy metals polluted soils.

Keywords: *heavy metals; mobility; bioaccumulation; bioavailability*

4.2 Introduction

There is growing awareness and concern about the adverse effects induced by elevated levels of heavy metals in the environmental systems and living organisms. However, there is also increasing recognition that elevated contaminant levels in themselves are not necessarily indicative of actually occurring negative effects. Observations in laboratory and field studies have demonstrated that the responses of at-risk populations are not affected by the total concentration of a contaminant in the soil (Alexander, 2000). Instead, they are affected by only that fraction that is biologically available to a population in a given time and under particular soil conditions (Harmsen et al., 2005). So, to define the environmental risks correlated to that heavy metals polluted soil it is necessary to consider the bioavailability, that is the extent to which living receptors are exposed to contaminants in soil or sediment (Ehers and Luthy, 2003). Bioavailability is a concept for which no simple generic definition can be formulated, although a large number of definitions can be found in literature. The lack of a universal expression of bioavailable metal fractions precludes the presentation of a detailed monitoring strategy for assessing potentially and actually available and accessible metal fractions in the environmental matrices of water, soil, and sediment that is broadly applicable (Peijnenburg and Jager, 2003). The translation of information on bioavailability into acceptable evaluations of “how clean is clean?” (e.g. site-specific limits for regulating the concentration level in a soil to which a contaminant has to be reduced), is essential for establishing realistic risk assessments and endpoints for remediation (Harmsen et al., 2005). Nevertheless, the scientific basis for the adequate use of “bioavailability” in the assessment of ecological and human risks is weak. Moreover, another important requisite for any further environmental evaluation of contaminated soils is the heavy metals mobility, because risks related to them depend highly on their mobile fractions. The bioavailability and mobility of these pollutants are controlled by many chemical and biochemical processes such as precipitation–dissolution, sorption– desorption, complexation-dissociation, and oxidation–reduction. Therefore, it is crucial to understand some major reactions in soils control the release of a specific trace element in the soil and the environment in order to overcome problems related to deficiency and contamination of these elements (Violante et al., 2010). So, adequate methods for the assessment of the mobility and bioavailability of heavy metals in soil are decisive for the determination of the soil quality, the potential risk derived from the presence of heavy metals, and the development of sustainable remediation strategies (Soriano-Disla et al., 2010). In this context, it is important to improve knowledge on soil microorganisms and heavy metals relationship.

All microorganisms contain biopolymers such as proteins, nucleic acids, and polysaccharides which provide reactive sites for binding metal ions. Cell surfaces of all bacteria are negatively charged containing different types of negatively charged functional groups, such as carboxyl, hydroxyl and phosphoryl that can adsorb metal cations, and retain them by mineral nucleation. Intact bacterial cells, live or dead, and their products are also highly efficient in accumulating both soluble and particulate metals forms. Therefore, bacteria play an important role in the speciation, fate and transport of heavy metals in soil and associated environments. The present study is an interdisciplinary research based on the three different methods. The methodologies proposed are used for a general monitoring strategy to assess the mobile fractions in the soils and the potential accumulation of heavy metals into soil microorganisms.

4.3 Specific aims

The present study aimed to investigate the mobility of heavy metals (HM) and the accumulation of these inorganic pollutants into soil microbial pool. It is important to define the heavy metals mobility and the capacity of soil microorganisms to accumulate them, as these two factors are considered important variables to define a suitable remediation strategy and to determine the heavy metals bioavailability. Three methods were applied to estimate the mobile and available fractions of metals: the fumigation extraction method, the leaching test and the metal uptake test.

The fumigation extraction (FE) method was applied to quantify the HM accumulation into soil microbial biomass. The second methodology represented an alternative screening method to determine the mobility of heavy metals. Finally, the metal uptake test was carried out to study the growth dynamics of the bacteria in presence of Zn, to assess the interactions between the bacteria and zinc and evaluate the bioaccumulation and biosorption of this metal.

4.4 Materials and methods

- **Soil samples**

Twelve soils were collected in different Italian regions (Fig. 4.1). Seven of these twelve soils were naturally contaminated (NC) and collected from two abandoned mines areas located in Central Italy (Tolfa- 2 and Ingurto-5). The other five soils were artificially contaminated (AC), polluted during laboratory incubations with a solution containing $ZnSO_4$, $CuSO_4$ and $CdSO_4$ to give a final soil concentration of 340 mg Zn kg^{-1} soil, 190 mg Cu kg^{-1} soil and 10 mg Cd kg^{-1} soil. The FE method (Vance et al., 1987) was applied to these soils in order to verify its reliability to quantify the heavy metals accumulated into soil microbial pool. Successively, the FAN, RIZ and UMB soils collected from the mining dump of Ingurto (5) were analyzed through the leaching test in order to obtain a rapid evaluation of heavy metals mobile fractions in these soils. Finally, a bacterial consortium comprising four different species isolated from the FAN and RIZ soils was tested *in vitro* to verify its capacity of Zn accumulation. The chemical properties of soils are reported in Table 4.1.



Figure 4.1 The soil sampling areas

Table 4.1 The main characteristics of different soils

Area of soil sampling	Soil	Organic Carbon (%)	pH (H ₂ O)	CEC (cmol(+) kg ⁻¹)	Clay (%)	Silt (%)	Sandy (%)
1	Mongl1 (AC)	3.92	8.07	28	24	28	48
1	Mong 12 (AC)	6.41	6.59	44	14	17	69
2	Tolfa B (NC)	3.18	6.70	27	14	22	63
2	Tolfa C (NC)	2.19	5.50	24	14	22	63
3	Rutigliano (AC)	1.13	7.90	15	35	48	16
3	Montepietroso (AC)	2.20	7.10	26	28	57	14
4	Metaponto (AC)	1.10	8.40	18	50	40	10
5	Gennamari (NC)	2.11	7.10	15	7	10	83
5	Piezometro (NC)	1.3	8.00	9	10	8	83
5	Riz (NC)	0.57	8.30	8	4	1	95
5	Fan (NC)	1.4	6.50	9	23	42	35
5	Umb (NC)	1.04	7.60	7	6	6	88

*CEC: Cation exchangeable capacity; AC: artificially contaminated soil; NC: naturally contaminated soil

- **Heavy metals**

Total content

In most European countries, the quality standards are based on the pseudo total metal fraction such as boiling nitric acid (HNO₃) or *aqua regia*. These values are excellent criteria to define the extent of metal build up or contamination in soil. It provides at least an estimate of the portion of total cation exchange capacity saturated by metals. But for the prediction of ecological impact the total content is of little value (Gupta et al., 1996). In the present study, the total content of Cu and Zn were determined in all soils under study. The soil (1g oven-dry soil) was mixed with 5 ml of nitric acid (HNO₃) and 2 ml of perchloric acid (HClO₄) and then put in a Block digester with the following cycle of time and temperature: 2 hours at 90 °C, 2 hours at 140 °C and 1 hour at 190 °C. Then, the extracts were made to volume (50 ml) with deionised water, filtered and analyzed by the Inductively Coupled Plasma Atomic Emission Spectrometry (LIBERTY AX – Sequential ICP-AES – Varian) (AOAC, 1990).

DTPA and NH₄NO₃-extractable fraction

The DTPA (diethylene triamine pentaacetic acid) -extractable fraction of heavy metals (Cd, Co, Cr, Cu, Mn, Ni, Pb, and Zn) gives a good indication of the amount of metals available to plant roots (Brun et al., 2001). Extraction of soil with 1 M NH₄NO₃-solution is used to determine readily soluble trace element contents. In Germany this method has been published as an official standard (DIN 197301997) and is used to estimate the transfer of heavy metals and arsenic from soils to plants (Gryschko et al., 2004). For determining these fraction, 15 g (oven-dry soil) was extracted with 0.005 M DTPA or 1 M NH₄NO₃ in ratio 1:2.5 (w:v) for 60 min by oscillating shaking at 200 rpm and filtered (Whatman no. 42). The DTPA and NH₄NO₃ soil extracts were acidified to avoid the metal precipitation with 0.5 ml of HNO₃ 69%. Then, they were analyzed by the Inductively Coupled Plasma Atomic Emission Spectrometry (LIBERTY AX – Sequential ICP-AES – Varian) (Quevauviller, 1998; Gleyzes et al., 2002).

CHCl₃-labile zinc and copper

CHCl₃-labile Zn and Cu were quantified through the fumigation-extraction (FE) method. This fraction could correspond to the Cu and Zn accumulated into soil microbial biomass (Khan et al., 2009). The basic principle of the FE method is that soil microorganisms die after their cell membranes are attacked by chloroform (CHCl₃) and a part of the microbial constituents, especially in the cytoplasm, is degraded by enzymatic autolysis and transformed into extractable components (Joergensen, 1996). As well the metals contained inside the microbial cells are released through soil matrix and so it is possible to quantify them. All soils were sieved (< 2 mm) and given a preliminary incubation at 60% water holding capacity (WHC) for 7 days before use (Brookes et al., 1985). Twelve subsamples of 10 g moist soil were taken from each soil. Three of twelve replicates were extracted with 25 ml of 1 M NH₄NO₃ (1:2.5 w:v), and three replicates extracted with 25 ml of 5mM DTPA (1:2.5 w:v) by 60 min horizontal shaking at 200 r.p.m. and filtered through a Whatman no. 42. The other six replicates were fumigated for 24 h with ethanol-free CHCl₃ in vacuum-sealed desiccators at room temperature. Following fumigant removal, these samples were extracted as described for the non-fumigated replicates. After filtration, the soil extracts were acidified with 0.5 ml 65 % HNO₃ and stored at 4 °C. Then, in all extracts, Cu and Zn were measured by ICP-AES. *CHCl₃-labile Cu and Zn* were calculated as the difference between Cu or Zn extracted from fumigated soil and those extracted from non fumigated soil (Khan et al., 2009). No conversion values to total microbial biomass were applied as the percentage of the CHCl₃-extractable fraction is unknown (Khan et al., 2009).

Soil mobile fraction of heavy metals

A simple screening method, applicable even “in field”, was used in order to obtain a rapid evaluation of heavy metals mobility in polluted soils, mainly focused on the fraction associated to Fe and Mn oxide/hydroxides more dependent on the pH and oxidising variation in the soil environment (Alloway, 2005) and, qualitatively and quantitatively, more representative of the risks related to HM mobility (Pinto et al., 2010). The leaching test was applied on the soils Fan, Riz, Umb collected for the experimental trials conducted under the UMBRELLA project. For this “mobility test”, a single-step leaching test was performed: 0.5 g sample (soil) was used with 30-mL buffer solution. The extraction solution was prepared with 141 g of trisodium citrate $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ and 19 g of hydroxylammonium hydrochloride $\text{NH}_2\text{OH} \cdot \text{HCl}$ dissolved in 1 l water and the pH adjusted to 9.3 with NH_3 . The soil extract was stirred using an end-over-end shaker at a speed of 30 rpm. Successively, the extract was separated from the solid residue by centrifugation at 4,000 rpm for 10 min and then filtered through 0.45 μm filter membranes. Then, it was titrated with dithizone (DZ) to determine the content of Zn, Cu, Pb and Cd. The proposed method generically measures, in few minutes, the concentration of total extractable of Zn, Cu, Cd and Pb, expressed as mol l^{-1} without distinguishing between elements. The heavy metals content was calculated using a calibration curve obtained from the titration of multi-element standard solutions in the range from 0.004 to 0.012 μmol (Pinto et al., 2010).

Zinc accumulated into bacterial biomass

Soil microorganisms not only store organic C, N, P, and S components in their biomass, but also macronutrients and heavy metals which are also transferred into microbial cells by expending metabolic energy (Gadd, 2004; Khan et al., 2009). In particular, accumulation of metals into soil microbial biomass can occur either by metabolism-independent passive sorption (biosorption) on the microbial cell membranes or by intracellular, metabolism-dependent active uptake (bioaccumulation) (Gadd, 1992; Malik, 2004). The bacterial consortium (made by the four strains isolated from Fan and Riz soils) was used in metal uptake tests and for the bioaugmentation purpose in the pot experiment (for more details see the chapter 6). Each strain was grown as a pure culture in liquid medium (Standard 1-St1) at 25 °C overnight. After determining the concentration of the single strains by the cell count in a Neubauer chamber, the individual suspensions were pooled in equal proportions in the Erlenmeyer flasks, inoculated at a final concentration of 10^4 CFU ml^{-1} and incubated in a rotating shaker at 180 rpm and 25 °C.

The growth of bacterial consortium was carried out in Mineral Medium (MM) with and without zinc. This metal which presents the highest concentration in Inguertosu, was added to the medium at concentration 5mM in the form of salt (ZnSO_4). The MM with Zinc was filtered through a 0.2 μm pore size filter (cellulose-acetate membrane). The real concentration of zinc was calculated on basis of the effective concentration measured in the blank without the bacterial consortium. The Erlenmeyer (250-ml) flasks, filled with 100 ml of Mineral Medium (MM), were prepared: 3 flasks were added with 5 mM ZnSO_4 , 6 flasks without zinc used as negative control. The flasks were incubated in a rotating shaker at 180 rpm and 25 °C. During the incubation time, the number of cells per ml of bacterial suspension was regularly determined for monitoring the growth of consortium. The bacterial growth was checked 0, 24, 48, 72, 144 and 168 hours after the inoculation on aliquots of samples by different methods: optical density (OD) at 600 nm of spectrophotometer, the cell count on ST1 plates and by a Neubauer chamber on microscope. Once it was reached the OD to the stationary phase of the growth curve of the bacterial consortium (168 hours), the incubation time was stopped. Viable (V) and viable non-proliferating (VNP) cells were employed to carry on biosorption experiments. The microbial cells from 4 flasks grown in MM free of metal up to the stationary phase, were harvested by centrifugation. The pellet from 2 flasks was suspended in 100 ml of MM+ 5 mM ZnSO_4 in order to obtain the viable cells (V) and the pellet of the other two flasks was suspended in the equal volume (100 ml) of 0.1M Tris-HCl pH 7+ 5 mM ZnSO_4 in order to obtain the viable non proliferating cells (VNP). The two sets of microbial cultures (V and VNP) were incubated in a rotary shaker at 25 °C for 48 h for metal sorption. The bacterial suspensions of all flasks (the negative controls as well) were filtered through a 0.2 μm pore size filter (cellulose-acetate membrane) to separate the bacterial biomass and the supernatant. The supernatants were acidified with 1% of HNO_3 69% and stored at 4°C. The filters were weighted and put in drying oven at 80°C overnight, and successively weighted again. Biomass content in each culture was measured as dry weight and analysed for the content of zinc. Both filters and supernatants were subjected to acid digestion with 5 ml of HNO_3 69% and 2 ml of H_2O_2 30% in teflon vessel using a microwave oven. The program of mineralization was performed in three steps of 10, 10, and 5 min at 100, 300, and 600 W, respectively. After cooling, the solutions were completely transferred in a volumetric flask and made up to the final volume with ultra pure water to perform the analysis to ICP-MS. In the final solutions, Zn concentrations were measured at 213.8 lines (Sprocati et al., 2006). The capacity of consortium to accumulate zinc at the stationary phase was expressed as zinc uptake efficiency through the coefficient $Q = \text{mg Zn gdw}^{-1}$, calculated as the ratio between the total Zn and the bacterial biomass.

- **Microbial biomass carbon**

The size of the microbial biomass is much used to evaluate detrimental effects of heavy metals (Murrieta et al., 2006) and in this study it was quantified as microbial carbon (C_{mic}) according to the fumigation-extraction (FE) method (Vance et al., 1987). Six subsamples of moist soil (20 g) were weighed, three replicates (non-fumigated) was immediately extracted with 80 ml of 0.5 M K₂SO₄ (1:4 w:v) for 30 min by oscillating shaking at 200 rpm and filtered (Whatman no. 42); the remaining samples were fumigated for 24 h at 25 °C with ethanol-free CHCl₃ and then extracted as described above. The carbon was determined in the extracts after oxidation with 0.4 N K₂Cr₂O₇ and titration with (NH₄)₂SO₄FeSO₄ 0,0333 N. The microbial biomass content was calculated as follows:

$$C_{mic} = EC * k_{EC}, \quad (b)$$

where EC is the difference between organic C extracted from fumigated soils and organic C extracted from non-fumigated soils and $k_{EC} = 2.64$ (Vance et al., 1987; Martens, 1995).

4.5 Results and discussions

- **Heavy metals**

Total content, DTPA and NH_4NO_3 -extractable fractions

Increased heavy metal concentrations in the soil (mostly from anthropogenic activities) are considered to pose possibly serious hazards in the soil-plant-animal system. This has created a demand for an intensive research effort aimed at predicting the availability of heavy metals in the soil environment (Qian et., 1996). Heavy metals exist in the soil in immobile (sulphides, phosphates, silicates, etc.) and mobile forms and the determination of these latter is important for understanding their migration patterns in the soil and their uptake by plants and organisms. Soil extraction is the method of isolating functionally defined forms of metal. The notion “form of heavy metal” defines the function of matter in the soil, as, for example, ‘plant available form’, ‘exchangeable cations’ or ‘labile form’ (Sabienė and Brazauskienė, 2004). The content of the mobile forms of heavy metals depends on the nature of metal ion, the nature of extractant and pH (Sabienė and Brazauskienė, 2004). In the table 4.2, the different pools of Cu and Zn are reported. Additionally to the soil total content of Cu and Zn, in this study 1M NH_4NO_3 and 5 mM DTPA were used as extraction solutions to evaluate the mobilisable fractions available to soil microbial biomass. Fan had the highest values of total content of Cu (495 mg kg^{-1}). Although Tolfa B, Tolfa C and Riz soils were collected from the ex-mine dumps, they showed a minimum level of Cu contamination ($87, 92, 79 \text{ mg kg}^{-1}$, respectively). As regards the total content of Zn, Fan had the highest value (30700 mg kg^{-1}). Instead, a minimum level of Zn was in Metaponto (339 mg kg^{-1}). However, all soils from Ingurtosu had higher content of Zn than the other soils because it is the most diffuse heavy metal in this Italian mining dump.

Table 4.2 Soil fractions of Cu and Zn

Soil	Total Cu (mg kg ⁻¹)	DTPA-Cu (mg kg ⁻¹)	DTPA-Cu/ Total Cu (%)	NH ₄ NO ₃ -Cu (mg kg ⁻¹)	NH ₄ NO ₃ -Cu/ Total Cu (%)	Total Zn (mg kg ⁻¹)	DTPA-Zn (mg kg ⁻¹)	DTPA-Zn/ Total Zn (%)	NH ₄ NO ₃ -Zn (mg kg ⁻¹)	NH ₄ NO ₃ -Zn/ Total Zn (%)
Monghidoro11 (AC)	196	16	8.1	0.42	2.16	370	52	14.0	0.80	2.16
Monghidoro 12 (AC)	135	11	8.4	0.30	2.22	410	62	15.2	0.83	2.02
Tolfa B (NC)	87	5	5.3	0.16	1.80	745	43	5.8	2.64	3.54
Tolfa C (NC)	92	4	4.5	0.35	3.75	660	34	5.2	23.08	34.98
Rutigliano (AC)	239	99	41.4	0.24	0.99	473	217	45.9	0.55	1.16
Montepietroso (AC)	228	102	44.5	0.32	0.14	421	161	38.2	11.26	26.74
Metaponto (AC)	178	69	39.0	0.63	3.53	339	111	32.6	0.36	1.06
Gennamari (NC)	145	11	7.4	0.32	2.18	2750	601	21.9	44.75	16.27
Piezometro (NC)	270	6	2.3	0.55	2.02	13500	827	6.1	49.85	3.69
Riz (NC)	79	4	0.8	0.11	2.21	7950	1043	3.4	32.63	1.06
Fan (NC)	495	31	39.0	0.91	11.52	30700	1473	18.5	561.35	70.61
Umb (NC)	270	9	3.4	1.22	4.51	14050	1079	7.7	77.80	5.54
Mean	201	31	15	0.52	3.25	6031	475	18	67	9.96
CV (±%)	55	117	107	70	104	148	103	77	225	109

CV=mean coefficient of variation between replicate extractions (n=3); AC: artificially contaminated soil; NC: naturally contaminated soil

The DTPA-extractable Cu ranged from 4 mg kg⁻¹ (Riz) to 102 mg kg⁻¹ (Montepietroso) and the NH₄NO₃-extractable Cu ranged from 0.11 mg kg⁻¹ (Riz) to 1.22 mg kg⁻¹ (Umb). The DTPA extractability of the Cu with respect to the total content of Cu ranged from 0.8 % (Riz) to 44 % (Montepietroso). Instead the NH₄NO₃ extractability of Cu with respect to the total content of Cu was markedly lower than the DTPA extractability and ranged from 0.14 ‰ (Montepietroso) to 11 ‰ (Fan). Both extraction solutions showed a high coefficient of variation (107 % and 104 %, respectively). As reported by several authors, extraction with DTPA has been shown to affect the Cu solubility differently at differing pHs dependent on the amount of DTPA relative to Cu (Vulava et al., 1997; Reichman, 2002). Since the DTPA extraction solution was originally developed to identify micronutrient deficiencies in near neutral and calcareous soil, caution is required when deviating from the original design (O'Connor, 1988). Methods such as DTPA extraction are frequently criticized because they can modify the soil chemistry (McBride et al., 2003). Indeed, any procedure that separates the solution from the solid phase inevitably disrupts the physical–chemical equilibrium, which may affect the distribution of the metal species in solution (Zhang et al., 1998). As regards the Zn, the DTPA-extractable Zn ranged from 34 mg kg⁻¹ (Tolfa C) to 1473 mg kg⁻¹ (Fan) and the NH₄NO₃-extractable Zn ranged from 0.36 mg kg⁻¹ (Metaponto) to 561 mg kg⁻¹ (Fan). The DTPA extractability of the Zn with respect to the total content of Zn ranged from 3.4 % (Riz) to 46 % (Rutigliano). As well as Cu, the NH₄NO₃ extractability of Zn was markedly lower than the DTPA extractability and ranged from 1.06 ‰ (Riz) to 70 ‰ (Fan). Both extractant solutions as for the Cu had a coefficient of variation very high (77 % and 109 %, respectively). The results showed also that the fractions of NH₄NO₃ and DTPA-extractable Zn were higher than those of Cu, suggesting that Zn was more mobile than Cu. As reported Peijnenburg and Jager (2003), Cu is more strongly bound to dissolved organic carbon (DOC) than zinc, and hence copper is in general less available for uptake by organisms. Among the twelve soils, it is very interesting to analyze the Fan soil. Fan resulted the most polluted soil, presenting the highest total content of Zn and Cu, it had also the highest values of the DTPA and NH₄NO₃-extractable Cu and Zn. So, a higher environmental risk in the Fan soil occurred for a higher availability of heavy metals, probably favored by its chemical characteristics. Indeed, the Fan soil had a low pH (6.5) and was constituted by the mud of flotation, very rich in waste products of mine dump. In environmental studies, the determination of these forms (DTPA and NH₄NO₃-extractable) gives more information on trace metal mobility, as well as on their availability and toxicity, in comparison to the total element content.

In soil science, it is a usual approach to use selective chemical extraction for understanding the chemistry of heavy metals in their interaction with other soil components such as clay minerals, organic matter and soil solution, or to assess their mobility and retention as well as their availability to plants (Ure, 1996). For this study 1 M NH_4NO_3 and 5 mM DTPA extraction solutions were chosen because generally they are used as suitable methods for the prediction of readily soluble and plant available trace element contents. However it should be considered, that these methods are not equally suitable for all elements (Gryschko et al., 2004). DTPA (diethylene triamine pentaacetic acid) is a chelating agent first proposed by Lindsay and Norvell (1967), and has received extensive validation (ten published papers referenced in Lindsay and Norvell, 1978) as being a good indicator of plant-available Zn, Fe, Mn, and Cu. The DTPA soil test has potential relevance to the ecological direct contact exposure pathway, since it attempts to extract the labile fraction of various trace element cations in soil, and the results have been correlated to the fraction that is bioavailable to plants. It has been widely used due to its ability to form very stable, water-soluble and well-defined complexes with metal cations, to determine the soil solution plus the ability of the soil to replenish metals, to release the mobilisable forms (complexed, adsorbed and carbonate forms) (Lindsay and Norvell, 1978; Haq et al., 1980; Norvell, 1984; Brun et al., 2001; Reichman, 2002). Chelating extractant tests were developed for soils with low organic matter (Lindsay and Norvell, 1978) and have not been correlated for soils high in organics. Moreover, the chelator extraction methodology requires buffering at near neutral pHs (Lindsay and Norvell, 1978; Clayton and Tiller, 1979). Such buffering would change metal-soil interactions as a result of the marked effect of pH on metal solubility (Carter, 1993). So, the applicability of the method at more acidic pH values may be limited due to the potential for the DTPA to become saturated by calcium freed from the dissolution of calcium carbonate and bicarbonate. Detailed chemical speciation modelling would likely be required before attempting to adapt the parameters of the extracting solution (Carter, 1993; Axiom Environmental, 2009). Concerning the soil extraction of heavy metals with 1 M NH_4NO_3 -solution, the German DIN 19730 (1997) describes this method for the extraction of readily available trace elements from soil. Further chemical mechanisms involved when soils are extracted with 1 M NH_4NO_3 are a moderate decrease in pH and an increase in ionic strength. Most of the colloids and parts of soluble metal organic complexes are precipitated due to the high ionic strength. High ionic strength also decreases the activity of metal- OH^+ species and the electrostatic potential of the particle surfaces, which in turn, increases desorption of heavy metal cations from negatively charged soil surfaces. The chemical soil extraction process may cause misleading predictions of the transfer of trace elements to plants for some soil properties. This knowledge should be used to

improve risk assessment of soil contaminations (Gryschko et al., 2004). The variety of extraction procedures used in environmental studies makes it very difficult to compare the results obtained. Therefore, harmonization and standardization is required. In this study both the extraction solutions showed very high coefficient of variation. As reported by several authors (Dube et al., 2000; Chaignon et al., 2003), the chemical properties of soils such as pH, texture, CSC are very important factors, which influence the bioavailability and mobility of heavy metals.

CHCl₃-labile zinc and copper

Soil microorganisms not only store organic C, N, P, and S components in their biomass, but also macronutrients and heavy metals. Mobile heavy metals are not only taken up by plants but also by microorganisms. A large part of the metals is adsorbed by cell-wall components, but a significant part is also transferred into microbial cells (Gadd, 2004; Khan et al., 2009). After soil fumigation with chloroform, it may be possible to extract these as CHCl₃-labile metals of microbial origin with extraction solutions. For this study, 1 M NH₄NO₃ and 5 mM DTPA were tested as extraction solutions. Data for comparison do not exist for soil microorganisms, so that the present data can only be indirectly tested for plausibility (Khan et al., 2009). Unknown is the exact location of CHCl₃-labile metals within the microbial cells. Even if it is generally assumed that more cytoplasmatic than cell-wall material is rendered extractable after 24 hours of autolysis with the fumigation, the exact percentages are unknown (Joergensen and Mueller, 1996). However large parts of mobile metals are not transferred into the cell but adsorbed by the cell walls, precipitated as oxalates or sequestered by exopolysaccharides and other extracellular metabolites (Baldrian, 2003). Unknown is also the contribution of the cell-wall bound metals to CHCl₃-labile fraction. As regards the DTPA extraction, the results of the present study showed it was possible to quantify the CHCl₃-labile Cu and Zn just for three soils (Fig. 4.3) and so this extraction solution did not seem to be a good solution for this test. The DTPA is a test developed for soils with low organic matter and for near neutral and calcareous soils (Lindsay and Norvell, 1978). Therefore, the chemical properties of the soils under studies could have influenced negatively the extraction of the CHCl₃-labile Cu and Zn with DTPA solution.

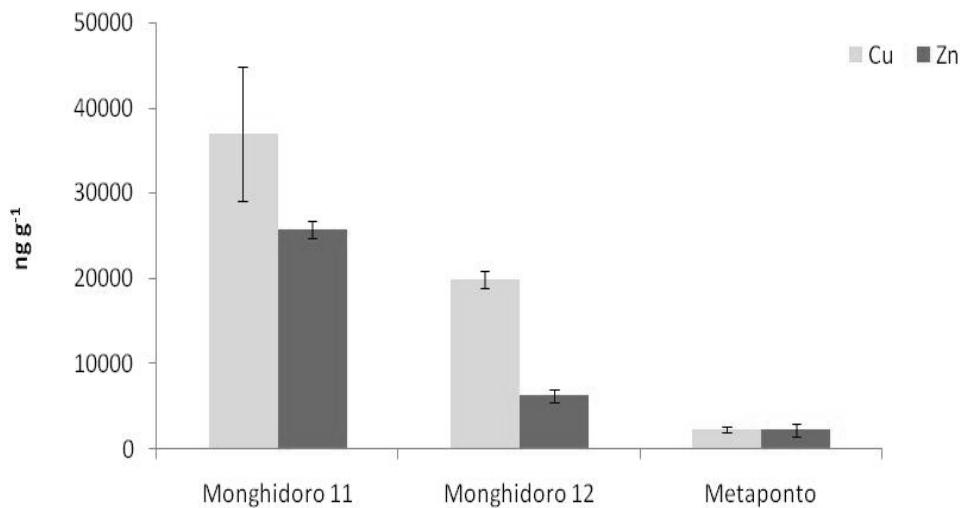


Figure 4.3 CHCl₃-labile Cu and Zn (DTPA extraction)

Moreover, the CHCl₃-labile zinc measured using 1 M NH₄NO₃ (Fig.4.4) was not detectable. The lack of values and their high variability could be caused by the large background concentration of Zn in extracts from the non-fumigated soils.

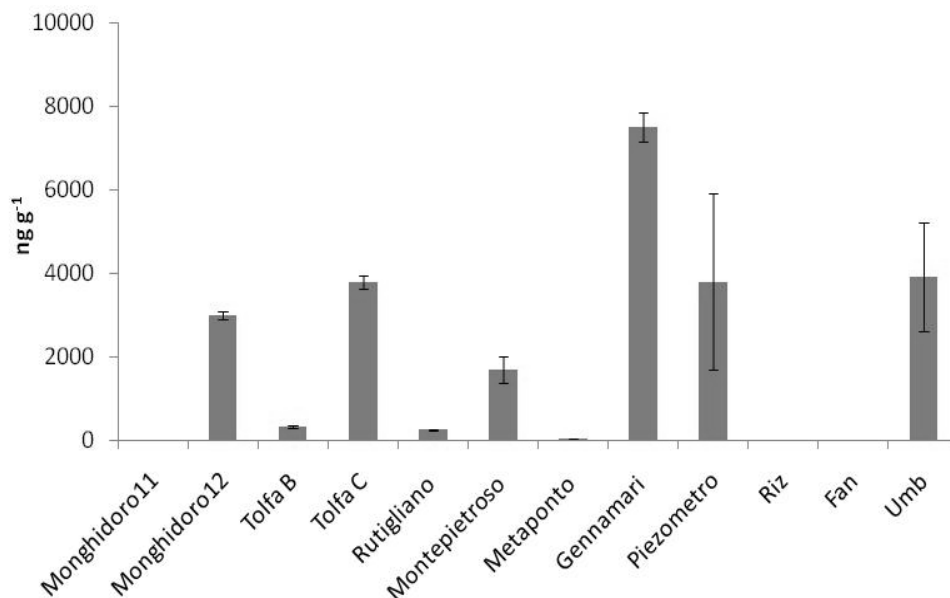


Figure 4.4 CHCl₃-labile Zn (NH₄NO₃ extraction)

Conversely to Zn, all soils under study showed a certain fraction of CHCl_3 -labile Cu additionally extractable with 1 M NH_4NO_3 after fumigation even if with high variation among twelve soils (Fig. 4.5). The highest value of CHCl_3 -labile Cu was registered in Metaponto (1035 ng g^{-1}) with a high content of clay and Monghidoro 11 (860 ng g^{-1}), with a high content of soil organic matter. The lowest fraction of bioaccumulated Cu was in Montepietroso (75 ng g^{-1}).

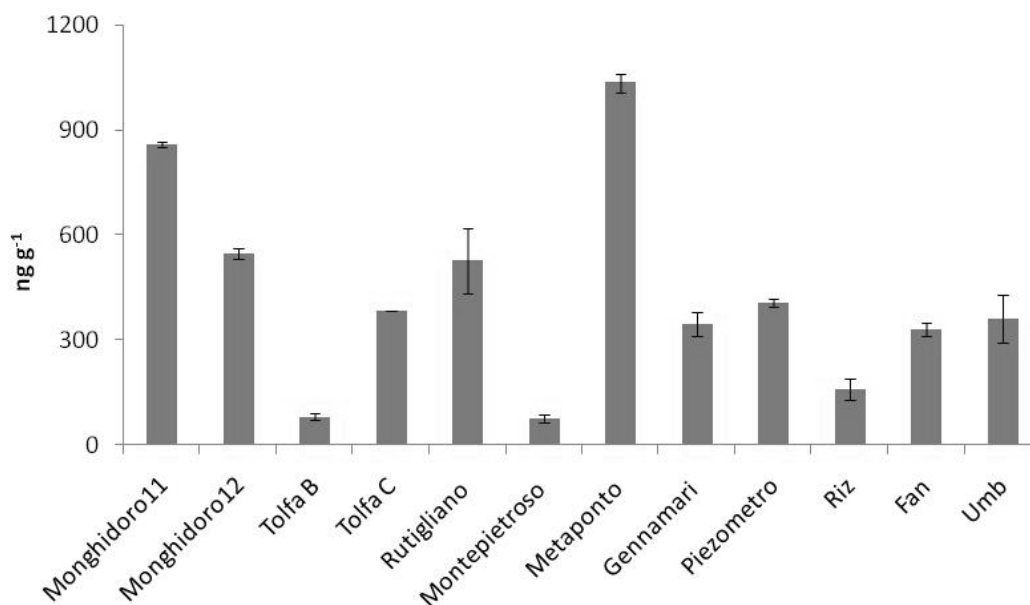


Figure 4.5 CHCl_3 -labile Cu (NH_4NO_3 extraction)

The mean contribution of CHCl_3 -labile Cu in relation to the NH_4NO_3 -extractable fraction from non-fumigated soils ranged from 15% (Riz) to 239% (Montepietroso) (Fig. 4.6). Similar range and mean value were reported by Khan et al. (2009). However, no correlation was found between the CHCl_3 -labile Cu and the Cu pools extracted with NH_4NO_3 , indicating that the microbial uptake is not controlled by the availability of this elements but probably by the microbial demand for micronutrients.

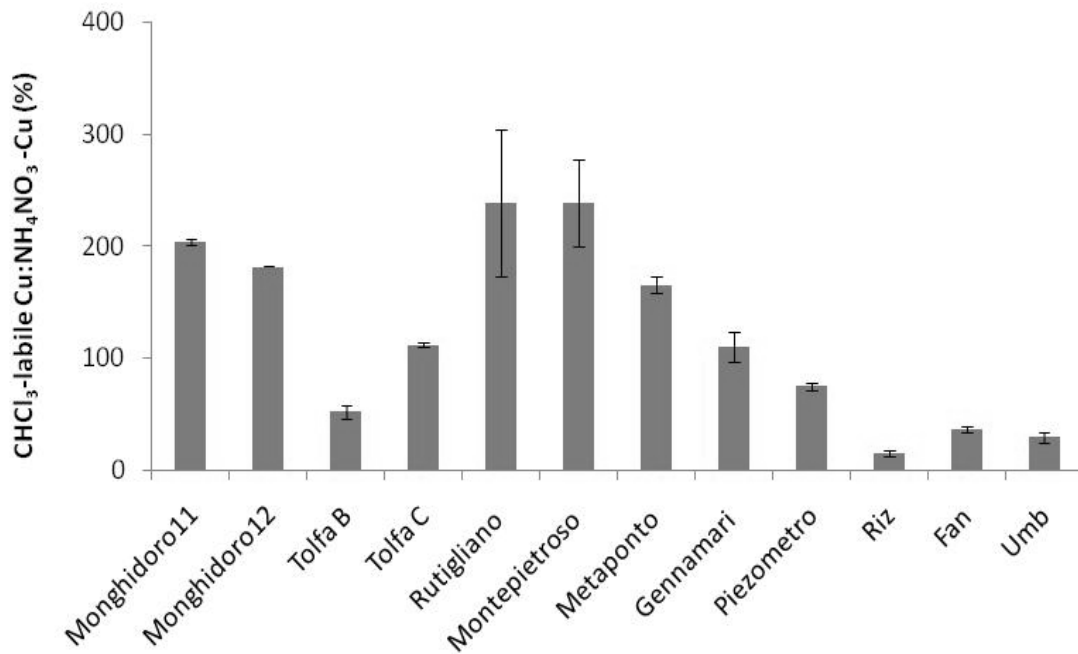


Figure 4.6 Contents of CHCl₃-labile Cu in % of the NH₄NO₃-extractable fraction from non fumigated soil samples

As regards the ratio CHCl₃-labile Cu to microbial biomass C ranged from 0.16 ‰ (Tolfa B) to 7.40 ‰ (Rutigliano) (Fig. 4.7). The large ratio indicates a high bioaccumulation of HM into microbial biomass. The mean of CHCl₃-labile Cu to microbial biomass C (2.64 ‰) were slightly lower than the mean one (3.9‰) reported by Khan et al. (2009). However, not significant positive correlations between microbial biomass C and CHCl₃-labile Cu were observed. In the present set of soils, up to 7.402 ng Cu g⁻¹ microbial biomass C (Rutigliano) was measured as CHCl₃-labile copper. It has to be noted that the range of values known from the limited literature is just on fungi grown on heavy metal contaminated soils. Wallander et al. (2003) measured a mean of 100 µg Cu g⁻¹ dry weight in fungal mycelia. In fungal fruit bodies, copper concentration was measured between 5 and 450 µg g⁻¹ dry weight (Tyler, 1980; Gadd, 2007).

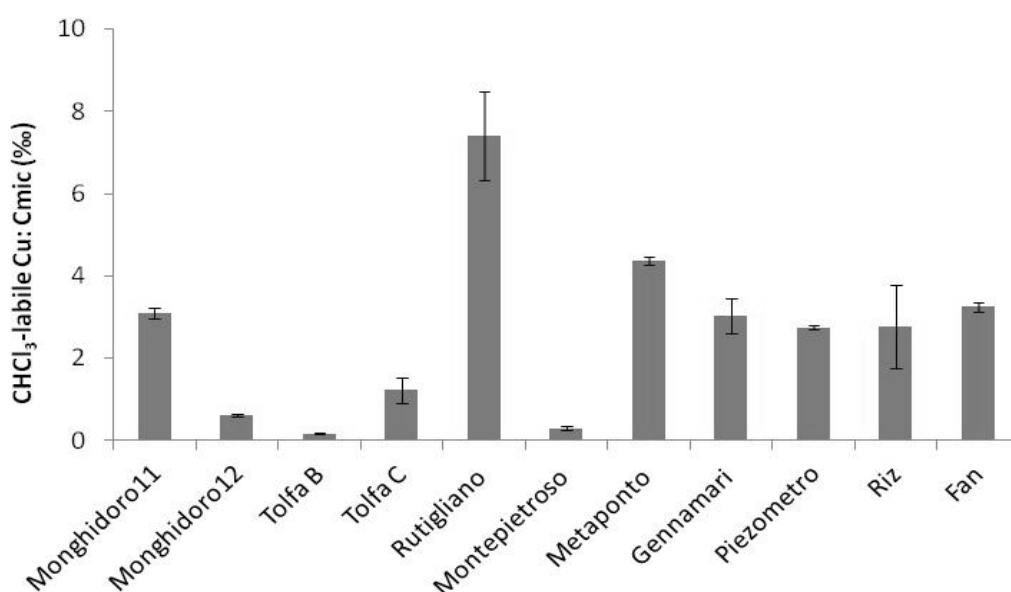


Figure 4.7 The CHCl₃-labile Cu in % of soil microbial carbon (Cmic)

The soil chemical properties were correlated with the different pools of Cu (Table 4.3). In particular, the organic carbon (Corg) and the cation exchangeable capacity (CEC) have a significant effect on the total content of Cu. The physical properties of soils such as soil texture have affected the ratio of DTPA-extractable Cu to total Cu content as well as the ratio of CHCl₃-labile form to NH₄NO₃-extractable Cu. In fact positive correlation coefficient was found with clays and silt. The soil texture in this study seems to have a greater effect on the Cu pools than the pH.

Table 4.3 The correlation between the different soil characteristics with the fractions of Cu (Total, DTPA-extractable and CHCl₃-labile Cu)

	<i>Cmic</i>	<i>Cu Tot</i>	<i>DTPA-Cu^a</i>	<i>CHCl₃-labile Cu^b</i>
Corg	0.91***	0.66**	n.s.	n.s.
pH	n.s.	n.s.	n.s.	n.s.
CEC	0.92***	0.55*	n.s.	n.s.
Clay	n.s.	n.s.	0.82***	0.611**
Silt	n.s.	n.s.	n.s.	0.77***
Sandy	n.s.	n.s.	-0.92***	-0.73***

* The significance probability levels of the results are given at the $P < 0.1$ (*); $P < 0.05$ (**) and $P < 0.01$ (***) respectively; ^a % DTPA-Cu to total Cu; ^b % CHCl₃-labile Cu to NH₄NO₃-Cu

It is apparent that more refined interpretation of soil properties is required to further characterize heavy metals. As reported from several authors, from the particle-size fractions - sand, silt, and clay – the finer particles show higher concentration of heavy metals due to increased surface areas, higher clay minerals and organic matter content, and the presence of Fe-Mn oxide phases (Fiirstner, 1980; Haque and Subramanian, 1982). However, most frequently metal availability from the different size fractions of soil has been neglected. The distribution of heavy metals with particle size is primarily a function of mineral composition and amount of adsorption sites in each size fraction. The accumulation of metals in the clay fraction was in agreement with the findings reported by several works, which were attributed to the high surface area and the presence of clay minerals, organic matter, Fe-Mn oxides and sulphides (Fiirstner, 1980; Haque and Subramanian, 1982). In addition, increasing metal concentrations with decreasing particle sizes indicated that in this size range, the behavior of the metals was governed by sorption processes. In general, concentration maxima in the clay fractions gave an indication that most of the metals studied were probably present in an adsorbed form on clay minerals or other clay materials, present in the crystal lattice of clay minerals or in the structure of heavy minerals (Qian et al., 1996).

Soil mobile fraction of heavy metals

Risks associated to heavy metals soil contamination depend not only on their total content but, mostly, on their mobility and, consequently, on bioavailability (Alloway, 2005). The knowledge of trace elements mobility in contaminated soils is becoming an important requisite for any further environmental evaluation (Pinto et al., 2010). A simple screening method based on a single step leaching test was applied to three soils (Fan, Riz and Umb), analyzed also through the fumigation extraction method, to evaluate the mobility of Cu, Pb, Zn and Cd. The three soils were characterized by different total content of the four metals considered (Table 4.4). Fan soil showed a markedly higher total content of these heavy metals than the other two soils. Instead, Riz had the lowest ones.

Table 4.4 The total content of Cu, Pb, Zn and Cd in the three analyzed soils

Soil/Metal	Cu mg kg⁻¹	Pb mg kg⁻¹	Zn mg kg⁻¹	Cd mg kg⁻¹
Riz	79	1350	7950	54
Fan	495	5910	30700	95
Umb	270	1910	14050	78

The leaching test was applied to these three soils and it was performed for 5 and 60 minutes since it seemed to be interesting to observe metal leacheability at the beginning and at pseudo-equilibrium. As it can be observed in Table 4.5, the metals in the samples showed a relative order of leacheability in which the Fan sample had the highest fraction of “mobile” metals and Riz sample showed the lowest values after 5 and 60 minutes.

Table 4.5 Results of the leaching test performed on the soil samples. The extracted metals M⁺⁺ (sum of Zn, Cu, Pb and Cd) are expressed as mmol kg⁻¹

Soil/Time of reaction	mmol kg⁻¹ extract		% extract to total	
	5'	60'	5'	60'
Riz	21	25	16	19
Fan	86	172	17	34
Umb	34	61	15	27

The kinetical approach indicated that it can be possible to have a useful preliminary evaluation of HM mobility in almost 60 min. The proposed screening method gives reliable and rapid information on the amount of the metals of toxicological interest that can be in natural conditions mobilised from the soil generating risks for the environment. The Fan soil showed the highest total content and also the highest fractions of heavy metals extracted during the kinetical curve. Then, the highest mobility of these four heavy metals in the Fan soil occurred (Fig. 4.9).

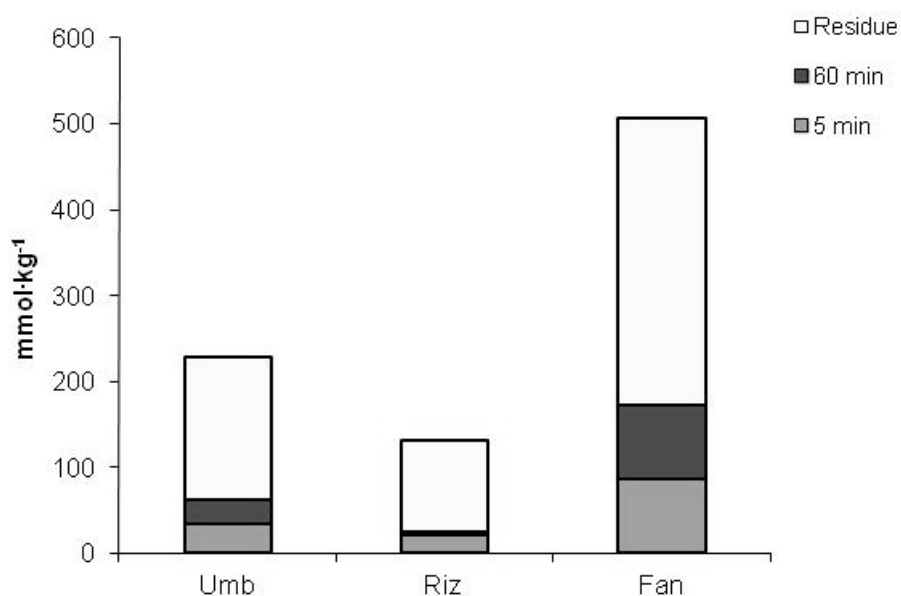


Figure 4.9 Results of the leaching test performed on the soil samples. The extracted metals M^{++} (sum of Zn, Cu, Pb and Cd) are expressed as mmol kg^{-1}

Nevertheless, it is necessary to remind that the higher total content does not correspond necessarily to the higher mobility of heavy metals as reported by several authors (Singh, 1997; Kabala and Singh, 2001; Alloway, 2005). Knowledge of the total contents of heavy metals present in soil horizons provides limited information about their potential behavior and bioavailability. Heavy metals are associated with various soil components in different ways, and these associations determine their mobility and availability (Kabata-Pendias and Pendias, 1992). Indeed, the evaluation of heavy metals mobility in soils should be correlated with the soil chemical and physical characteristics.

In this case, an important difference between Fan and Riz was the pH (6.5 and 8.3, respectively), which could have determined the highest mobility of heavy metals in Fan. Normally, the effect of pH dominates because it has a major influence on most of the chemical species (especially DOM and carbonate), but it is also important to make the distinction among the various chemical species present in solution. Moreover, the importance of pH on metal solubility is well recognized but difficult to segregate from the influence of other soil characteristics that are often inter-correlated (Sauvé et al., 2000).

Zinc accumulated in bacterial biomass

Since microorganisms have a generic character for survival strategies in heavy metal polluted habitats, a consortium of growing metal-resistant cells could ensure better removal through a combination of biosorption and continuous metabolic uptake of metals during a bioremediation process (Khan, 2005). However, sensitivity of living cells to extremes of pH or high metal concentration and need to furnish metabolic energy are some of the major constraints of employing growing cells for bioremediation (Malik, 2001). In this study, the growth of bacterial consortium was tested following the growth curves performed either in the presence or in the absence of zinc. The growth curve, reported in Fig.4.10, shows the inhibiting effect of the zinc on the development of the consortium. The presence of Zn reduced by 80% the growth of bacteria.

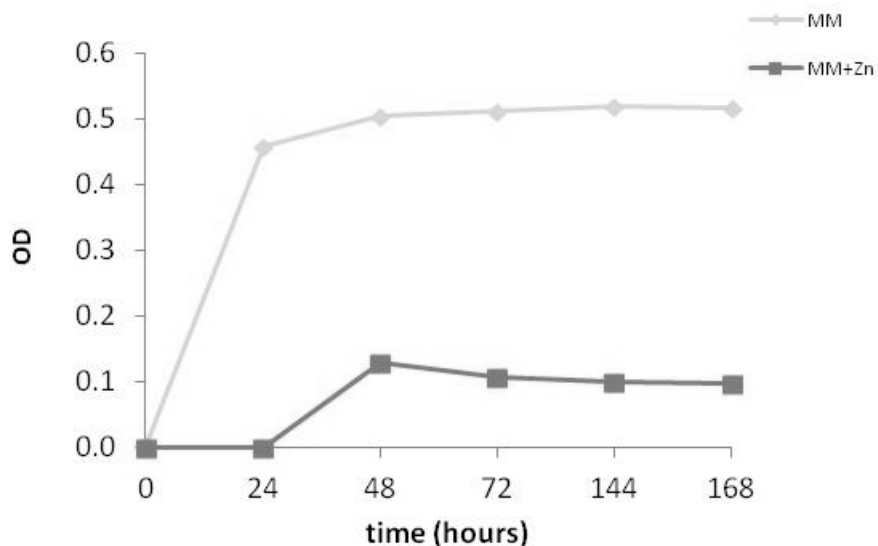


Figure 4.10 The growth curve of the consortium in presence and in absence of the 5mM ZnSO₄

The phenomenon of microbial resistance to heavy metal toxicity allows no simple explanation. This is undoubtedly caused by the multiplicity of interactions that can occur among microbial cells, heavy metal ions, and other environmental constituents. Even though microorganisms have specific uptake systems, high concentrations of nonessential metals may be transported into the cell by a constitutively expressed unspecific system. This “open gate” is the one reason why metal ions are toxic to microorganisms (Nies, 1999). From the plates in St1, it was possible to monitor the resistance of the single strains. It seemed that just *Pseudomonas sp.* (Riz 7) was able to survive in presence of Zn. On the other hand, the strain *Bacillus cereus* (Fan 5) from the MM without Zn, dominated during the growth of the consortium on the plates St1. So, it is probable that the bacteriostasis among the four strains occurred. More research is necessary to explain this phenomenon that is probably related to the growth medium, its components and its carbon source. It is indispensable also to deepen if the *Pseudomonas sp.* could be able to produce some molecules, which could slow down the growth of the other strains. Indeed, an early antibiosis test realized using the St1 plates, didn't show these inhibitions between the four strains. So, probably the MM exerted a selection on the bacteria, favouring the most suitable one for these nutrients. Some laboratory experiments in which resistant microbial strains have been isolated and identified must be open to question, especially when the isolation or maintenance medium contains peptones, yeast extracts, or certain buffer solutions known to react chemically with heavy metals. It is not unlikely that under conditions such as these, resistance is only apparent and may be merely an indirect result of the interference with the availability of other nutrients to "metal-sensitive" cells (Gadd et al., 1978). In this regard, MM was chosen to avoid this problem, containing nutrients that minimize the interactions with heavy metals (Sprocati et al., 2006). The capacity of bacterial biomass to accumulate zinc was expressed as zinc uptake efficiency through the coefficient Q measured at the stationary phase of the consortium growth (Table 4.6). The value of Q calculated for the viable cells (V) in MM + 5mM ZnSO₄ was 6.10 mg Zn gdw⁻¹ and for the non viable proliferating cells (VNP) in TRIS 0.1 M + 5 mM ZnSO₄ was 31.84 mg Zn gdw⁻¹.

Table 4.6 “Q the accumulation coefficient of zinc”

Microbial cells	Q= Total Zn (filter)/ microbial biomass (mg Zn gdw⁻¹)
V	6.10
VNP	31.84

The Q of viable cells was markedly lower than the Q of viable not proliferating cells, probably due to the negative effects of Zn which reduced the bacterial growth. The increase of this coefficient Q for VNP suggests the involvement of a metabolism-independent mechanism in the metal uptake. So, it is probable that instead active bioaccumulation, a passive mechanism of Zinc biosorption on binding sites located on the outer of cells surfaces occurred. Zinc is a trace element that seems to be necessary for every form of life since it is found in proteins and enzymes of nucleic acid metabolism, in many other enzymes, and in some ribosomal proteins. However, high concentrations of zinc are toxic as zinc can interact with thiols and block essential reactions in the cell. Cells can achieve the delicate balance between the requirement for metals and their toxicity in several ways: storage mechanisms that safely deposit loosely bound metals for later use, export systems to rid the cells of surplus metals, and high- and low-affinity uptake systems. Only recently, with the availability of molecular genetic methods, have further details of bacterial Zn^{2+} transport and Zn^{2+} homeostasis been revealed. The zinc concentration in the cell is tightly regulated since zinc-binding sites are in excess. Nevertheless, the binding of zinc to inappropriate sites is harmful to the cell. How the metal-binding specificity of proteins is determined is not understood. (Hantke, 2001, 2005). Specific transporters active under Zn^{2+} -replete conditions have not been identified. Only a few unspecific systems are known to transport Zn^{2+} among other divalent cations. Although the outer membrane has many binding sites for divalent cations, little is known how Zn^{2+} passes through this membrane. Gram-positive bacteria, which do not have an outer membrane, have many binding sites for divalent cations in the teichoic acids, the polymeric ribitol phosphates bound to the thick peptidoglycan layer. These systems seem to act as cation exchangers on the surface of bacteria (Hantke, 2001, 2005). However, many environmental factors, pH, ionic strength, temperature, and the presence of other metals and organic compounds influence the chemical reactivity of the binding sites on bacterial cell surfaces and the subsequent biosorption of metals, which could represent a fast and reversible process for removing toxic metal ions from soil solution (Violante et al., 2010).

4.6 Conclusions

The aim of the present study was to investigate the mobility of heavy metals (HM) and the accumulation of these inorganic pollutants into soil microbial pool. The three methods applied to evaluate the heavy metals mobility and bioaccumulation (the fumigation extraction method, the leaching test and the metal uptake test) showed several interesting results. We can conclude that the CHCl_3 -labile heavy metal, determined according to the fumigation-extraction method, may represent a new approach to give information on the heavy metals availability and toxicity for soil microbial biomass. The results showed that the fumigation extraction method is more suitable for Cu than for Zn detection, as well as the NH_4NO_3 extraction showed less variability in comparison to DTPA one. The chemical, physical and biochemical characteristics of soils, in particular the organic matter content and the soil texture are important elements to be considered in this study. So, CHCl_3 fumigation and NH_4NO_3 extraction in combination with ICP-AES can be successfully used to determine heavy metals stored in the microbial biomass but further research is needed to improve and verify this methodology. The leaching test can provide a useful preliminary evaluation of HM mobility in almost 60 min and the use of toxic extractants and solvents is avoided. So, this screening method could be used for in-field tests, and it could be very useful, both to guide the sampling activity on site and to monitor the efficacy of the subsequent remediation action. The leaching test together with the fumigation extraction method applied to three soils under study, provided more detailed information about the mobility and the bioavailability of heavy metals. The metal uptake test was an explorative trial for evaluating the capacity of Zn bioaccumulation and biosorption of the bacterial consortium used for the pot experiment. From these results, it was not possible to elaborate a precise conclusion. Certainly, it is evident the behaviour of the consortium in the presence and in absence of Zn and the interactions among the strains. It is more complicated to express a reflection about the bioaccumulation and the biosorption. However, the results showed that the bacterial cells of the consortium should interact with Zn with a passive mechanism of biosorption rather than with an active process of bioaccumulation. These two microbial mechanisms occurred but their entity is to be verified and a more adequate and exhaustive research about the nutritional requirements of the single strains should be carried out. In conclusion, it is evident as it could be carried out an interdisciplinary research to investigate the mobility and bioavailability of heavy metals.

Different scientific disciplines should interact to create a common database with reference values for the environmental risk analysis. Further studies should lead to a better understanding of the soil chemistry of heavy metals and to the development of improved techniques for measuring metal availability in soil. Chemical, biochemical and biological parameters strongly influence the speciation of heavy metals, their availability to biological systems and, consequently, the possibilities to use bioremediation as a cleanup tool for heavy metal polluted sites.

Chapter 5- Changes of soil quality in a phytoremediation system of a former dump

5.1 Abstract

Phytoremediation could represent an unconventional and sustainable solution to restore a good level of soil quality in polluted areas, such as abandoned dumps. This study aimed to evaluate the effects of a phytoremediation system on soil quality of a heavy metals (HM) contaminated area used as a former illegal dump. The phytoremediation system was based on the combination of three Mediterranean species (*Populus nigra var. italica* L., *Paulownia tomentosa* (Thunb) Sieb. & Zucc. ex Steud and *Cytisus scoparius* L.). In order to achieve this aim, soil chemical and biochemical properties were monitored during two years of the field experiment. A second objective of this study was to quantify the CHCl_3 -labile metals through the fumigation-extraction method using 1 M NH_4NO_3 as extracting solution to evaluate the HM accumulation into microbial biomass pool. The results of soil analysis obtained in the first two years of field experiment (2008-2010) showed that the system was able to reduce the total content of soil HM. However, while the microbial biomass content per unit of soil total organic carbon ($\text{C}_{\text{mic}}:\text{C}_{\text{org}}$) in 2010 was lower than in 2008, a general increase of microbial metabolism was observed in terms of specific respiration (qCO_2) and hydrolytic enzyme activity. Moreover, the increase of the DTPA-extractable HM suggested a higher HM mobility which caused probably their accumulation into microbial biomass, as it was revealed by the increase of CHCl_3 -labile Cu. These results suggested a positive effect of plant by root exudates on soil HM availability, which lead to an eco-physiological stress of microbial biomass. In conclusion, CHCl_3 -labile form of metals could represent a new approach to assess HM availability to microbial biomass. In this case of study, the phytoremediation system caused a significant reduction of soil pollution but the increase of HM availability determined an eco-physiological stress of microbial biomass at least in the medium-term period.

Keywords: *phytoremediation; heavy metals; soil quality; soil microbial biomass; microbial functional diversity*

5.2 Introduction

Soil is precious natural resource on which rely the sustainability of agriculture and the civilization of mankind. Unfortunately, it is subjected to maximum exploitation and severely degraded or polluted due to anthropogenic activities (Lone et al., 2008). Each source of contamination has its own damaging effects to plants, animals and ultimately to human health, but those that add heavy metals to soils are of serious concern due to their persistence in the environment and carcinogenicity to human beings. They cannot be destroyed biologically but are only transformed from one oxidation state or organic complex to another (Garbisu and Alkorta, 2001). Therefore, heavy metal pollution poses a great potential threat to the environment and human health. In order to maintain good quality of soils and keep them free from contamination, continuous efforts have been made to develop technologies that are easy to use, sustainable and economically feasible, but metal-contaminated soils are notoriously hard to remediate. Physicochemical approaches (soil excavation and either landfilling or soil washing followed by physical or chemical separation of the contaminants) have been widely used for remedying polluted soil, especially at a small scale. However, they experience more difficulties for a large scale of remediation because of high costs and negative effects on soil functions and structure (Alkorta et al., 2004). The use of plant species for cleaning polluted soils named as phytoremediation has gained increasing attention, as an emerging cheaper technology. Due to its great potential as a viable alternative to traditional contaminated land remediation methods, phytoremediation is currently an exciting area of active research. The phytoremediation is based on the capability of particular vegetable species, to remove, to degrade and to make less dangerous the pollutants (Cunningham et al., 1993). Phytoremediation is reported to be an effective, non-intrusive, inexpensive, aesthetically pleasing, socially accepted technology to remediate polluted soils (Alkorta & Garbisu 2001; Weber et al. 2001; Garbisu et al. 2002) and so it is widely viewed as the ecologically responsible alternative to the environmentally destructive physico-chemical remediation methods currently practiced (Meagher, 2000). In order to improve phytoremediation of heavy metal polluted sites, several important points relevant to the process have to be elucidated. These include the bioavailability of the heavy metals in soil, which should be considered as the most important factor from ecological, toxicological and health standpoints (Schwitzguebel et al., 2002). A need exists for rapid, cost-effective systems that reliably predict this parameter. Many studies have been conducted in field in the last two decades. Numerous plant species have been identified and tested for their traits in the uptake and accumulation of different heavy metals. Mechanisms of metal uptake at whole plant and cellular levels have been investigated.

Progresses have been made in the mechanistic and practical application aspects of phytoremediation (Lone et al., 2008). Nevertheless, more demonstration projects are needed to prove that phytoremediation is effective in order to rigorously measure its underlying economics, and to expand its application. More fundamental research is also required to better understand the complex interactions between pollutants, soil, plant roots and microorganisms at the rhizosphere level, to control the bioavailability of pollutants, to fully exploit the metabolic diversity of plants and, thus, to successfully implement this new green technology (Schwitzguebel et al., 2002).

5.3 The aim of the study

The aim of this study was to evaluate the medium-term effects of a phytoremediation system on soil quality restoration in heavy metals (HM) contaminated area used as a former illegal dump. Soil biochemical properties, such as microbial biomass content, its activity and functional diversity, were used as bioindicators in order to evaluate soil quality improvement after two years of phytoremediation. A second objective of this study was to quantify the CHCl_3 -labile metals through the fumigation-extraction method using 1 M NH_4NO_3 as extracting solution to evaluate the HM accumulation into microbial biomass pool.

5.4 Materials and methods

- ***Experimental field and soil sampling***

The phytoremediation system was established in a heavy metals contaminated area, located in Madonna dell'Acqua, Municipality of San Giuliano Terme - Pisa (Italy). In the last decade this area, a level land extended to 10.000 m² (100x100), was used as illegal dump for different kinds of waste such as plastic, electric and iron material, oil and fuel. Before the phytoremediation, in October 2007 the soil was removed until the clay basement, the wastes were removed with sieve and then the soil was replaced *in situ*. In March 2008 the selected plants: Lombardy poplar (*Populus nigra* var. *Italica* L.), Scotch broom (*Cytisus scoparius* L.) and Princesstree (*Paulownia tomentosa* Thunb Sieb. & Zucc. Ex Steud) were bedded out with a planting layout of arboreous species (2 x 2 m) with the interposed shrubby species (1 x 1 m). Twelve soil samples were collected from the whole area at depth 0-30 cm before planting tree/shrubby species and after two years (February 2008 and 2010). Then, the following analyses were performed on soil samples: HM content, microbial biomass content, microbial activity and functional diversity.

- **Heavy metals**

Total content

The determination of the total content of heavy metals in soils is particularly useful to collect information on the genesis of the soil and is widely used to assess soil pollution (Sheppard et al., 1992). However, it does not give valuable information on the ability of the elements to be absorbed by plants, nor does it predict the transfer of toxic elements in the food chain that is the total content of heavy metals is not suitable to quantify their bioavailability (Morel, 1997). Nevertheless, the total content of HM defines the extent of metal build up or contamination in soil and an estimate of the portion of total cation exchange capacity saturated by metals. In this study, the soil (1g oven-dry soil) was mixed with 5 ml of nitric acid (HNO_3) and 2 ml of perchloric acid (HClO_4) and then put in a Block digester with the following cycle of time and temperature: 2 hours at 90 °C, 2 hours at 140 °C and 1 hour at 190 °C. Then, the extracts were made to volume (50 ml) with deionised water, filtered and analyzed by the Inductively Coupled Plasma Atomic Emission Spectrometry (LIBERTY AX – Sequential ICP-AES – Varian) (AOAC, 1990).

DTPA and NH_4NO_3 -extractable fractions

A complexing agent, DTPA (diethylene triamine pentaacetic acid) is used to release the mobilisable forms of heavy metals (complexed, adsorbed and carbonate forms) (Maiz et al., 1997). This fraction is useful for studies of metal mobility, soil-plant transfers and study of physic-chemical processes of HM (Gleyzes et al., 2002). Extraction of soil with 1 M NH_4NO_3 -solution is used to determine readily soluble trace element contents. In Germany this method has been published as an official standard (DIN 197301997) and is used to estimate the transfer of heavy metals and arsenic from soils to plants (Gryschko et al., 2004). For determining these fraction, 15 g (oven-dry soil) was extracted with 0.005 M DTPA or 1M NH_4NO_3 in ratio 1:2.5 (w:v) for 60 min by oscillating shaking at 200 rpm and filtered (Whatman no. 42). The DTPA and NH_4NO_3 soil extracts were acidified to avoid the metal precipitation with 0.5 ml of HNO_3 69%. Then, they were analyzed by the Inductively Coupled Plasma Atomic Emission Spectrometry (LIBERTY AX – Sequential ICP-AES – Varian) (AOAC, 1990).

CHCl₃-labile copper

Mobile heavy metals are not only taken up by plants but also by microorganisms. Soil microorganisms not only store organic C, N, P, and S components in their biomass, but also macronutrients and heavy metals.

A large part of the metals is adsorbed by cell-wall components, but a significant part is also transferred into microbial cells (Gadd, 2004). However, data on the storage of heavy metals in soil microorganisms are currently rare, although they would give important information on the effects of heavy metal toxicity on the microbial turnover (Khan et al., 2009). Through the fumigation-extraction (FE) method, it is possible to quantify the called CHCl₃-labile metals which could represent the heavy metals accumulated into soil microbial biomass. After fumigation of soil with chloroform (CHCl₃), it may be possible to extract the HM as CHCl₃-labile metals of microbial origin with 1 M NH₄NO₃ (Khan et al., 2009). Briefly, the basic principle of the FE is that soil microorganisms die after their cell membranes are attacked by CHCl₃, and a part of the microbial constituents, especially in the cytoplasm, is degraded by enzymatic autolysis and transformed into extractable components and so also the metals contained inside the microbial cells (for more details see chapter 4). In this study, the accumulation of Cu into soil microbial pool was evaluated through the fumigation extraction method for quantifying the CHCl₃-labile Cu (Khan et al., 2009). The air-dry soil samples were sieved (<2 mm) and the moisture content adjusted to 60% of their water holding capacity (WHC), then soil samples were left at 28 °C for seven days. Two portions of moist soil (15 g air-dry soil) were weighed, the first one non-fumigated (NF) was immediately extracted with 1 M NH₄NO₃ (1:2.5 w:v) for 60 min by oscillating shaking at 200 rpm and filtered (Whatman no. 44); the second one (F) was fumigated for 24 h at 25 °C with ethanol-free CHCl₃ and then extracted as described above. After filtration, the extracts were acidified with 0.5 ml 65% HNO₃ and stored at 4 °C until Cu was measured by ICP-AES (LIBERTY AX - Inductively Coupled Plasma-Atomic Emission Spectrometry- Varian). CHCl₃-labile Cu (mg CHCl₃-labile Cu kg⁻¹ soil) was calculated as described by Khan et al. (2009), reported in the following equation (a):

$$\text{CHCl}_3\text{-labile Cu} = (\text{Cu extracted from fumigated soil}) - (\text{Cu extracted from non fumigated soil}) \text{ (a)}$$

- ***Microbial biomass content and respiration***

Soil microbial biomass, a small fraction of soil organic matter (1-3%), is of fundamental importance in the biological cycles of all major plant nutrients (De Haan et al., 1989). It is ideal to monitor of soil pollution, having both mass and activity and being in intimate contact with the soil microenvironment. Combining microbial activity and content appears to provide more sensitive indications of soil pollution by heavy metals (Brookes, 1995). Abiotic stress caused by the addition of heavy metals, in inorganic and organic forms, affects the growth, morphology, and metabolism of microorganisms in soils, through functional disturbance, protein denaturation, or destruction of the integrity of cell membranes. Consequently, soil pollution by heavy metals can reduce the size and activity of the microbial biomass (Nannipieri et al., 1990; Chander and Brookes, 1991). Two of the most commonly used parameters for the study of soil microflora are the microbial carbon, which permits an evaluation of the size of the living soil components (Insam et al., 1988) and the soil respiration (as mineralization of soil organic C), considered an useful index of the activity of soil microflora (Anderson and Domsch, 1990). The size of the microbial biomass is much used to evaluate detrimental effects of heavy metals (Murrieta et al., 2006) and is quantified as microbial carbon (C_{mic}) according to the fumigation-extraction (FE) method (Vance et al., 1987). Two portions of moist soil (20 g oven-dry soil) were weighed, the first one (non-fumigated) was immediately extracted with 80 ml of 0.5 M K_2SO_4 for 30 min by oscillating shaking at 200 rpm and filtered (Whatman no. 42); the second one was fumigated for 24 h at 25 °C with ethanol-free $CHCl_3$ and then extracted as described above. The carbon was determined in the extracts after oxidation with 0.4 N $K_2Cr_2O_7$ and titration with $(NH_4)_2SO_4FeSO_4$ 0.0333 N.

The microbial biomass content was calculated as reported in the following equation (b):

$$C_{mic} = EC * k_{EC}, \quad (b)$$

where EC is the difference between organic C extracted from fumigated soils and organic C extracted from non-fumigated soils and $k_{EC} = 2.64$ a conversion coefficient for computing biomass C (Vance et al., 1987; Martens, 1995). The activity of the soil microbial biomass is usually evaluated by measuring CO_2 evolution. It is determined using a slightly modified version of the Isermayer method (1952). The soil samples (20g) were conditioned at 60% of the water holding capacity (WHC) and pre-incubated for 3 days at 4 °C; then each sample was placed in a hermetically sealed container (1000 ml), which was fitted with a beaker containing 2 ml of 1 M NaOH to trap the evolved CO_2 . The CO_2 production is determined by titration of the excess NaOH with 0.1 M HCl (Badalucco et al., 1992). The basal C- CO_2 value of each soil samples ($mg\ C-CO_2\ g^{-1}soil$) was computed as the average of the values measured during the 28-day period of analysis, at 1-3-7-10-14-21-28days.

- **Microbial indices**

Several authors have used the microbial quotient (C_{mic}:C_{org}) and the metabolic quotient (qCO₂) as two derived parameters in attempting to gain a better understanding of the relationships between the function of the microbial community and environmental conditions (Leita et al., 1995; Dai et al., 2004; Murrieta et al., 2006). Brookes and McGrath (1984) suggested that these two quotients are better indicators for evaluating the effects of toxic metals on soil microorganisms than either microbial activity or biomass measurements alone (Murrieta et al., 2006).

The microbial quotient (C_{mic}:C_{org}) is the ratio of biomass C to soil organic C as described in equation (c).

$$C_{mic}:C_{org} = \mu\text{g of C-mic} / \mu\text{g total organic carbon}^{-1} * 100 \text{ (Anderson and Domsch, 1989)} \quad (c)$$

The metabolic quotient (qCO₂) is the CO₂-C production per unit biomass C and unit time as described in equation (d).

$$qCO_2 = \mu\text{g CO}_2\text{-C g}^{-1} \text{ h}^{-1} / \mu\text{g C}_{mic} \text{ g}^{-1} \text{ (Dilly and Munch, 1998)} \quad (d)$$

- **Enzyme activity**

Soil enzyme activities have been suggested as suitable indicators of soil quality in both natural and agro-ecosystems because: (i) they are measures of the soil microbial activity and therefore they are strictly related to nutrient cycles and transformations; (ii) they may rapidly respond to changes in soil caused by both natural and anthropogenic factors; (iii) they are easy to measure (Gianfreda and Bollag, 1996; Nannipieri et al., 2002). In this study, enzyme activity was measured according to the methods of Marx et al. (2001) and Vepsäläinen et al. (2001), based on the use of fluorogenic methylumbelliferyl (MUF)-substrates. Soil was analysed for β-cellobiohydrolase (CELL) (EC 3.2.1.91), N-acetyl-β-glucosaminidase or chitinase (CHIT) (EC 3.2.1.30), β-glucosidase (β-GLU) (EC 3.2.1.21), α-glucosidase (α-GLU) (EC 3.2.1.20), acid phosphatase (PHOS) (EC 3.1.3.2), arylsulfatase (ARYL) (EC 3.1.6.1), xylosidase (XYL) (EC 3.2.2.27) and butyrate esterase (BUT) (EC 3.1.1.1) using 4-MUF-β-D-cellobioside, 4-MUF- N-acetyl-β-glucosaminide, 4- MUF-β-D-glucoside, 4- MUF - α-D-glucoside, 4- MUF-phosphate, 4- MUF-sulphate, 4-MUF-7-β-D-xyloside and 4- MUF-butyrates as substrates, respectively.

A moist sample (equivalent weight to 1 g oven-dry material) was weighed into a sterile jar and 50 ml of Na-acetate buffer pH 5.5 was added. A homogenous suspension was obtained by homogenising with UltraTurrax at 9600 rev min⁻¹ for 3 min. Aliquots of 100 µl were withdrawn and dispensed into a 96 well microplate (three analytical replicates sample⁻¹substrate⁻¹). Finally, 100 µl of 1 mM substrate solution were added giving a final substrate concentration of 500 µM. Fluorescence was measured after 0, 30, 60, 120, 180 min of incubation at 30 °C. Fluorescence (excitation 360 nm; emission 450 nm) was measured with an automated fluorimetric plate-reader (Fluoroskan Ascent). After the measurements of eight enzyme was possible to calculate the Synthetic Enzyme Index that is the sum of the single enzyme activities on soil mass base (Dumontet et al., 2001).

- ***Community level physiological profiles***

The community level physiological profiles (CLPPs) are used to determine the functional diversity in soil, which gives information on the functioning of those members of the soil microflora involved in carbon cycling (Chapman, 2007). In this study, the MicroResp™ was chosen to assess the CLPPs of soils (Campbell et al. 2003). C-substrates for the analysis of CLPP were selected depending on their ecological relevance and the objective of the experiment. Substrates consisted of four carbohydrates: α-D-glucose (GLU), D-Galactose (GA), D-fructose (FRU), L-arabionose (ARA), six amino acids : L-leucine (LEU), L-arginine (ARG), Glycine (GLI), L-aspartic acid (ASP), γ-amino-butyric (BUT) and glutamic acid (ACGLU), three carboxylic acids: citric acid (CIT), oxalic acid (OX) and L-ascorbic acid (ASC), and two phenolic acids: vanillic (VAN) and syringic acid (SIR). Each C-substance was dissolved in deionized water to deliver 30 mg of C g soil water⁻¹, with the exception of Glycine, L-arginine and oxalic acid (7.5mg C g soil water⁻¹); L-leucine (3mg C g soil water⁻¹); phenolic acids (0.3 mg C g soil water⁻¹); L-aspartic acid (0.75 mg C g soil water⁻¹). Substrate concentrations were chosen from values utilized in previous CLPP studies with the MicroResp method (Campbell et al., 2003; Lalor et al., 2007). The MicroResp™ method works as a multi-SIR; a detection plate positioned over the deep-well plate with soil was filled with a gel containing the cresol red and the sodium carbonate that changes colour with the acidification process as the microorganism-produced CO₂ reacts with the gel mixture. Colour development of the gel was measured as absorbance (ABS) at 590 nm using a Biolog MicroStation (Microlog- Release 4.20.04, Biolog, Inc., Hayward, CA).

Measured values were then converted to % CO₂ by applying a least squares fitting curve on experimental data that relates absorbance measured to % CO₂ of equilibrium with gel after 6 hours of incubation, following the rectangular hyperbole: % CO₂ = -0.3409-1.4604/ (1-7.882*ABS). Finally, the SIR data (Substrate Induced Respiration) were converted to a flux of µg C-CO₂ g⁻¹ soil h⁻¹. The discussed data were obtained after subtracting the control value (deionized water only), and dividing by the mean respiration of the samples (Campbell et al., 2003).

- ***Microbial functional diversity***

Functional diversity is a component of overall diversity in soil that possibly provides a more practical and ecologically relevant measure of microbial diversity (Zak et al., 1994). This component of diversity is different from that obtained by measuring species diversity in that it concerns the range and evenness of functions expressed in situ by the microbial community, rather than the species present in soils.

Microbial functional diversity includes a vast range of activities including: nutrient transformations, decomposition, plant growth promotion/suppression and modification of soil physical processes (Giller et al., 1997; Wardle et al., 1999). In the current literature functional diversity of soil microbial communities is determined alternatively either through enzyme activities or by means of community level physiological profile (CLPP) techniques, such as MicroRespTM. In this study the microbial functional diversity was measured using both methodological approaches. By data of these two methodologies, catabolic evenness or Simpson-Yule index (a component of microbial functional diversity defined as the uniformity of substrate use) was calculated as a measure of functional diversity (Degens et al., 2001; Bending et al., 2004). Catabolic evenness (E) by MicroRespTM was calculated using the Substrate-Induced Respiration (SIR) data of all substrates as described in equation (e), where p_i is summed for all substrates and p_i=r_i/∑r_i (defined as the respiration response of each substrate (r_i) as a proportion of total respiration responses summed over all substrates (∑r_i)).

$$E=1/\sum p_i^2 \text{ (Magurran, 1988)} \quad (e)$$

The catabolic evenness was also computed from enzymatic rates as a measure of functional diversity where p_i is the ratio of the activity of a particular enzyme to the sum of all enzymatic activities (Degens et al., 2000).

- *Statistical analyses*

Statistical analyses were carried out with Systat version 7. The means and least significant differences between the soil samplings with time were calculated by a one way ANOVA. The significance probability levels of the results are given at the $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***), respectively.

5.5 Results and discussions

- *Heavy metals*

In most European countries, the quality standards are based on the total metal fraction such as boiling nitric acid HNO_3 , or *aqua regia* (Rao et al., 2008). These values are excellent criteria to define the extent of metal build up or contamination in soil and an estimate of the portion of total cation exchange capacity saturated by metals. In this study the results obtained after two years of phytoremediation showed that this soil-plant-microorganism system was able to reduce the total content of HM by the average of 17% in the polluted soil (Fig. 5.1). All metals analysed decreased with time, from 10% of Zn to 32% of Cd, as reported in Table 5.1. These results suggest a positive effect of phytoremediation on HM soil decontamination, so that the industrial utilization of this area was possible, according to the Italian law 152/2006 which establishes the thresholds of soil pollutants (Table 5.2).

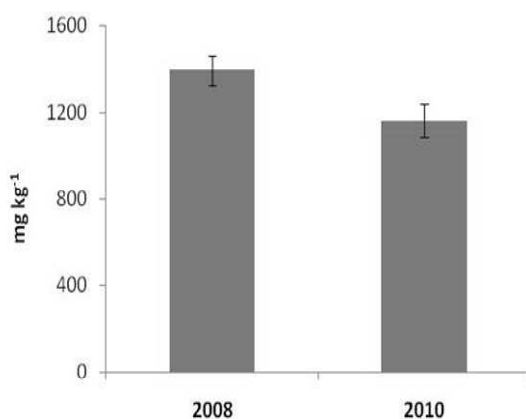


Figure 5.1 Total heavy metal amount (Cr, Cd, Cu, Pb, Ni, Zn) measured in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

Table 5.1 The concentrations of the single heavy metals and their percentage variations with time, measured in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

Pollutants (mg kg ⁻¹)	Total content of pollutants		Variations (%)
	2008	2010	
Cr	66	51	-24
Cd	3.54	2.39	-32
Cu	186	151	-18
Pb	526	412	-22
Ni	116	99	-15
Zn	499	450	-10

Table 5.2 Limits of the soil heavy metals content for the civil and industrial destination of the polluted area according to the Italian law 156/2006.

Pollutants (mg kg ⁻¹)	Limits according to the Italian law 156/2006 about the pollutants levels in the soil	
	Civil destination	Industrial destination
Cr	150	800
Cd	2	15
Cu	120	600
Pb	100	1000
Ni	120	500
Zn	150	1500

The total content of HM in soil reduced with time might be due different process: (i) the phytoextraction capacity of plants, or (ii) the HM leaching in the waters, collected in the loglines of the phytodepuration system. In fact, it is known that the Lombardy poplar and the Princess tree are fast growth species with a developed shoot and root biomass, for these peculiarities they have a better phytoextraction capacity and resistance to metals than other species such as Scotch broom (Giachetti e Sebastiani, 2005; Liu Y et al., 2007). Both processes, phytoextraction and leaching, which will be quantified by the HM determination in the plants tissues and in the leaching water, could be favored by a higher HM mobility due to the root exudation. However, the determination of total soil metal content alone is not a very useful tool to quantify contamination and potential environmental and human health risks (Harmsen, 2007). There are important phases for metal ions in the soil system: the solid and the solution phase. In the solid phase metals occur e.g. adsorbed at clay particles, organic matter and iron hydroxides.

In the soil solution metals may form organic complexes with dissolved organic matter, inorganic complexes with dissolved anions and occur as free hydrated metal ions (Kalis, 2006). Several speciation techniques have been developed that measure only a certain defined or undefined fraction of the total metal concentration (Kalis, 2006). The free ion concentration in soil solution and the labile amount in the solid phase represent the real potentially toxic amount (Sauvé et al., 1996; Cattani et al., 2006), that is, the bioavailable concentration of metals. Indeed, it is generally accepted that the risk factor associated with this kind of pollution is related to their bioavailability rather than their total concentrations. The bioavailability is available elemental pools for organisms and determines the diffusion and accumulation of these inorganic pollutants in the ecosystems through the food chain (Alkorta et al., 2004). In order to verify the HM mobility and bioavailability, two different fractions of copper were taken into account: the DTPA-extractable Cu and the CHCl_3 -labile Cu. While the total content of Cu was reduced (-18%) (Fig. 5.2), the DTPA-extractable Cu was increased (+14%) with time (Fig. 5.3), suggesting an increase of Cu mobility and availability (Lindsay et al., 1978; Ernst, 1996).

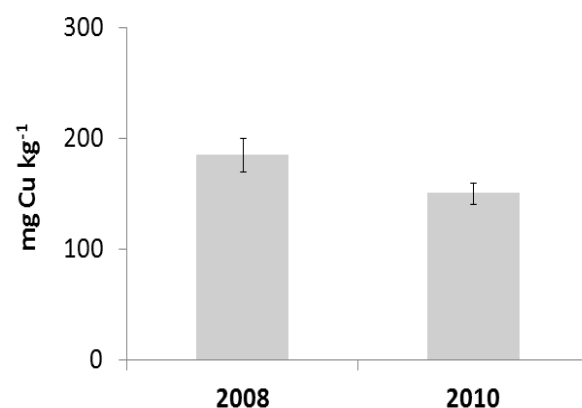


Figure 5.2 The soil total content of Cu in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

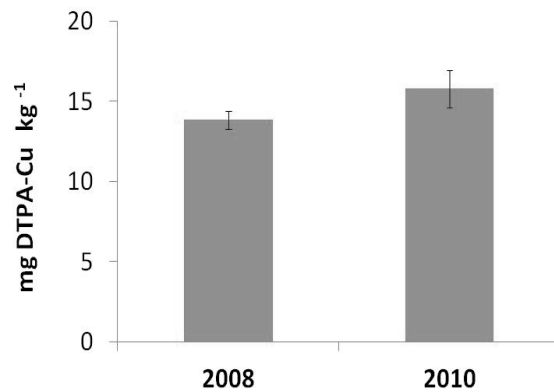


Figure 5.3 The DTPA-extractable Cu in soil in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

In literature, DTPA-extractable metal is regarded as a mixture of both mobile and potentially mobilisable species (Lindsay and Norvell, 1978; Wu et al., 2006). Moreover, the results of ecotoxicological evaluations carried out in precedent researches show the DTPA-extractable fraction correlates well with HM toxicity on soil microorganisms (Prokop et al., 2003). In this study, the increase of Cu mobility could be determined by the root exudates. The root exudates exercised probably a higher pressure on the heavy metals mobility than the decrease of soil pH (McGrath et al., 2001), since a slight increase of soil pH was observed after two years of phytoremediation. These organic compounds mainly carbohydrates, carboxylic acids and amino acids, passively released by roots along concentration gradients, promote the microbial activity in the rhizosphere (Marschner, 1986). The root exudates are of prime importance for microorganisms since they are readily assimilable without synthesis of exoenzymes (Bremer and van Kessel, 1990; Bremer and Kuikman, 1994). Thus, these exudates represent a convenient source of carbon (and possibly nitrogen) and energy and are likely to favor fast growing microbes in the rhizosphere (Baudoin et al., 2003). The release of organic substances from roots is a key process influencing nutrient availability in the rhizosphere. Rhizodeposition, including root exudation can influence plant growth directly by making cations available for uptake through processes such as chelation and by influencing soil microbial activity. The stimulation of specific populations in contaminated soil by organic substrates exuded by plant roots may be an important result of phytoremediation (Kozdrój and van Elsas, 2000). It is important to gain knowledge about the range of compounds released and the factors influencing their release, to understand their effects on the microbial community and enable development of techniques to enhance microbial activity (Grayston et al.,

1996). Moreover, these organic compounds can mobilize different metals strongly adsorbed to the soil particles through weathering and chelation. The mobility and bioavailability of trace elements in the soil, particularly at the root–soil interface (rhizosphere) where root uptake or exclusion takes place, is a critical factor affecting the outcome and success of phytoremediation. Developing new methods to either enhance (for phytoextraction) or reduce (for phytostabilisation) the bioavailability of metal contaminants in the rhizosphere could significantly improve the efficiency of this technique for soil remediation (Sørensen, 1997; Kozdrój and van Elsas, 2000). As a consequence of soil-plant system effect on HM mobility, an increase of the HM bioaccumulation was expected. For this reason the fumigation-extraction was applied as a new methodology to quantify the CHCl_3 -labile HM representing the fraction accumulated into soil microbial pool. The results showed that the CHCl_3 -labile Cu increased from 2008 to 2010 (+100%) (Figure 5.4), suggesting a growing accumulation into soil microbial biomass (Khan et al., 2009).

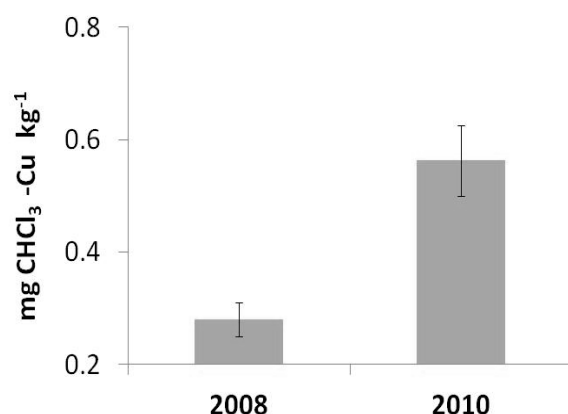


Figure 5.4 CHCl_3 -labile copper in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

Copper revealed a certain fraction of CHCl_3 -labile components additionally extractable with NH_4NO_3 after fumigation, but currently no conversion values to total microbial biomass were applied as the percentage of the CHCl_3 -extractable fraction is unknown. Also unknown is the exact location of CHCl_3 -labile metals within the microbial cells. It is generally assumed that more cytoplasmic than cell-wall material is rendered extractable after fumigation and the 24 h period of autolysis (Jenkinson, 1988; Joergensen and Mueller, 1996), but the exact percentages are unknown.

- **Microbial biomass and indices**

Two important biological parameters can be used to assess the adverse effects of heavy metals on soil microbial biomass and its activity, namely, the microbial quotient ($C_{mic}:C_{org}$) and metabolic quotient (qCO_2). Anderson and Domsch (1990) applied these ecophysiological parameters to study environmental effect on the microbial pool in terms of microbial carbon loss and energy demand. In many precedent studies, data showed a significant decrease of C_{mic}/C_{org} ratio and an increase of qCO_2 due to higher metal concentrations (Wardle, 1992; Yao et al., 2003; Renella et al., 2005; Murrieta et al., 2006). As well in this study, the amount of microbial carbon on mass soil basis (C_{mic}) and the microbial quotient ($C_{mic}:C_{org}$) decreased after two years of phytoremediation (Fig. 5.5 and 5.6).

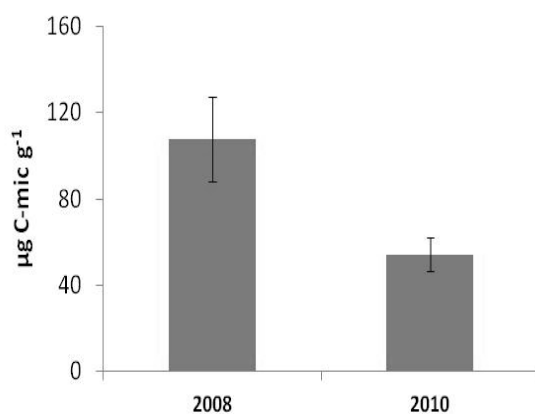


Figure 5.5 Microbial carbon (C_{mic}) in two soil samplings (February 2008 - February 2010- before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

The $C_{mic}:C_{org}$ ratio has been proposed as a useful measure of pollution (Brookes, 1995). It clearly revealed the heavy metal effects and the ratio decreased with the increase of heavy metal mobility (higher DTPA-extractable fraction of HM), as reported also by other authors (Dahlin and Witter, 1998). A lower $C_{mic}:C_{org}$ ratio primarily indicates a low availability of organic matter to soil microorganisms and additionally explains as a result of reduced substrate utilisation efficiencies (Anderson and Domsch, 1990). This means that more substrate is diverted towards catabolic processes at the expense of anabolic processes, leading to reduced microbial biomass levels (Chander and Brookes, 1991).

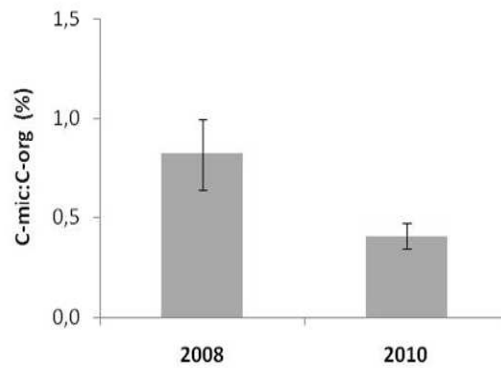


Figure 5.6 Microbial quotient $C_{mic}:C_{org}$ (ratio to the microbial carbon C_{mic} to the organic carbon C_{org}) measured in two soil samplings (February 2008-February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

Conversely, the metabolic quotient (qCO_2) increased with time (Fig. 5.7). According to precedent studies, the higher qCO_2 emphasized the need of living organisms to expend more energy to survive (Yao et al., 2003). Enhanced qCO_2 has been attributed to a low efficiency of substrate utilization for growth when microorganisms are under environmental stress and to the increase in the maintenance energy requirements, this indicated more CO_2 evolved per unit of biomass and thus a lower efficiency for utilization of carbon substrates (Murrieta et al., 2006). The metabolic quotient (qCO_2) undoubtedly provides a useful measure of microbial efficiency, increase in qCO_2 indicating reduced microbial efficiency, which appeared to be related to stress (Leita et al., 1999). However, contrasting results of qCO_2 in relation to environmental stress have been observed, leading to controversy (Insam, 1996; Giller, 1998). Results obtained from studies on the influence of heavy metals on CO_2 evolution in contaminated soils have sometimes been at variance, due to the qCO_2 is able to react quite differently, depending on the actual supply of energy and probably also on the structure of the microbial communities present.

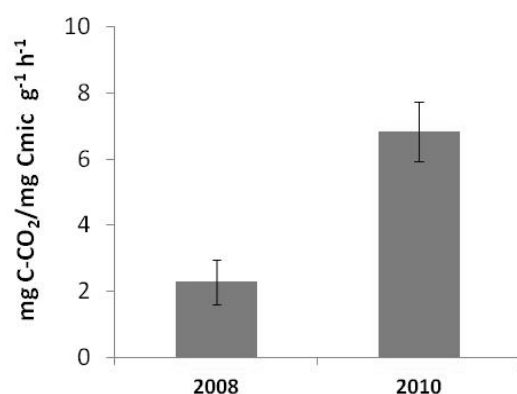


Figure 5.7 Metabolic quotient qCO_2 (CO₂-C produced per C_{mic} per hour) measured in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

According to the previous studies (Leita et al., 1999; Kunito et al., 1999; Yao et al., 2003), the results obtained after two years of phytoremediation, revealed a possible ecophysiological stress for the soil microbial communities, causing a reduction of the biomass magnitude with an enhanced basal metabolism. The shift towards catabolic processes is often reflected by increased metabolic quotients, qCO_2 , which shows ideally a strong negative relationship to the biomass C/soil C ratio (Anderson and Domsch, 1990; Chander et al., 2001). Probably, there was a greater demand for energy by microorganisms in order to cope with the toxicity of pollutants. In the study, since the DTPA-extractable fraction and the CHCl₃-labile form of some HM increased, the observed ecophysiological stress was probably due to the increasing mobility and bioavailability of HM in soil solution which may lead to their accumulation into microbial biomass (Leita et al., 1995). Therefore, it is supposed that microorganisms increased their activity due to the defensive metabolic mechanisms to contrast the high and growing availability of metals, which determined a quantitative loss of the soil microbial biomass (Gadd et al., 1978; Haferburg et al., 2007).

- ***Enzyme activity***

Combining soil enzyme activities with estimates of microbial biomass may provide a more sensitive indication of soil pollution than either enzyme activity or microbial biomass measurement alone (Kandeler et al., 1996). Soil enzyme activities are the direct expression of the soil community to metabolic requirements and available nutrients (Caldwell, 2005).

Furthermore, Kandeler et al. (1996) have indicated that the composition of the microbial community determines the potential of that community for enzyme synthesis, and thus any modification of microbial community due to environmental factors should be reflected on the level of soil enzyme activities. In this study, all enzyme activities (β -cellobiohydrolase (CELL), N-acetyl- β -glucosaminidase or chitinase (CHIT), β -glucosidase (β -GLU), α -glucosidase (α -GLU), acid phosphatase (PHOS), arylsulfatase (ARYL), xylosidase (XYL) and butyrate esterase (BUT) measured by a fluorimetric assay increased after two years of phytoremediation as showed by the Synthetic Enzyme Index (SEI), calculated as the sum of the eight hydrolytic enzyme activities. This index in 2010 reached a double value with respect to that obtained in 2008 (Fig. 5.8).

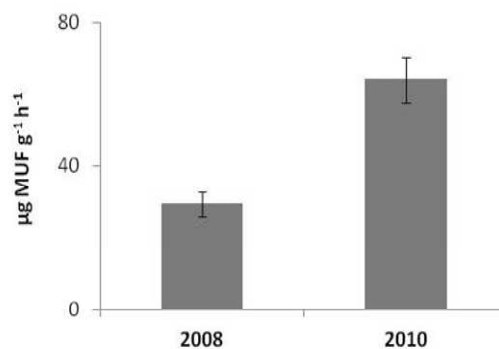


Figure 5.8 SEI (Synthetic Enzyme Index) measured in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

The enzyme activities with respect to 2008 were increased with the following percentages: CELL, 102%; CHIT, 79%; β -GLUC, 218%; α -GLU, 2197%; PHOS, 151%; ARYL, 170%; XYL, 84%; BUT, 69% (Fig. 5.9). These enzymes are involved in metabolic processes as C cycling (e.g. β -glucosidase, catalyzes the final step of cellulose degradation), N and P cycling (e.g. β -N-acetylglucosaminidase, hydrolyzes the glucosamine and acid phosphatase hydrolyzes organic-P compounds).

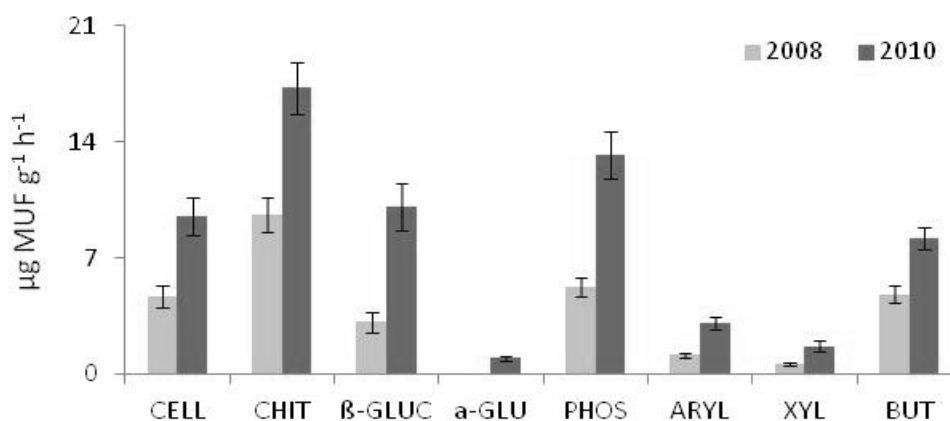


Figure 5.9 Soil enzyme activities measured by fluorimetric method in two soil samplings (February 2008 - February 2010 before and after phytoremediation) at depth 0-30 cm. The value of the butyrate esterase was divided by 10, due to the value scale of the other enzymes. Error standard bars (n=12)

The significant increase of soil enzyme activities after two years of phytoremediation was caused probably by the plant growth and root expansion of the tree species, Lombardy poplar and Princess tree, which influenced the extracellular enzymatic activity of the hydrolytic enzymes analysed (Reboreda and Caçador, 2008). Indeed, the root activity may cause a positive feedback to microbial activity through new organic anions and sugars coming from rhizodeposition (Sequi, 1989). For example, the release of soluble sugars by roots supply the substrates needed by β -glucosidase and chitinase (Burke et al., 2002). Certainly, aboveground plant and belowground microbial communities of terrestrial ecosystems are closely related. Plants provide a source of carbon (C) and other nutrients for the soil decomposer community in the form of plant litter and root exudates; changes in aboveground plant diversity can alter belowground soil microbial diversity (Bartelt-Ryser et al., 2005; Rodríguez-Loinaz et al., 2008). In turn, belowground microbial communities decompose soil organic matter (OM), stabilize soil structure and, through its essential role in the cycling of elements, release nutrients for plant growth (Porazinska et al., 2003), thus affecting vegetation structure (Epelde et al., 2010). Otherwise, since the HM inhibit enzyme activity, the reduction of total HM content in soil after two years of phytoremediation reestablished optimal conditions for enzyme activity (Nannipieri et al., 1994).

- ***Community-level physiological profile***

When the soil quality and its activity are assessed, a basic method is to measure the amount of carbon dioxide (CO₂) that is being respired by soil microorganisms that are decomposing organic substrates within the soil. It is also possible to measure the substrate-induced respiration (SIR) by measuring the amounts of CO₂ before and after addition of a substrate, such as glucose. The individual species that comprise a soil microbial community have different abilities to respire different substrates, so that by adding different substrates it is possible to obtain a catabolic fingerprint of the community or a community-level physiological profile (CLPP) (Campbell et al., 2003). In this study, the CLPP of soil microorganisms was measured by MicroResp™. The SIR data (µg CO₂ g⁻¹ h⁻¹) obtained by this methodology showed an increase just for the C-source group of carboxylic acids and a slight decreasing utilization of the other three functional groups (aminoacids, phenolic acids and carbohydrates) (Fig. 5.10).

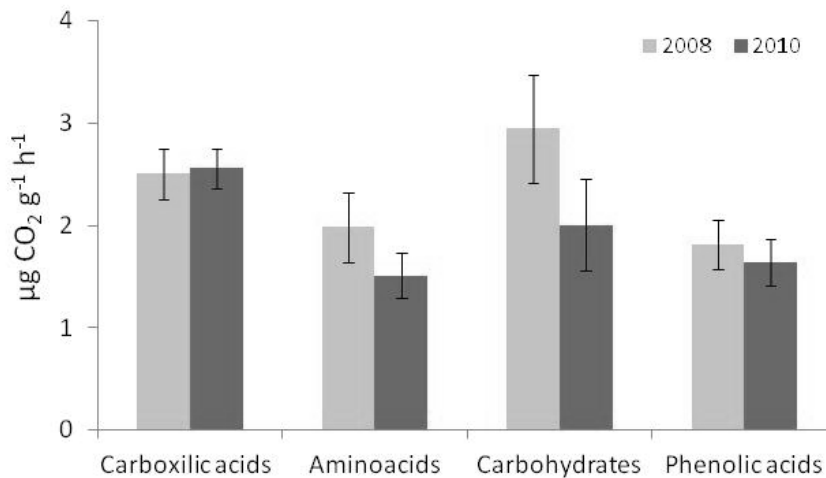


Figure 5.10 CLPP for classes of C-substrates measured by Microresp™ in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

As single C-source regards, the production of CO₂ slightly decreased from 2008 to 2010 in all cases with exception of three substrates (oxalic acid OX, arginine ARG and the glutamic acid ACGLU) which showed a significant increase of SIR (Fig.5.11).

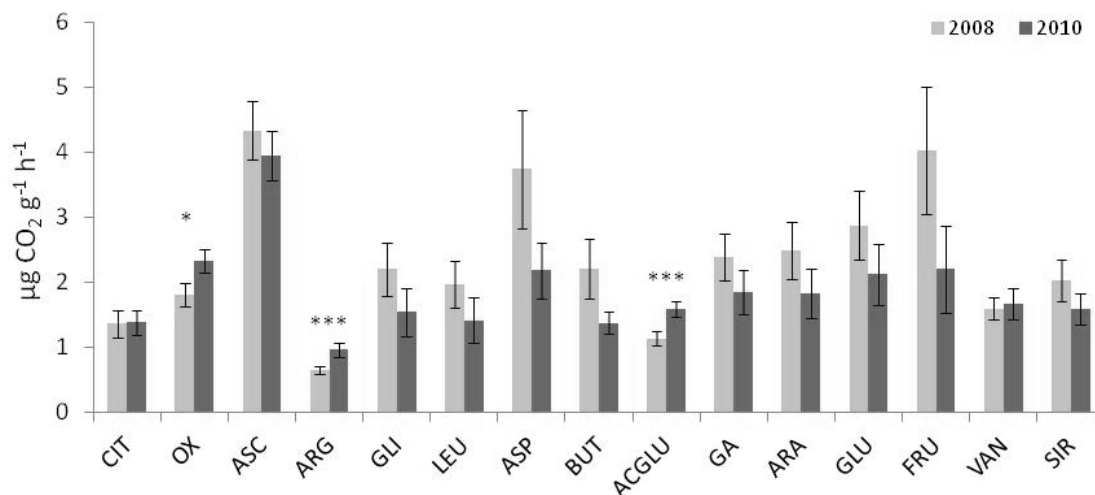


Figure 5.11 CLPP for each substrate (four carbohydrates: α-D-glucose GLU, D-Galactose GA, D-fructose FRU, L-arabionose ARA), six amino acids (L-leucine LEU, L-arginine ARG, Glycine GLI, L-aspartic acid ASP, γ-amino-butyric BUT and glutamic acid ACGLU), three carboxylic acids (citric acid CIT, oxalic acid OX and L-ascorbic acid ASC), and two phenolic acids (vanillic VAN and syringic acid SIR), measured by Microresp in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12) the starred bars denote significant (* P < 0.05;*** P<0.001)

Successful application of plants for remediation of contaminated soils depends on the phenotype and genotype of the plants, but interactions between rhizospheric organic matter and heavy metals are also of great importance due to the lower solubility and availability of metals to plant uptake caused by the strong fixation of heavy metals by soil organic matter, oxides and clays. It is also reported that the excretion products of roots, especially low molecular weight organic acids, many of which (such as acetic, oxalic, fumaric, citric, and tartaric acids) are able to form soluble complexes and chelates with metal ions (Mench and Martin, 1991; Robert and Berthelin, 1994; Stevenson and Fitch, 1994), modifying the fixation and mobility of heavy metals in soils (Krishnamurti et al., 1997; Chen et al., 2003). Then, the root exudates increased the mobility of heavy metals after two years of phytoremediation, which caused the ecophysiological stress of soil microbial biomass, which reduced consequently its catabolic activity.

- ***Microbial functional diversity***

Information on functional diversity (metabolic potential) is essential for understanding the role of microbial communities in different environments. There is now a plethora of information on microbial diversity in a vast range of environments. Most of this concerns genetic and taxonomic diversity, but to understand the role of microbial communities in different environments it is essential to have knowledge of microbial community function and functional diversity. Microbial community function implies actual catabolic activity expressed. In contrast, functional diversity indicates its potential activity, i.e. the capability of the community to adapt metabolism (catabolism) and/or the relative composition and size of constituent populations to varying abiotic conditions (microclimate and added substrates). In this study functional diversity of soil microbial communities was assessed by the catabolic evenness or Simpson-Yule index (CE), a component of microbial functional diversity defined as the uniformity of substrate use. The CE was calculated from catabolic response profiles, using data of enzyme activities and a community level physiological profile (CLPP - MicroResp™). The CE obtained using enzyme activities was higher in 2010 than 2008 (Fig.5.12). This means that the phytoremediation process improved the soil functional diversity probably due to the additional hydrolytic activity of root system.

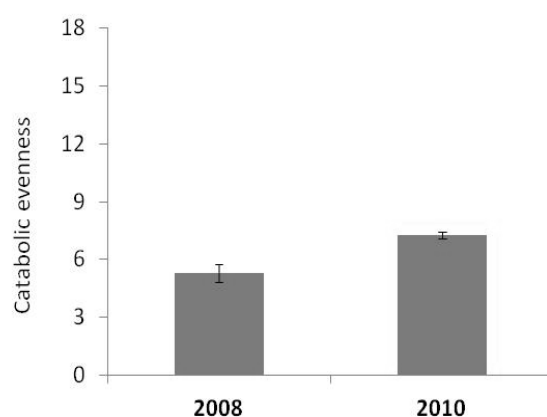


Figure 5.12 The Catabolic evenness or Simpson-Yule index measured by the activity of eight enzyme in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

The CE was also measured by data of SIR revealed by Microresp™. In this case the CE decreased with time (Fig. 5.13) suggesting a reduction of microbial functional diversity after two years of phytoremediation.

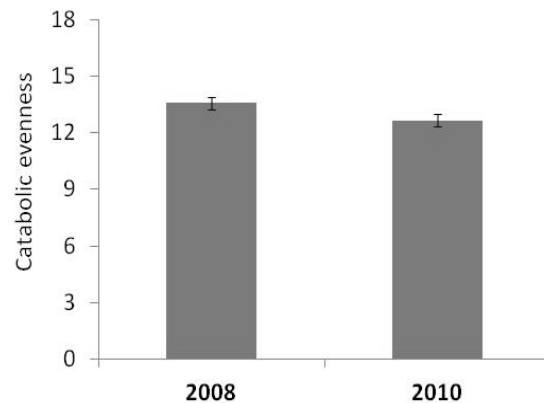


Figure 5.13 The Catabolic evenness or Simpson-Yule index by data of the SIR measured by Microresp in two soil samplings (February 2008 - February 2010-before and after phytoremediation) at depth 0-30 cm. Error standard bars (n=12)

Also in other case studies this diversity index calculated using enzyme activities and CLPP gave opposite results suggesting that these two methodologies probably point to different components of microbial functional diversity. A hypothesis is that measuring microbial functional diversity by means of enzymes or CLPP methods provides information on sequential processes occurring in soil: firstly the exemplification of complex organic substrates obtained after enzymatic hydrolysis and secondly the direct utilization of simple substrates, derived from the previous step, by microorganisms (CLPPs). However, in this study the root activity may determine the improvement of the hydrolytic functional diversity, while the catabolic function of microbial population was probably inhibited by the increased HM availability in soil.

5.6 Conclusions

Two years of phytoremediation caused a significant reduction of heavy metals total content in soil. However, soil-plant-microorganism interactions increased their mobility and availability with time probably due to the effect of root exudates. This increase probably determined an ecophysiological stress of soil microbial pool, which accumulated increasing concentrations of heavy metals into its biomass and as well reduced quantitatively, increased its basal respiration and reduced its catabolic activity. Moreover, the reduction of the heavy metals total content in soil and a higher concentration of root exudates favored the enzyme activities. Then, it is fundamental to study the interactions between microorganisms and plants in the polluted soils, because they are a critical element for the control of the heavy metals bioavailability. Furthermore, it is necessary to analyse all components of ecosystem involved in the phytoremediation as the leaching water and plants. These analyses are fundamental to define the pathways of the heavy metals during the phytoremediation process.

For this reason, the results of the present research will be completed and discussed with the other results obtained by the chemical analysis of plants harvested in the test field and waters collected in the phytodepuration system. The aim will be to supply a complete and exhaustive description of the heavy metals destiny. The phytoremediation system showed its capacity to reduce significantly the soil pollution in a sustainable environmental way, improving at the same time the esthetical value and recovering the whole area for the industrial utilization. It supposes that the ecophysiological stress for the microbial communities is just concerning the first period of the phytoremediation and in the long term probably there will be significant positive effects also for the soil microorganisms. In conclusion, the soil biochemical properties result good indicators of soil quality during phytoremediation process and the CHCl_3 -labile metals could represent a new methodology for evaluating the accumulation of these inorganic pollutants into soil microbial biomass.

Chapter 6- Use of Plant Growth-Promoting Bacteria to enhance heavy metals phytoremediation in mining soils: a preliminary study under Umbrella project

6.1 Abstract

The soils of abandoned mining areas, characterized by high heavy metals content and often mobility, represent a significant problem all over Europe. It is urgent to choose sustainable methods for their remediation aimed to avoid heavy metals diffusion in the environment and uptake into the food chain. This study was carried out under the EC 7FP UMBRELLA project and aimed to evaluate the effects of the bioaugmentation on soil quality improvement in terms of microbial biomass content, activity and diversity. For this purpose a pot experiment was carried out by inoculating four strains of PGPB, isolated from the test site of Ingurto (Italy), in a soil collected from the test site of Ronneburg (Germany). Ten weeks later, the results showed an increase of microbial biomass content and an improvement of microbial activity and functional diversity measured in the inoculated soil by MicroResp and enzyme activities. Furthermore, the biodiversity Richness Index obtained by DGGE data, showed an evident increase of genetic diversity, while the physiological profiling at community level, measured by Biolog system did not reveal a significant difference between inoculated and uninoculated pots. In conclusion, bioaugmentation produced positive effects on soil microbial biomass, on functional diversity and genetic microbial diversity, which may lead to the improvement of a phytoremediation process.

Keywords: *mining soil, bioaugmentation, microbial functional and genetic diversity*

6.2 Introduction

Abandoned mining areas are a significant problem all over Europe. The closure of mining activities presents serious threats as a result of insufficient attention to the possible environmental impacts and inadequate definition of pollution containment plans. In these abandoned mines, heavy metals concentration is high and toxic to many organisms and plants, as shown by the absence of vegetation over most of the basin surface (Cao A. et al., 2009). The high levels of heavy metals (HM) from metalliferous mines are found in and around the mines due to the discharge and dispersion of mine-waste materials into the ecosystem. As a result, the surrounding large areas can be contaminated. Migration of contaminants into non-contaminated sites, as dust or leachate through the soil, and the spreading of sewage sludge are examples of events that contribute towards contamination of our ecosystems. Thus, it is necessary to plan some suitable projects to remediate and reevaluate these areas, choosing methods necessary to avoid the heavy metals dissemination in the environment and/or the food chain (Malik, 2004; Singh and Cameotra, 2004). Among the sustainable methods for the soil remediation of these particular heavy metals polluted areas, the phytoremediation could represent an alternative to physico-chemical treatments (Lebeau et al., 2008; Khan et al., 2004). It is an emerging and inexpensive strategy which uses plants to extract, mitigate, and stabilize contaminants. Benefits from successful approaches of phytoremediation include healthier soil, promoting and sustaining indigenous microbial communities that are essential for long-term bioremediation of the soil, and creation of a more pleasing landscape, compared with ugly contaminated areas (Mendez and Maier, 2008; de-Bashan et al., 2011). Many successful examples of phytoremediation are documented and employed in the environmental cleaning industry (Macek et al., 2000; Suresh and Ravishankar, 2004). However, often the plants needed for phytoremediation cannot establish in degraded systems. Also, when established, they do not perform well under adverse environmental conditions, such as excessive concentrations of contaminants, extreme high and low pH, deficient supply of nutrients, poor or no soil structure, lack of clay and organic matter to retain water, lack of a seed source from nearby native plants and also due to the severely damaged microbial communities. Moreover, the major disadvantage of phytoremediation is that it requires a long-term economic commitment and patience by project managers for results because the process is dependent on plant growth, tolerance to contaminants, and bioaccumulation capacity, all of which are slow processes (Kuiper et al., 2004; Hernandez et al., 2006; Grandlic et al., 2008). These limits of the phytoremediation could be overcome if it is applied in association with bioaugmentation.

Indeed, bioaugmentation, which corresponds to the inoculation of appropriate bacterial strains in soil, can enlarge the microbial genetic and functional diversity with augmentation of catabolically-relevant organisms, which contributes to soil remediation process (Bashan et al., 1999; Vogel and Walter, 2001; de-Bashan et al., 2002; Duponnois and Plenchette, 2003). It has been studied extensively for the remediation of environmental pollution by recalcitrant chemicals (Van Limbergen et al., 1998; Lebeau et al., 2008; Ma et al., 2011). Synergistic use of plants and microbes has been profitable for cleanup of metalliferous soils (Jing et al., 2007; Glick, 2010). The beneficial partnerships between plants and their associated bacteria can be exploited as a strategy to accelerate plant biomass production and influence plant-metal accumulation or stabilization with better performance abilities such as adaptive strategies, metal mobilization, and immobilization mechanisms (Ma et al., 2011). Plant Growth-Promoting Bacteria (PGPB) are capable of stimulating plant growth, producing indole acetic acid, siderophores and 1-aminocyclopropane-1-carboxylate deaminase, solubilizing phosphate, fixating the atmospheric nitrogen, and are able to change the mobility of metals making phytoremediation a faster, efficient and more attractive process to the public (Glick, 2003; de-Bashan et al., 2011).

6.3 The aim of the study

The research for the present study was carried out under the EC 7FP of the European project UMBRELLA (the acronym UMBRELLA stands for Using MicroBes for the REgulation of heavy metaL mobiLity at ecosystem and landscape scale). The international UMBRELLA team, coordinated by Prof. Dr. Erika Köthe, the Professor for Microbial Phytopathology at the Friedrich Schiller University in Jena, is composed by six European countries (Italy, Sweden, Germany, United Kingdom, Poland, and Romania). The Italian partner is represented by Prof. Dr. Anna Rosa Sprocati, head of the WP1 (Soil microbiology of metal contaminated sites) at ENEA- Casaccia (Rome). The general goal of this European project is to finalize a tool-box which will allow a cost-efficient and sustainable technique for soil remediation, through the use of suitable plants and microorganisms, which allows unrestricted land-use after recultivation of mining sites or other areas with metal contamination throughout Europe. The specific aim of the present study was to evaluate the effects of the bioaugmentation, performed with the Plant Growth-Promoting Bacteria (PGPB) on soil quality improvement in terms of soil microbial biomass content, activity and diversity. For this purpose four species of PGPB were isolated from the ex-mine dump of Montevecchio–Ingurtosu (Cagliari-Italy). These strains were used for the soil bioaugmentation in a pot experiment. The soil microbial biomass, its functional and genetic diversity were measured in inoculated and in control soils.

6.4 Materials and methods

- *Soil samples*

Two soils, called Fan and Riz, were collected in the former mining complex of Montevecchio–Ingurtosu (Cagliari-Italy) on September 2009. The mine dump Ingurtosu is an abandoned mine of blend and galena located in southwestern Sardinia (Italy, Central Mediterranean Sea) at 150 m s.l. and 5 km from the coast. It was one of the most important mining districts for Pb and Zn in Sardinia in production from the beginning of the last century up to 1968. Currently, it is one of the priority sites for intervention of restoration and remediation. Fan soil was the mud of flotation of the Rio Naracauli and Riz soil represented a rhizospheric soil. Fifteen samples (for a total of 750 g) for both soils were mixed and collected in sterile plastic bags. Fan and Riz were kept refrigerated and transported to the laboratory, where were divided in two sub-samples, for the microbiological and biochemical analysis. The general characteristics of these soils are reported in table 6.1.

Table 6.1 The main characteristics of two soils, Fan and Riz

Parameter	Fan	Riz
Total Carbon (%)	1.63	1.07
Organic Carbon (%)	1.40	0.57
Total Nitrogen (%)	0.14	0.03
pH (H ₂ O)	6.50	8.30
*CEC (cmol(+) kg ⁻¹)	9.00	7.79
Clay (%)	23.06	3.73
Loam (%)	41.79	1.40
Sandy (%)	35.14	94.87
Humidity (%)	20.13	11.78
*Cmic (µg C g ⁻¹)	102.12	65.89
*Nmic(µg N g ⁻¹)	11.30	12.90
Total Copper (mg kg ⁻¹)	495	79
Total Lead (mg kg ⁻¹)	5910	1350
Total Zinc (mg kg ⁻¹)	30700	7950

*CEC: Cation Exchangeable Capacity; Cmic: microbial Carbon; Nmic: microbial Nitrogen

- ***Isolation and selection for Plant Growth-Promoting Bacteria (PGPB)***

Successful application of bioaugmentation techniques is dependent on the identification and isolation of appropriate microbial strains, and their subsequent survival and activity, once released into the target habitat (Kuiper et al., 2004). The selection of strains should be taken on the basis of some understanding of the kind of microbial communities present in the source habitat, and preferably with some knowledge of the type of organisms that are common in the target habitat, that is fundamental to overcome the ecological barriers. This suggests that such dominant populations at polluted sites could be good candidates as hosts for desired catabolic activities that are to be exogenously introduced for bioremediation in situ (Thompson I.P. et al., 2005). From the two soils Fan and Riz, different Plant Growth-Promoting Bacteria (PGPB) were isolated and tested for their capacity to resist the HM pollution and to promote the plants' growth. The heterotrophic microbial community was extracted from 10 g of fresh soil mixture, added with 40 ml of pyrophosphate and stirred with glass beads for 2 h in orbiting shaker (180 rpm) at 25 °C. The slurry was let stand overnight and then the supernatant containing the microorganisms was recovered in sterile tubes. After the serial dilutions, the growth of culturable microorganisms was carried out on Standard 1 (St1) and on Mineral Medium (MM) agar plates (Schmidt and Schlegel, 1989) at 25 °C. The St1 medium comprised the following (per liter): peptone, 15g; yeast extract, 3g; NaCl, 6g; Glucose, 1g; agar, 15g. The medium's pH was adjusted to 7.5±0.2 with 10 M NaOH. Next, the medium was autoclaved at 117 °C for 25 min. The MM medium comprised the following (per liter): TRIS, 6g; NaCl, 4.68g; KCl, 1.49g; NH₄Cl, 1.07g; Na₂SO₄, 0.43g; MgCl₂·6H₂O, 0.2g; CaCl₂·H₂O, 0.03g; Na₂HPO₄·2H₂O, 0.11g; Fe(NH₄) citrate 0.005g. After the medium's pH was adjusted to 7.0 with 37% HCl, it was autoclaved at 121 °C for 20 min. Successively, MM medium was supplemented with trace element solution SL7 (1 ml l⁻¹). The components of the SL7 (per liter) were: ZnSO₄·7H₂O, 100 mg, MnCl₂·1H₂O, 30 mg, H₃BO₃, 300 mg, CoCl₂·6H₂O, 10 mg, CuCl₂·2H₂O, 10 mg, NiCl₂·6H₂O, 20 mg, NaMoO₄·2H₂O, 30 mg. The CFUs (Colony-Forming Units) were calculated for the estimation of total bacterial cell numbers in soil and strains isolation was carried out. Successively, the bacterial strains were selected to establish a microbial consortium for bioaugmentation purpose. The selection depended on their characteristics as PGPB and so the following properties were tested: heavy metals resistance, N₂ fixation, siderophores production, PO₄ mobilization, phytohormone production.

- ***Selection of PGPB resistant to heavy metals***

Although many soil bacteria are tolerant to heavy metals and play important roles in mobilization or immobilization of heavy metals (Gadd, 1990), only a few attempts have been made to study the rhizosphere bacteria of metal accumulating and hyperaccumulating plants and their role in the tolerance to and uptake of heavy metals by the plants (Belimov et al., 2009). In this study resistance of the isolated bacterial strains was tested as the MIC (Minimum Inhibitory Concentration). MIC defines the lowest concentration that causes the total growth inhibition. The bacterial growth and the colony morphology in the presence of different heavy metals were compared to a metal-free control. Two different approaches were carried out to test the metal resistance, a qualitative and quantitative test.

Qualitative metal resistance test:

A qualitative screening was performed to assess the metal resistance of each bacterial strain. For agar diffusion assay, the square plates (12 cm) were prepared with sterilized minimal medium (per liter: L-Asparagin, 0.5 g; K₂HPO₄, 0.5 g; MgSO₄, 0.2 g; FeSO₄, 0.01 g; D-Glucose, 10 g; Agar, 18 g). The solid medium was cut to create a trough and then the strains were streaked vertically from the trough until the edge of the plate. The 3 mL of metal test solution was put in the trough with concentrations for the heavy metals: Ni: 20 mM, Cu: 5 mM, Zn: 50 mM, Cr: 5 mM, Cd: 5 mM, Pb: 5 mM. As control and for estimating growth, trough of the other plate filled also with distilled aqua. The plates were incubated for 7 days at 25 °C. Then, the growth distance from the trough of the strains were measured as suggested in Figure 6.1.

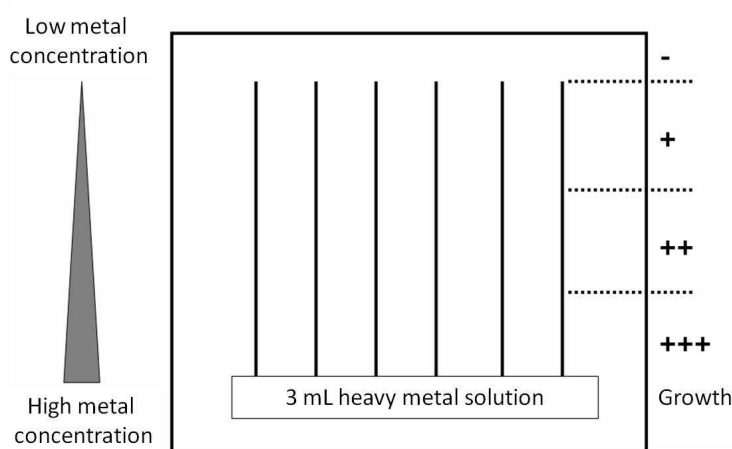


Figure 6.1 Plate trough assay for the qualitative metal resistance test

Quantitative metal resistance test:

The most resistant strains from the qualitative test could be further analyzed in a quantitative test, which evaluates the limits of metal resistance. The minimal medium was prepared as described above, with heavy metals at different concentrations (Ni, 10 mM; Cu, 2.5 mM; Zn, 10 mM; Cd, 2.5 mM; Pb, 2.5 mM). The plates were streaked with bacterial strains and incubated for 7 days at 25 °C. Then, the growth of isolates was evaluated.

- ***Selection of PGPB able to fix atmospheric nitrogen***

The PGPB have a great impact on nitrogen nutrition of plants by increasing their NO₃⁻ uptake capacity, indirectly as a consequence of stimulated lateral root development and possibly directly by stimulating NO₃⁻ transport systems (Mantelin and Touraine, 2004). It is attractive to explain the positive effect of PGPB on plant growth by the provision of nitrogen fixed by the bacteria to the host plant. This 'biofertilization' hypothesis is all the more attractive since N availability is the main yield-limiting factor in many agricultural situations and the leaching of N fertilizers into groundwater causes environmental problems. In this study, the bacterial capacity to fix nitrogen was assessed by a nitrogen-free medium (Azotobacter medium). This medium comprised the following (per liter): CaCl₂·2H₂O 0.1 g; MgSO₄·7H₂O 0.1 g; Na₂MoO₄·2 H₂O 5 mg; K₂HPO₄ 0.9 g; KH₂PO₄ 0.1 g; FeSO₄·7H₂O 0.01 g; CaCO₃ 5 g; Agar 15 g; Distilled water 950 ml. After the medium's pH was adjusted to 7.3 and it was autoclaved at 121 °C for 20 min. The Azotobacter medium was successively supplemented with glucose and mannitol (Glucose 5 g l⁻¹; Mannitol 5 g l⁻¹) sterilized separately from the other compounds. Then, the bacterial strains were streaked on the agar plates prepared with the Azotobacter medium (four to eight stains per plate). The plates were incubated for 7 days at 25 °C and bacterial growth was observed as qualitative evidence of the atmospheric nitrogen fixation.

- ***Selection of PGPB able to produce siderophores***

Iron, an element essential for microbial growth, is mostly unavailable because it is mainly present in soil in highly insoluble form of ferric hydroxide (Fe³⁺). To sequester iron from the environment, numerous soil microorganisms secrete low-molecular-weight organic compounds, iron binding molecules, called siderophores, which have a high capacity for binding Fe³⁺ and render it available for reduction to Fe²⁺, which is preferred by plants. The now-soluble, bound iron is transported back to the microbial cell and is available for their growth.

Moreover, the siderophores play a significant role in metal mobilization and accumulation (Dimkpa, et al., 2009; Rajkumar et al., 2010), as these compounds produced by PGPB solubilise unavailable forms of heavy metal-bearing Fe but also form complexes with bivalent heavy metal ions that can be assimilated by root mediated processes (Carrillo-Castañeda et al., 2003; Braud et al., 2009). Moreover, siderophores secreted by PGPB may also reduce the growth of phytopathogens present in the rhizosphere (Ma et al., 2011). In this study, the chrome azurol S (CAS) assay was used to detect siderophores, according to Schwyn and Neilands (1987). On CAS agar plates, siderophore-producing bacteria form colonies with an orange halo. This occurs because iron is removed from the original blue CAS-Fe (III) indicator during siderophore production. Formation of siderophore halos was evaluated following 5 days of colony incubation at 28 °C. CAS-Fe-Indicator contained (per 100 ml): Chromazurol S (CAS) 60.5 mg and 10 ml (1 mM FeCl₃.6 H₂O in 10 mM HCl); CTAB (cetyl trimethyl ammonium bromide) 72.9 mg. The Buffer-solution (per 750 ml): PIPES 30.24 g, 50% w/w NaOH-solution 12 g, Agar 15 g. The buffer solution was adjusted to 750 ml with distilled water. The CAS-Indicator and buffer solution were autoclaved separately, and then mixed carefully by stirring with C-source and trace elements (500 ml). The plates were prepared with one half of this nutrient agar and the other half with the CAS-medium. Then, the bacterial strains were streaked on the part containing nutrient agar of plates, which were incubated for 7 days at 25 °C. Afterwards the color change of the CAS indicator was evaluated: if the indicator turns from blue to orange/yellow the strain produces and releases iron chelating compounds.

- ***Selection of PGPB able to mobilize phosphate***

Phosphorus is the second most limiting mineral nutrient affecting terrestrial plant growth. Although phosphorus is often abundant in soil, it mostly exists in insoluble form with only a very small fraction (~0.1%) available to plants. Plants can only take up phosphorus in monobasic (H₂PO₄⁻) or dibasic (HPO₄²⁻) soluble forms (Stevenson and Cole, 1999). The elevated levels of heavy metals in soil interfere with P uptake and lead to plant growth retardation (Zaidi et al., 2006). Under metal stressed conditions, most metal-resistant PGPB can either convert these insoluble phosphates into available forms through acidification, chelation, exchange reactions, and release of organic acids such as gluconic acid and 2- ketogluconic acid or mineralize organic phosphates by secreting extracellular phosphatases. An increase in P availability to plants through the inoculation of phosphate-solubilizing bacteria has been reported in pot experiments and under field conditions (Ma et al., 2011). The Pikovskaya's medium (PVK) (Pikovskaya, 1948) was used to identify isolates able to solubilize phosphates.

This medium comprised the following (per liter): Glucose 10 g; $\text{Ca}_3(\text{PO}_4)_2$ 5 g; $(\text{NH}_4)_2\text{SO}_4$ 0.5 g; NaCl 0.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g; KCl 0.2 g; Yeast extract 0.5 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.002 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.002 g; Agar 15 g. The medium's pH was adjusted to 7.0. The bacterial strains were streaked on the agar plates prepared with the Pikovskaya's medium. The plates were incubated for 14 days at 25 °C. The halo appearing around the colony after the incubation time indicates phosphate solubilization ability of strains.

- ***Selection of PGPB able to produce auxin***

The beneficial effects of PGPB on plants have also been attributed to the production of phytohormones that promote root development and proliferation resulting in efficient uptake of water and nutrients. The positive effects of PGPB on plant growth are always correlated with remarkable changes in root morphology, namely increased lateral root length and root hair number and length (Bertrand et al., 2000). It is generally assumed that these developmental responses are triggered by phytohormones produced by the bacteria (Bloemberg and Lugtenberg, 2001; Persello-Cartieaux et al., 2003). Among the plant growth regulators, auxin may play a major role (Mantelin and Touraine, 2004). The Salkowski assay was used for quantifying indole-3-acetic acid production (IAA) according to Patten and Glick (2002). The isolated bacterial strains were grown overnight in 5 ml of DF salts minimal media. DF salts minimal medium comprised the following (per liter) (Dworkin and Foster, 1958): $(\text{NH}_4)_2\text{SO}_4$, 2 g; KH_2PO_4 , 4 g; Na_2HPO_4 , 6 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg; H_3BO_3 , 10 µg; MnSO_4 , 10 µg; ZnSO_4 , 70 µg; CuSO_4 , 50 µg; NaMoO_3 , 10 µg. Then, 20-µl aliquots of bacterial precultures were transferred into 5 ml of DF salts minimal media supplemented with the following concentrations of L-tryptophan (from a filter-sterilized 2-mg ml⁻¹ stock prepared in warm water; Sigma): 0, 50, 100, 200, and 500 g ml⁻¹. After 42 h of incubation, the density of each culture was measured at spectrophotometer (600 nm), and then the bacterial cells were removed from the culture medium by centrifugation (5.500 g for 10 min). A 1-ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4 , 250 ml of distilled H_2O , 7.5 ml of 0.5 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Gordon and Weber, 1951). The tubes containing the mixture were allowed to stand at room temperature for 30 min, for color development. Salkowski reagent turns from yellow to pinkish in presence of indoles. Then, the absorbance at 535 nm was measured. The concentration of IAA in each culture medium was determined by comparison with a standard curve, prepared with indole-3-acetic acid (IAA).

- ***Identification of bacterial strains***

Four bacterial strains of the 21 isolated and selected as described above, were chosen in accordance to their performances to constitute the bacterial consortium for bioaugmentation purposes. The selected strains were identified by means of 16 S r-DNA sequencing. Single-colony 16S r-DNA amplification was performed with a GeneAmp PCR System 9700 thermocycler (Perkin Elmer, Norwalk, CT, USA), using the universal Eubacteria primers P0 (5'-GAG AGT TTG ATC CGT GCT CAG- 3') and P6 (5'-CTA CGG CTA CCT TGT TAC GA- 3'), yielding a fragment of about 1500 bp. Each PCR reaction (50 µl) contained 2 µl of cell lysate (obtained from a single colony of each isolate, according to the procedure described by Di Cello et al., 1997), 25 µl 2× Master Mix (Fermentas, Burlington, Ontario, Canada), 50 pmol of each primer and 0.5 µl formamide. The PCR thermal cycling scheme consisted of 1 min at 95 °C, followed by 30 s at 95 °C, 30 s at 60 °C, 4 min at 72 °C (5 cycles); then 30 s at 95 °C, 30 s at 55 °C, 4min at 72 °C (5 cycles); then 30 s at 95 °C, 30 s at 50 °C, 4min at 72 °C (25 cycles), followed by a final extension at 72 °C (10 min) and at 60 °C (10 min). PCR products were purified and concentrated with Microcon 100 (Millipore, BiollERICA,MA, USA), following the manufacturer's instructions. The sequencing was performed by Genechron (ENEA, Italy) using the same pair of primers. The sequences obtained were compared to data-base sequences using the BLAST system (<http://www.ncbi.nlm.nih.gov/BLAST>) and deposited in the GenBank® genetic sequence database (Nucleic Acids Research, 2008; 36 (Database issue): D25-30). All primers were purchased from Metabion (Martinsried, Germany).

- ***Pot experiment design***

Soil sampling

The bioaugmentation can be exploited to enhance the phytoremediation potential through the augmentation of catabolically-relevant organisms improving the soil quality restoration. A pot experiment was carried out to assess the effects of bioaugmentation on the microbial functional and genetic diversity in a heavy metals polluted soil. This pot experiment was a preliminary test for the Umbrella project and it was set up on basis the aims and protocols established for this European project. The bacterial strains isolated from the Fan and Riz soil and selected for their characteristics as PGPB, were tailored to the microbial formula for the bioaugmentation. The bioaugmentation technique was applied to the soil, collected from the test site Gessenwiese (Thuringia, Germany) in October 2010. Gessenwiese, one of the six sites chosen for the Umbrella project, is located at the base area of the former uranium-leaching heap Gessenhalde near Ronneburg.

The Gessenhalde was a leaching heap that was built up by waste rocks with a low grade of uranium mineralisation (Rüger and Dietel, 1998). Between 1946 and 1990, the Eastern Thuringian mining district in Germany produced about 200,000t of uranium. Until 1978, uranium was leached with acid mine drainage from underground mines and subsequently with sulphuric acid (Wismut, 1994). During the leaching process, leachate infiltrated through the lining of the heap and accumulated in the glacial sediments underneath. In 2004, the test site Gessenwiese was created in the northern part of the base area of the former leaching heap. Soil samples from the top 30 cm were collected in sterile plastic bags; kept refrigerated and transported to the laboratory, where they were oven-dried at 40 °C, sieved (\varnothing 2 mm) and pooled. Some chemical and biochemical characteristics were determined. The general characteristics of the Ronneburg soil are reported in the following table (Table 6.2). The soil material at the former Gessenhalde site has been mapped as sandy loam (10–30% loam) and the total concentrations of the heavy metals in the soil are not extremely high.

Table 6.2 The main characteristics of Ronneburg soil

Parameter	Ronneburg soil
Total Carbon (%)	0.23
Organic Carbon (%)	0.05
pH (H ₂ O)	5.06
*CEC (cmol(+) kg ⁻¹)	12.15
Clay (%)	9.03
Loam (%)	27.39
Sandy (%)	63.58
Humidity (%)	0.32
*Cmic (µg g ⁻¹)	219
*Nmic(µg g ⁻¹)	8.6
Total Copper (mg kg ⁻¹)	46
Total Lead (mg kg ⁻¹)	21
Total Zinc (mg kg ⁻¹)	84

*CEC: Cation Exchangeable Capacity; Cmic: microbial Carbon; Nmic: microbial Nitrogen

Greenhouse and pots

The pot experiment was carried out in a greenhouse in Isserstadt (Thuringia, Germany), under defined climatic conditions: light/night ratio (16/8 h), humidity (60%), temperature (21 °C). 12 standard plant pots (height 11 cm, diameter pot at top 12 cm, diameter pot footprint 6 cm, diameter pot tray 12 cm) were prepared.

Six pots were filled with 1 kg of Ronneburg soil: three were prepared for testing the bioaugmentation (R+), three were as control soil without the bacterial inoculation (R-). The other six pots were filled with the quartz sand (3 pots with and 3 pots without bacteria, S+ and S-, respectively). The pots with the sand were useful to test the plant growth. The soil was irrigated with distilled water and the quartz sand with the Helriegel's nutrition solution, which comprised the following (per liter): $\text{Ca}(\text{NO}_3)_2$, 0.492g; KH_2PO_4 , 0.136g; KCl , 0.075g; MgSO_4 , 0.06g; FeCl_3 , 0.025g. The applied irrigation to plots was to 70% of the water-holding capacity of the two growth substrates.

Plants for microbial assisted phytoremediation in pot experiments

Euphorbia L., one of the most diverse genera of flowering plants, and the largest genus of the Euphorbiaceae, includes about 2000 species (Govaerts et al., 2000; Radcliffe-Smith, 2001). The genus has a nearly cosmopolitan distribution and is extremely variable in life form consisting of annual as well as perennial herbs, shrubs, succulents, lianas and trees (Chehregani and Malayeri, 2007). Some species are known as hyper-accumulators of heavy metals and are appropriate candidates in phytoremediation (Chehregani et al., 2009; Nouri et al., 2010; Salmakia et al., 2011). *Euphorbia helioscopia* L. (sun spurge) could represent a possible phytoremediation agent for the Italian mining site Montevecchio-Ingurtosu, being endemic and diffused in this area. The seeds of *Euphorbia helioscopia* L. (supplied by the IPK-Institut für Pflanzengenetik und Kulturpflanzenforschung) were incubated in growth chamber for 7 days at 24 °C on moistened layers of filter paper. Then, four seedlings per pot were bedded out.

Establishing a microbial formula for bioaugmentation

Bioaugmentation was performed using the strains isolated from the Fan and Riz soils and selected on purpose for multiple resistances to heavy metals and for characteristics as PGPB. Each strain of the consortium was grown as a pure culture in 100 ml of St1 liquid medium in 500-ml Erlmeyer flask for 1 day at 28 °C in orbiting shaker (180 rpm). The bacterial cultures were centrifuged and twice washed. After washing, the biomass was suspended in 30 ml of NaCl 0.9%. The appropriate serial dilutions were performed to determine the CFU of every strain. The individual suspensions were then pooled in equal proportions to setup the consortium for bioaugmentation. The microbial consortium was inoculated in soil at a final concentration of 10^7 CFU g^{-1} . Cell suspension was applied in the pots close to the seedlings by pipetting.

- ***Microbial biomass content***

Microbial biomass C (C_{mic}) and microbial biomass N (N_{mic}) are affected by management practices and can be used as sensitive indicators of ecological stability. Although the soil microbial biomass C (C_{mic}) constitutes only 1–3% of total soil C and the biomass N (N_{mic}) up to 5% of total soil N, they are the most labile C and N pools in soils (Jenkinson and Ladd 1981). Therefore, nutrient availability and productivity of agroecosystems mainly depend on the size and activity of the soil microbial biomass (Friedel et al. 1996). The turnover time for N immobilized in the microbial biomass was found to be about ten times faster than that derived from plant material (Smith and Paul 1990). The determination of N_{mic} is, therefore, important for the quantification of N dynamics in agricultural ecosystems because it controls soil inorganic N availability and loss, especially in high input systems. The C and N in the microbial biomass were determined using the fumigation-extraction method (Vance et al., 1987; Brookes et al., 1985) with an extracting solution of 0.5 M K₂SO₄ (for more details see the paragraph 5.4). Microbial biomass N was analyzed as ninhydrin-reactive nitrogen in fumigated and non-fumigated samples following the method of Joergensen and Brookes (1990). The tubes with the soil extracts and the ninhydrin reagent (1 ml) were placed in a boiling water bath for 15 min, shaken for 30 s, and cooled to room temperature. Then, 4 ml of ethanol-to-water mixture 1:1 (v:v) were added and the samples were analyzed against L-leucine-N standards as described Amato and Ladd (1988) at the spectrophotometer (570 nm). Calculation of microbial ninhydrin-reactive N (N_{mic}) is as reported in equation (a), where 5 is a correction factor.

$$N_{mic} = (N_{nin} \text{ extracted from the fumigated soil}) - (N_{nin} \text{ extracted from the nonfumigated soil}) * 5 \text{ (a)}$$

- ***Enzyme activity***

The final goal of any remediation process must be not only to remove the contaminants from the polluted soil but also, most importantly, to restore the capacity of the soil to function according to its potential (Epelde et al., 2009). The measurements of microbiological and biochemical parameters such as microbial biomass, enzyme activities and the diversity of soil microbial communities are a good index of the variations occurring in the soil system (Labud et al., 2007). Since the enzymatic activities in the soil are mainly of bacterial and fungal origin, the characterization of soil enzyme patterns can improve knowledge on microflora activity, soil productivity and the impact of pollutants (Pankhurst et al., 1995).

Kandeler and Böhm (1996) suggested that the enzyme diversity of a soil provides an effective approach to examine its functional diversity. The pot experiment was also established to evaluate the effect of microbial bioaugmentation in terms of hydrolytic enzyme activity. In this study, enzyme activity was measured according to the methods of Marx et al. (2001) and Vepsäläinen et al. (2001), based on the use of fluorogenic methylumbelliferyl (MUF)-substrates. Soil was analysed for β -cellobiohydrolase (CELL) (EC 3.2.1.91), N-acetyl- β -glucosaminidase or chitinase (CHIT) (EC 3.2.1.30), β -glucosidase (β -GLU) (EC 3.2.1.21), acid phosphatase (PHOS) (EC 3.1.3.2), arylsulfatase (ARYL) (EC 3.1.6.1), xylosidase (XYL) (EC 3.2.2.27), butyrate esterase (BUT) (EC 3.1.1.1) and acetate esterase (ACET) using 4-MUF- β -D-cellobioside, 4-MUF-N-acetyl- β -glucosaminide, 4-MUF- β -D-glucoside, 4-MUF- α -D-glucoside, 4-MUF-phosphate, 4-MUF-sulphate, 4-MUF-7- β -D-xyloside, 4-MUF-butyrat and 4-MUF-acetate as substrates, respectively (for more details see the paragraph 5.4).

- ***Community level physiological profiles CLPP***

The diversity in decomposition functions performed by heterotrophic microorganisms represents one of the important components of microbial functional diversity. A simple approach to measure functional diversity is to examine the number of different C-substrates utilized by the microbial community. So, soil microbial functional diversity can be determined through the utilization of community level physiological profiles (CLPPs). In this study CLPPs were assessed using the MicroResp™ and Biolog® techniques. The metabolic profile, obtained by a Biolog® assay and MicroResp™, provides a physiological fingerprinting of the potential functions of the microbial community (Garland and Mills, 1991; Campbell et al., 2003).

MicroResp™

The MicroResp™ was chosen to assess the variations of CLPPs occurred in the inoculated soil. This technique is based on assessing the ability of soil microbial communities to metabolize a range of organic substrates that vary in structural complexity (Campbell et al. 2003) and the catabolic conversion can be used to assess catabolic diversity in soil microbial communities. C-substrates were selected depending on their ecological relevance and the objective of the experiment. Substrates consisted of four carbohydrates: α -D-glucose (GLU), D-Galactose (GA), D-fructose (FRU), L-arabionose (ARA), six amino acids : L-leucine (LEU), L-arginine (ARG), Glycine (GLI), L-aspartic acid (ASP), γ -amino-butyric (BUT) and glutamic acid (ACGLU), three carboxylic acids:

citric acid (CIT), oxalic acid (OX) and L-ascorbic acid (ASC), and two phenolic acids: vanillic (VAN) and syringic acid (SIR) (for more details see the paragraph 5.4).

Biolog®

The Biolog® Ecoplates, specifically designed for community analysis and microbial ecological studies, were used to measure the variations of the substrate utilization patterns that occurred in the bioaugmented soil. The assay is based on the oxidative catabolism of the substrates to generate patterns of sole carbon source utilization. Communities of organisms will give a characteristic reaction pattern called a metabolic fingerprint or profile (Garland and Mills, 1991). This technique is simple, uses an automated measuring apparatus and provides a more meaningful assay of community structure than isolate-based methods. In this study, the heterotrophic microbial community was extracted from 10 g of fresh soil mixture, added with 40 ml of pyrophosphate and stirred with glass beads for 2 h in orbiting shaker (180 rpm) at 25 °C. The slurry was diluted 10 times in PBS and inoculated into Biolog® ECOPlates that contain 31 of the most useful carbon sources for soil community analysis. Then, plates were incubated at 25 °C in the dark and analysed by the Microplate Reader (dual wavelength data: OD590–OD750) once a day until the twentieth day. Kinetic analysis was performed using AWCD (Average Well Colour Development) as parameter that enables to capture an integral picture of differences in carbon sources utilisation. AWCD was calculated as the arithmetic mean of the OD values of all of the positive wells in the plate per reading time (Garland, 1996).

- ***Microbial functional diversity***

The functional diversity of microbial communities includes a vast range of activities including: nutrient transformations, decomposition, plant growth promotion/suppression and modification of soil physical processes (Giller et al., 1997; Wardle et al., 1999). The functional diversity of microbial communities has been found to be very sensitive to environmental changes (Kandeler et al., 1999). Among the functional diversity indicators, the carbon utilization pattern and the measurement of enzymatic activity profiles expressed by the whole bacterial community have been suggested as useful tools to evaluate the soils (Nielsen and Winding, 2002). In the current literature, functional diversity of soil microbial communities is determined alternatively either through enzyme activities or by means of community level physiological profile (CLPP) techniques, such as MicroResp™.

In this study the microbial functional diversity was measured using these two methodological approaches. By data of these two methodologies, catabolic evenness or Simpson-Yule index (a component of microbial functional diversity defined as the uniformity of substrate use) was calculated as a measure of functional diversity (Degens et al., 2001; Bending et al., 2004) (for more details see the paragraph 3.2.1).

- ***Molecular profiling at community level (PCR-DGGE)***

In the last 20 years, molecular techniques have allowed the investigation of bacterial communities without culturing, which gives a more reliable view, particularly of the richness component of diversity (Pickup, 1991; Stackebrandt et al., 1993; Amann et al., 1995; Holben and Harris, 1995). The denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting technique, by means of it the double-stranded DNA fragments with the same length but different basepair sequence, obtained after PCR of rRNA genes, are separated in a denaturant polyacrylamide gel (Muyzer et al., 1993; Dejonghe et al., 2001). It represents a powerful tool to study the bacterial community structures in complex environments as well as in enrichment cultures (Muyzer and Smalla, 1998). In this study, the two complementary fingerprinting techniques denaturing gradient gel electrophoresis (DGGE) and community level physiological profiles (CLPP) are useful to evaluate the difference in the genetic and functional diversity of soil microbial communities in the bioaugmented soil (R+) in respect to the control soil. All genomic DNAs were extracted using ZR Soil Microbe DNA kit (Zymo Research, CA, USA) according to the manufacturer's protocol, using 1 g of soil in triplicate for each sample. For DGGE analysis, 16S rDNA from bacterial community DNA and from the single strains was amplified with the universal primers 9bfm GAGTTTGATYHTGGCTCAG and 1512ru ACGGHTACCTTGTTACGACTT as described in (Mühling et., 2009). Amplification was performed with a denaturing step of 4 min at 96 °C, followed by 30 cycles of 1 min at 96 °C, 1 min at 52 °C, 1.5 min at 72 °C and a final extension at 72 °C (10 min). A nested PCR was performed on these PCR products using primers for the V3 region (338f 5'-CCTACGGGAGGCAGCAG with a GC clamp attached to the 5' end and 534R 5'-ATTACCGCGGCTGCTGG). PCR mixtures (50 µl) contained 5–50 ng of template DNA, 25 µl 2× Master Mix (Bioline, London, UK) and 20 pmol of each primer. The PCR program is a modification of Muyzer protocol: the denaturing step (96 °C, 4 min), was followed by 15 cycles of 30 s at 96 °C, 30 s of touchdown (decreasing the annealing temperature from 62 °C to 55 °C, 1 °C every two cycles), 45 s at 72 °C, then 15 cycles at 55 °C, and a final extension at 72 °C for 7 min.

PCR products were resolved on 8% polyacrylamide gels with a denaturing gradient 35–60% (urea and formamide) in a DCode apparatus (Bio-Rad, CA, USA) run at a constant voltage of 100 V for 16 h. As a reference, a standard was assembled with the strains of the microbial formula, and run in the same gel along with the samples. After electrophoresis, the gels were stained with GelRed (Biotium, CA, USA) and analysed with the 1-D Quantity One (Bio-Rad) software (Sprocati et al., 2011).

- ***Microbial genetic diversity***

The genetic diversity of soil microorganisms is an indicator that provides the basis for all actual and potential functions. Diversity is a function of two components: (i) the total number of species present, known as species richness or species abundance; and (ii) the distribution of individuals among those species, known as species evenness or equitability. In microbial ecology, in view of the difficulty of estimating the different biomass levels of each species, diversity often relates to species richness. Different ecological indices were determined: Simpson's diversity index (1-D), range-weighted richness (Rr), Simpson's evenness index (Ed) and functional organization (Fo), according to Simpson (1949) and Marzorati et al. (2008) (for more details see the paragraph 3.2.2).

- ***Statistical analyses***

Statistical analyses were carried out with Systat version 7. The means and least significant differences between the soil without and with bioaugmentation (R- and R+) were calculated by a one way ANOVA. The significance probability levels of the results are given at the $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***), respectively.

6.5 Results and discussions

- ***Plant Growth-Promoting Bacteria (PGPB)***

Successful application of bioaugmentation techniques is dependent on the identification and isolation of appropriate microbial strains, and their subsequent survival and activity, once released into the target habitat. However, the selection of strains should be taken on the basis of some understanding of the kind of microbial communities present in the source habitat, and preferably with some knowledge of the type of organisms that are common in the target habitat (Thompson et al., 2005). This suggests that such dominant populations at polluted sites could be good candidates as hosts for desired catabolic activities that are to be exogenously introduced for bioremediation in situ (Van der Gast et al., 2004; Alisi et al., 2009). Consequently, bioaugmentation should aim at the rearrangement of the group of organisms dominantly involved in the overall energy flux, so that specific catabolic traits necessary to clean up pollutants are part of that active group. For soil ecosystems, the capacity of plant roots as creators of physical and chemical discontinuity should be used more strategically to bring about such rearrangements (Dejonghe et al., 2001). The best-known microorganisms with PGPR activity are bacteria belonging to the group of *Pseudomonas* species and a wealth of literature has accumulated on the mechanisms underlying their plant growth-promoting activity, as well as on their ecological performance. In addition to strains of the genus *Pseudomonas*, other bacteria such as *Bacillus* spp. have been indicated as effective PGPR organisms (Van Veen et al., 1997). For this study, the two soils Fan and Riz were collected from Ingurtosu to isolate the suitable PGPB for bioaugmentation purpose. Fan and Riz soils had a bacterial population estimated as 2×10^3 UFC g⁻¹ soil and 1.2×10^5 UFC g⁻¹ soil, respectively. Different bacteria were isolated (Table 6.3). Many strains were resistant to heavy metals, produced siderophores and fixed N₂, but only a few mobilized PO₄ and produced the IAA, almost all RIZ. Among these bacterial strains, RIZ 7 (*Pseudomonas* sp.), RIZ 15 (*Pseudomonas koreensis*), RIZ 16 (*Variovorax* sp.), and FAN 5 (*Bacillus cereus*) were chosen to prepare the bioaugmentation formula as they showed the highest resistance to heavy metals and the best performances as PGPB.

Table 6.3 The bacterial strains isolated from Fan and Riz soils, tested for their heavy metal resistance and their characteristics as PGPB for the bioaugmentation formula

Strains	Identification 16S rDNA sequence	Ni	Cd	Pb	Zn	Cu	N ₂ fixation	PO ₄ mobilisation	Siderophore production	IAA production
Riz 6	<i>Arthrobacter sp.</i> 100%	-	+	+	+	-	+	-	+	+/-
Riz 7	<i>Pseudomonas sp.</i> 98%	+	+	+	+	+	+	-	+/-	+
Riz 8	<i>Herminiimonas glacei</i> 100%	-	+	+	-	-	-	-	+/-	+
Riz 13	<i>Pseudomonas sp.</i> 99%	+/-	+	+	-	-	+	+	+/-	+/-
Riz 15	<i>Pseudomonas koreensis</i> 100%	+	+	+	+	-	+	+	+/-	+/-
Riz 16	<i>Variovorax sp.</i> 99 %	-	-	+	-	+	+	+	+	-
Riz 18	<i>Pseudomonas putida</i> 99%	+	+	+	+	+	+	-	+/-	+
Fan 1	<i>Arthrobacter sp. (oxydans)</i> 98%	-	+	+	+	+	+	-	+	-
Fan 3	<i>Nocardia sp.</i> 99%	-	+	+	+	-	+	-	+	-
Fan 4	<i>Arthrobacter sp. (oxydans)</i> 98%	+	+	+	+	+	+	-	+	-
Fan 5	<i>Bacillus cereus</i> 100%	-	-	+	+	+/-	+	-	++	+

- **Pot experiment**

Ten weeks later, the soil samples from the pots were collected and the microbiological and biochemical analysis were carried out. After this period, the seedlings of *Euphorbia helioscopia* L. were still very small, not grown enough to be analyzed for their capability to absorb heavy metals. *Euphorbia helioscopia* L. was not probably able to adapt to Ronneburg soil and to the quartz sand. The quartz sand seemed to be not suitable for this experiment, it suggests to change it with the perlite, which supports better the plants growth and retains better the nutrients.

- **Microbial biomass**

After ten weeks the bioaugmentation determined a positive effect on the content of the microbial communities in soil. Indeed, the microbial carbon and nitrogen increased with bacterial inoculation (Fig. 6.2 and Fig. 6.3).

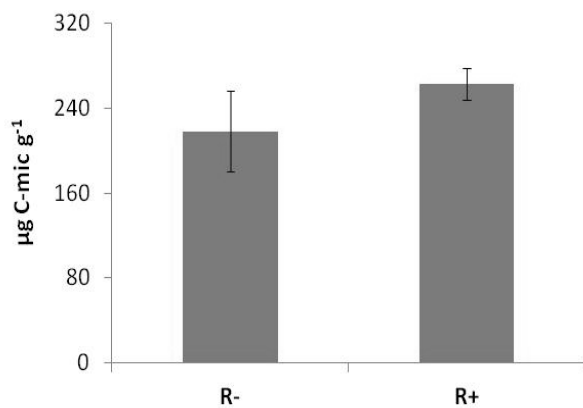


Figure 6.2 Microbial carbon (C_{mic}) in two soils R- and R+ (without and with bioaugmentation). Error standard bars (n=6)

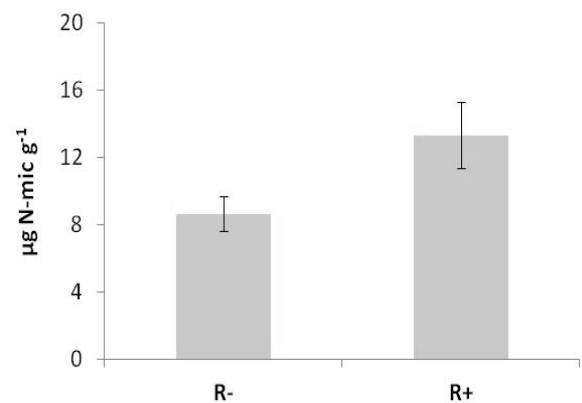


Figure 6.3 Microbial nitrogen (N_{mic}) in two soils R- and R+ (without and with bioaugmentation). Error standard bars (n=6)

- **Enzyme activity**

Soil enzyme activities have been reported to be useful as indicators of soil functional diversity and to provide a unique integrative biochemical assessment of soil function and condition (Dick, 1997; Naseby and Lynch, 2002; Bending et al., 2004; Epelde et al., 2008). The responsiveness of enzyme to environmental disturbance makes them a potential indicator of the soil biological quality (Dick, 1994). In this study the enzyme activities (β -cellobiohydrolase (CELL), N-acetyl- β -glucosaminidase or chitinase (CHIT), β -glucosidase (β -GLU), acid phosphatase (PHOS), arylsulfatase (ARYL), xylosidase (XYL), butyrate esterase (BUT) and acetate esterase (ACET) measured by a fluorimetric assay increased in Ronneburg soil with bioaugmentation, as showed by the Synthetic Enzyme Index (SEI), calculated as the sum of the eight hydrolytic enzyme activities. This index in R+ soil was 25% higher than that of R- soil (Fig. 6.5).

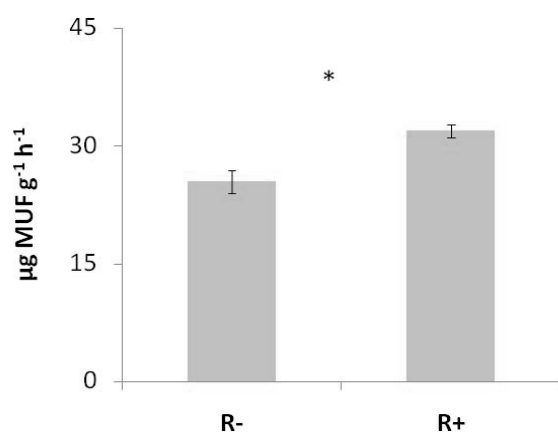


Figure 6.5 SEI (Synthetic Enzyme Index) in two soils R- and R+ (without and with bioaugmentation). Error standard bars (n=3). The starred bars denote significant P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***)

Thus, the enzyme activities in soil with the bioaugmentation (R+) in respect of control soil (R-) were increased with the following percentages: CELL, 41%; CHIT, 27%; β -GLUC, 24%; PHOS, 15%; ARYL, 9%; XYL, 2%; BUT, 23%; ACET, 38% (Fig.6.6).

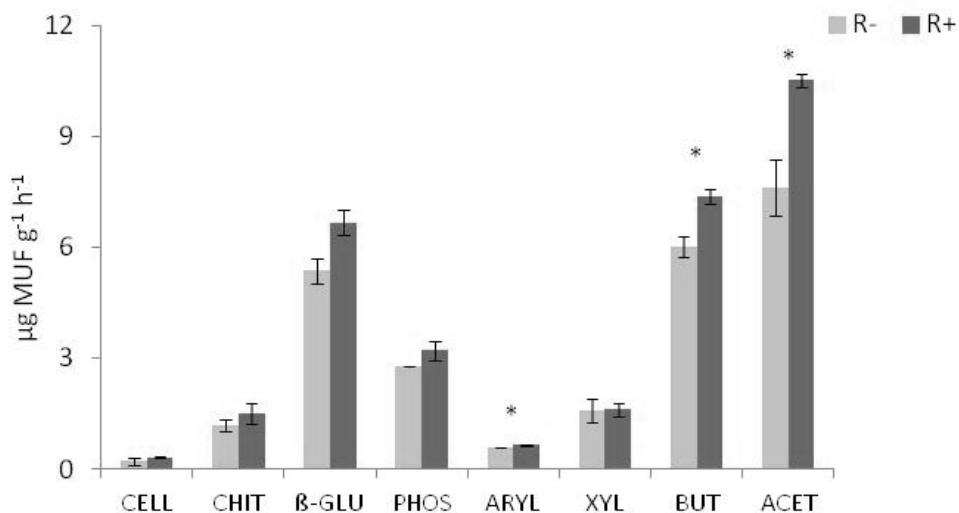


Figure 6.6 Soil enzyme activities measured by fluorimetric in two soils R- and R+ (without and with bioaugmentation). Error standard bars (n=3). The value of the acid phosphatase, butyrate esterase and acetate esterase was divided by 10, due to the value scale of the other enzymes. Error standard bars (n=3). The starred bars denote significant P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***)

- **Community level physiological profiles CLPP**

Soil microbial functional diversity can be determined through the utilization of community level physiological profiles (CLPPs) which reflect the potential of the microbial community to respond to carbon substrates (Bending et al., 2004; Epelde et al., 2008). In this study, the CLPPs of soil microorganisms were measured by MicroResp™ and Biolog® ECOPlates.

MicroResp™

The SIR data ($\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$) obtained by MicroResp™ revealed an increase for all of the C-source group except for the functional groups of carboxylic acids which showed a slight decrease in soil with bioaugmentation (R+) (Fig. 6.7).

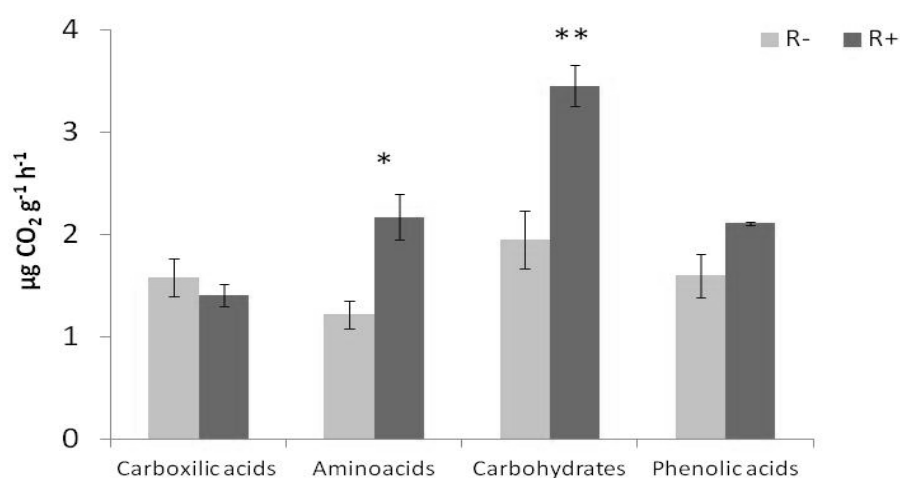


Figure 6.7 CLPP for classes of C-substrates measured by Microresp™ in two soils R- and R+ (without and with bioaugmentation). Error standard bars (n=3) the starred bars denote significant P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***)

As single C-source regards, the production of CO₂ increased with the bioaugmentation in all cases with exception of four substrates: citric acid (CIT), L-ascorbic acid (ASC), arginine (ARG) and the vanilic acid (VAN) which showed a small decrease of SIR (Fig.6.8). The other substrates, in particular those of the carbohydrates groups increased significantly ($P < 0.05$) in the bioaugmented soil (R+).

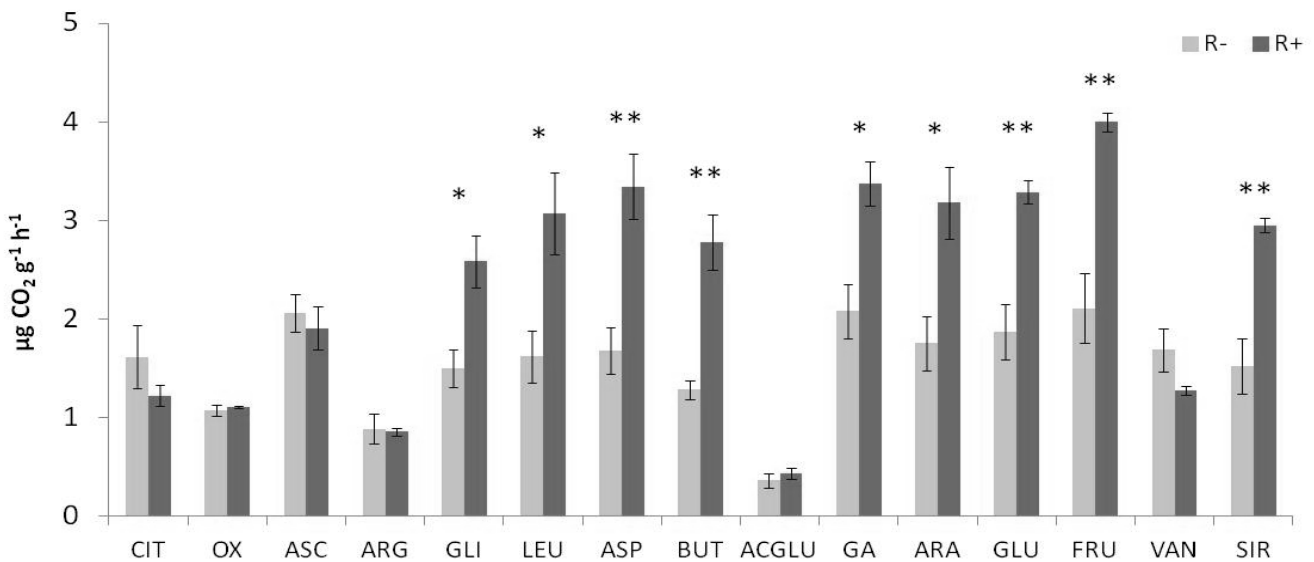


Figure 6.8 CLPP for each substrate (four carbohydrates: α -D-glucose GLU, D-Galactose GA, D-fructose FRU, L-arabionose ARA), six amino acids (L-leucine LEU, L-arginine ARG, Glycine GLI, L-aspartic acid ASP, γ -amino-butyric BUT and glutamic acid ACGLU), three carboxylic acids (citric acid CIT, oxalic acid OX and L-ascorbic acid ASC), and two phenolic acids (vanillic VAN and syringic acid SIR), measured by Microresp in two soils R- and R+ (without and with bioaugmentation). Error standard bars (n=3) the starred bars denote significant (* $P < 0.05$; *** $P < 0.001$)

In this study, Biolog® ECOplates were used to quantify the substrate utilization patterns of the microbial communities which characterized the inoculated soil (R+) in respect of the control soil (R-). The AWCD (Average Well Colour Development) of these two soils did not show significant differences (Fig.6.9).

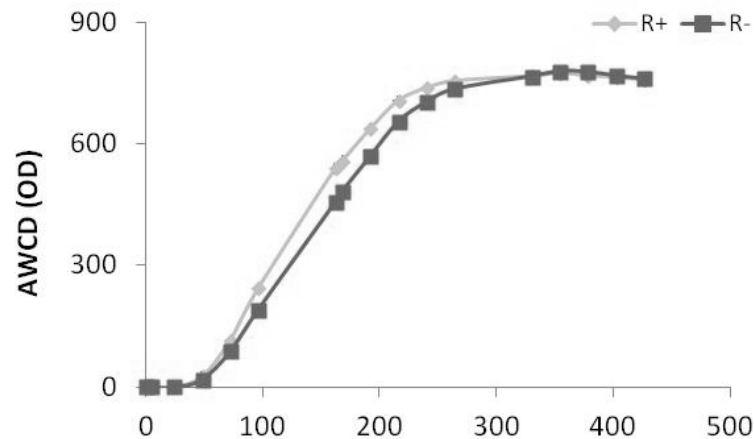


Figure 6.9 Average Well Colour Development (AWCD) curves in two soils R- and R+ (without and with bioaugmentation).

The comparison of AWCD curves (R+ and R-) showed a slight increase of the general metabolic capacity in soil samples R+. This increase of AWCD values in bioaugmented soil could be explained partly by the higher values of microbial carbon and nitrogen (C_{mic} and N_{mic}) measured in R+. However, almost no difference was between R+ and R-, because no substantial alteration of the native community occurred. In some studies is reported that AWCD provides information about differences in community structure and it can be related to microbial biomass (Bååth et al., 1998; Grayston et al., 1998; Yao et al., 2000). Differences between R+ and R- were observed for substrates that belonged to different classes (e.g., carbohydrates). Interestingly, three of the 31 substrates (D-Xylose, i-Erythritol, D-Malic Acid) showed an increase in the soil with bioaugmentation (R+). In particular, the D-Malic Acid was 108% higher in the inoculated soil (Table 6.4).

Table 6.4 Utilization of Biolog® ECOPlates substrates after 18 days and expressed as the percentage of the control

Substrates	R- (OD)	R+ (OD)	Variation (%)
D-Xylose	416±16	550±37	+32%
i-Erythritol	652±19	1181±34	+81%
D-Malic Acid	273±8	567±25	+108%

The ability to utilize a diverse range of nutrients has previously been correlated to competitive ability in the rhizosphere (Bakker et al., 1993; Oresnik et al., 1998). The authors concluded that, in some cases at least, the ability to grow rapidly on a more narrow range of substrates can confer a competitive advantage, if that compound is abundant in the habitat. However, the contrasting conclusions of these studies highlights the difficulty of pinpointing the specific traits that confer fitness on inocula, which are very much dependent on the characteristics of the habitat and strains, and their interactions, which in turn are unlikely to remain constant (Thompson et al., 2005). Finally, the results from Biolog® are concurring with the data of catabolic activity, measured by enzyme and MicroResp™. These three methodologies suggested that there were qualitative differences in the soil microbial communities between the different treatments of soil (R- and R+).

- ***Microbial functional diversity***

It is generally assumed that increased microbial diversity corresponds to increased catabolic potential and, hence, to better removal of metabolites and pollutants (Dejonghe et al., 2001). In this study functional diversity of soil microbial communities was assessed by the catabolic evenness or Simpson-Yule index (CE), a component of microbial functional diversity defined as the uniformity of substrate use. The CE was calculated from catabolic response profiles, using data of enzyme activities and of the community level physiological profiles (CLPPs) measured by MicroResp™. The CE obtained both by data of enzyme activities and the SIR data of Microresp showed a slight increase in the bioaugmented soil (R+) (Fig.6.10 and Fig.6.11, respectively). This means that the bioaugmentation can improve the soil functional diversity probably for the additional catabolic activity of the inoculated bacterial strains.

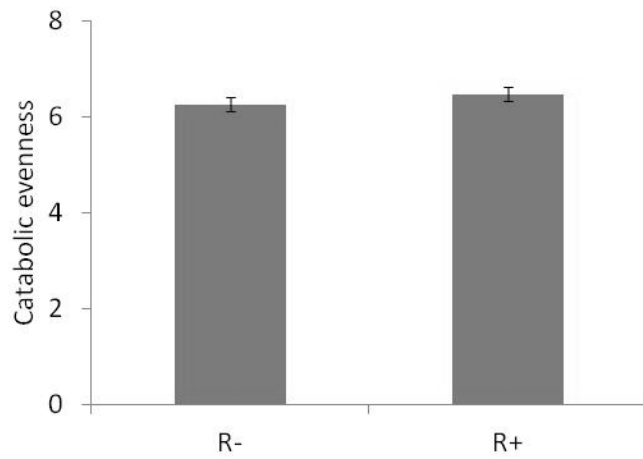


Figure 6.10 The Catabolic evenness or Simpson-Yule index measured by the activity of eight enzymes in two soils R- and R+ (without and with bioaugmentation). Error standard bars (n=3)

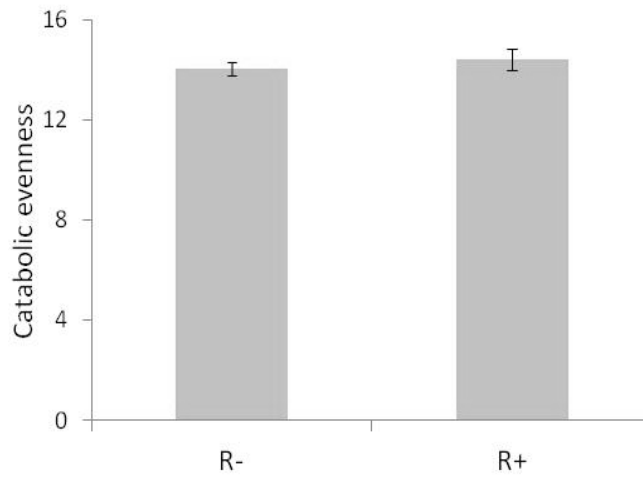


Figure 6.11 The Catabolic evenness or Simpson-Yule index by data of the SIR measured by Microresp in two soils R- and R+ (without and with bioaugmentation). Error standard bars (n=3)

- ***Molecular profiling at community level (PCR-DGGE)***

Further information concerning the microbial community comes from the PCR-DGGE banding profiles, obtained from two different soils (R+ and R-). Molecular fingerprinting techniques such as PCR in combination with denaturing gradient gel electrophoresis (DGGE) offer new approaches to the study of microorganisms in their habitat as they account for as yet uncultured organism. Validation of the successful outcome of a bioaugmentation program should be based on the soil quantitative performance, on the detection of the seeded microorganisms and on the tagging of the genes transferred. They are an important complement to conventional methods that require cultivation or that measure bacterial activities. Despite the phenomenally large and ever increasing resource of pollutant-degrading microbial isolates in laboratories around the globe, inoculums survival remains the ‘Achilles’ heel’ for bioaugmentation of contaminated land. Different strains, even when genetically very similar, have different competencies, in terms of survival, when introduced into the environment as inocula.

- ***Microbial genetic diversity***

In this study, ecological indices were calculated from the DGGE banding profiles, according to Marzorati et al. (2008) to evaluate the effects of bioaugmentation on the genetic diversity. The indices of the microbial genetic diversity used were: range-weighted richness (Rr), Simpson’s diversity index (1-D), the evenness (Ed) and the functional organization (Fo) (Table 6.5).

Table 6.5 Ecological indices calculated from the DGGE banding profiles. Range-weighted richness (Rr), Simpson’s diversity index (1-D), evenness index (Ed) and the functional organization (Fo)

Indices of genetic diversity	R-	R+
Rr	55.50	91.09
Simpson's diversity index (1-D)	0.94	0.97
Ed	0.99	1.01
Fo	45.75	45.95

Range weighted richness (R_r) can be defined in ecology as the carrying capacity of an environment (i.e. the number of individuals that the resources of an environment can support): applied to DGGE, it represents the probability to host a high number of bands with a wide GC variability. The R_r calculated from the DGGE profiles showed a higher richness in the bioaugmented soil (R_+) (91.09) than the R_r measured for the R_- (55.50). Such ecological considerations can be evaluated by using the criterion of whether bioaugmentation leads to an overall increase in the 'microbial richness' of the soil under remediation. Indeed, the introduction of allochthonous organisms or genes is an effective way of increasing species richness and has also been shown to be effective in terms of soil remediation. In a review of current and emerging strategies of soil bioaugmentation, Dejonghe et al. (2001) indicate that this richness could reflect not only an expanded catabolic repertoire but also an enhanced capacity of the exogenously introduced microorganisms to capture a major portion of the overall metabolic energy flux in relation to the autochthonous flora. Simpson's diversity index (1-D) measures the probability that two individuals randomly selected from a sample will belong to different species. The value of this index ranges between 0 and 1 and the greater the value, the greater the sample diversity. In this study the Simpson's diversity index of inoculated soil was not very different from its value in the control soil (0.97 and 0.94, respectively). The evenness (E_d) showed similar results for the soil with the bioaugmentation and the control soil (0.99 and 1.01, respectively). Functional organization (F_o) is the result of the action of the microorganisms that are most fitting to the ongoing environmental-microbiological interactions. Table 6.5 reports the percentages of deviation from the theoretical evenness line: the values for the inoculated soil and the control are 45.75 and 45.95, suggesting a similar distribution of the most fitting species that are dominant and present in high numbers. According to Marzorati et al. (2008) the high concentration of some species and the availability of many others allow the community to potentially deal with changing environmental conditions and preserve its functionality. The analysis of the ecological indices suggests that the microbial community of R_+ was characterized by a high richness (R_r and 1-D), but no significant changes in the functional structure were observed between R_+ and R_- . Finally, the higher richness in R_+ due to the inoculation of the bacterial consortium did not cause a higher and significant specialization of the soil microbial community. These results are positive, that means the inoculated bacteria did not interfere with the microbial autochthonous population in soil.

6.6 Conclusions

Ten weeks later, the bioaugmentation determined a positive effect on the microbial communities in soil. The content of soil microbial biomass, its hydrolytic and catabolic activities were higher in the soil inoculated with the bacterial consortium (R+) than in the control soil (R-). This increase was due to the bioaugmentation as shown also the results obtained by DGGE. However, an increase of the genetic diversity did not match a significant increase of the functional diversity and so the bacterial inoculation did not determine a higher specialization of the soil activities. This means that the bacteria did not alter the microbial ecosystem of the inoculated soil. Bioaugmentation produced some positive effects on soil microbial pool, and so it may lead to the improvement of a phytoremediation process. The soil microbial biomass, its activity, its functional and genetic diversity proved to be good indicators of soil quality variations generated by the bioaugmentation. The evaluation of the molecular and the physiological data suggests that the increase in metabolic activity highlighted in enzymatic and catabolic activities, is related to significant increase of genetic richness within the microbial community, but not to significant changes within the microbial community functionality. Controversies that concern the advantages of bioaugmentation as a viable methodology are indicative of the fragmented knowledge that still exists in the field of bioremediation science and technology and of the less than optimal interaction between industry and academia. Given the recent advances in microbiology, microbial ecology, molecular biology and bioengineering, the current decade will be crucial in giving clear-cut answers that concern the real potential of bioaugmentation. Obviously, the greatest problem in soil inoculations for beneficial purposes is the general complexity of the soil ecosystem, which normally acts as a buffer against incoming microorganisms. The relationship of the inoculated microorganism with its new biotic and abiotic environments, in terms of survival, activity and migration, can be decisive in the outcome of any bioaugmentation strategy. This is especially true in a complex and dynamic biotope such as soil. The rapid decline in population size of active exogenously inoculated microbial cells in soil ('microbiostasis' or 'obstinacy') is attributed to both biotic and abiotic factors. However, on balance, the recent studies are encouraging and thus bioaugmentation might well be used as a rational methodology for site remediation, subject to a thorough understanding of the site's ecology and of the local physicochemical constraints. On the applied side, and given the history of failures or variability of previous microbial releases, the selection and use of different mixtures of ecologically diverse strains with similar functions instead of single strains seems more promising.

The study showed that the microbial formula introduced as a bioaugmentation agent was able to promote the microbial community and its catabolic activity. In conclusion, the tailor-made microbial formula, composed of allochthonous strains previously isolated from a chronic polluted soil, was able to increase the metabolic competences of the system, without perturbing the native microbial community.

Chapter 7-General conclusions

The current remediation techniques of heavy metal polluted soils are expensive and environmentally destructive. The use of alternative and sustainable methods is essential to reclaim the soil, a non renewable resource, which regulates the most important ecosystem processes. The heavy metals are inorganic pollutants, which cannot be degraded and then they are accumulated in the natural ecosystems and also in the food chain. For the risk environmental assessment it is necessary to define their mobility and bioavailability, but currently no standardized methods are defined. This thesis represents an effort to supply new results about the efficiency of soil bioremediation applied to heavy metal polluted areas and new considerations concerning the heavy metal bioavailability, the most important characteristic to be considered. The principal conclusions of the thesis can be resumed in the following points:

- the selected soil microbiological and biochemical properties (microbial biomass, its activity, functional and genetic diversity) resulted sensitive to the variations of soil quality occurred after the phytoremediation and the bioaugmentation. Then, the sensitivity, rapid response and integrative character of the soil microbiological and biochemical properties make them invaluable bioindicators for the assessment of the efficiency of metal bioremediation processes;
- CHCl_3 -labile metals may represent a new methodology to quantify the accumulation of the heavy metals into soil microbial biomass, even if further research is necessary to test the reliability of this indicator;
- an interdisciplinary study is essential to investigate the heavy metals bioavailability. Soil chemical, biochemical and biological properties strongly influence the heavy metals availability to biological systems and, consequently, the possibilities to use bioremediation as a cleanup tool for heavy metal polluted sites;
- the phytoremediation process caused a significant reduction of soil pollution in a two years period as also reported in previous field experiment. However, the selected bioindicators showed that an eco-physiological stress on soil microbial biomass at least in the medium period occurred.

This stress was probably caused by increasing bioaccumulation of the heavy metals, which showed a high mobility probably due to the effects of the root exudates. For this reason, it is fundamental to study the interactions between microorganisms and plants in the polluted soils, because they are a critical element for the control of the heavy metals bioavailability. Moreover, it is necessary to analyse all components of ecosystem involved in the phytoremediation as the leaching water and plants. These analyses are fundamental to define the pathways of the heavy metals during the phytoremediation process;

- the bioaugmentation treatment caused a positive effect on soil microbial communities. Indeed, it caused an increase of the soil microbial biomass content, the extracellular enzyme and catabolic activities. Nevertheless, the functional diversity of the microbial communities was not increased by the bacterial inoculation. This means that the bacteria did not cause a higher specialization of the soil activities and they had not modified the microbial structure of inoculated soil. The positive effects on soil microbial pool produced by the bioaugmentation may be exploited to improve and speed up the phytoremediation process. Controversies that concern the merits of bioaugmentation are indicative of the fragmented knowledge that still exists in the field of bioremediation science and technology. It is fundamental more research to isolate many and more suitable bacterial strains, native from the soil to be reclaimed. This is probably the best solution to make successful bioaugmentation.

Soil bioremediation in a heavy metal polluted area can take, in many cases, unacceptably long periods of time, but the growth of the phytoremediating plants and the inoculation of the plant-growth promoting bacteria cause beneficial effects on soil quality in a short time, actually enhancing the activity and functionality of the soil microbial communities which are largely responsible for soil functioning. It should never be forgotten that the ultimate goal of any soil remediation process must be not only to remove the contaminants from the polluted soil but, most importantly, to restore the continued capacity of the soil to perform or function according to its potential (i.e. to recover soil quality). Indeed, the conventional treatments aim to remove the pollutants in the shortest possible period, considering the contaminated soil as waste to be disposed of rather than a valuable resource to be cleaned and reused. These treatments do not solve the problem, it merely transfers it to future generations. Differently to them, the bioremediation can be considered as sustainable technology, of course from an environmental point of view.

Certainly, the bioremediation aims to the recovery or improvement of soil quality to preserve the resource soil, where soil quality can be described as *“the continued capacity of a specific kind of soil to function as a vital living system, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, to maintain and enhance the quality of air and water environments, and to support human health and habitation”* (Doran and Parkin, 1996).

References

- Acton D.F., Padbury G.A. (1993) "A conceptual framework for soil quality assessment and monitoring". A Program to Assess and Monitor Soil Quality in Canada. Soil Quality Evaluation Summary. Res Branch Agric. Ottawa, Canada
- Adriano D.C., Weber J., Bolan N., Parasivam S., Koo B.J., Sajwan K.S. (2002) "Effects of high rates of coal fly ash on soil, turfgrass and ground water quality" *Water, Air, Soil Pollution* 139, 365–385
- Adriano D.C., Wenzel W.W., Vangronsveld J., Boland N.S. (2004) "Role of assisted natural remediation in environmental cleanup" *Geoderma* 122, 121–142
- Al Chami Z. (2006) "Effect of compost and manure amendments on zinc bioavailability, speciation and plant translocation in an artificially contaminated soil" Doctoral thesis, Università degli Studi di Bari
- Alef K., Nannipieri P. (1995) "Methods in applied soil microbiology and biochemistry" Academic Press, London, 49–116
- Ali Q., Ahsan M., Khaliq I., Elahi M., Ali S., Ali F., Naees M. (2011) "Role of Rhizobacteria in phytoremediation of heavy metals: An overview" *International Research Journal of Plant Science* Vol. 2(8), 220–232
- Alkorta I., Garbisu C. (2001) "Phytoremediation of organic contaminants in soils" *Bioresource Technol* 79, 273–276
- Alkorta I., Aizpurua A., Riga P., Albizu I., Amezaga I., Garbisu C. (2003) "Soil enzyme activities as biological indicators of soil health". *Rev Environ Health* 18, 65–73
- Alkorta I., Albizu I., Amezaga I., Onaindia M., Buchner V., Garbisu C. (2004b) "Climbing a ladder: a step-by-step approach to understanding the concept of agroecosystem health" *Rev Environ Health* 19, 141–159
- Alkorta I., Hernández-Allica J., Becerril J.M., Amezaga I., Albizu I., Garbisu C. (2004c) "Recent findings on the phytoremediation of soils contaminated with environmentally toxic heavy metals and metalloids such as zinc, cadmium, lead, and arsenic" *Reviews in Environmental Science and Bio/Technology* 3, 71–90
- Alisi C., Musella R., Tasso F., Ubaldi C., Manzo S., Cremisini C., Sprocati A.R. (2009) "Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance" *Science of the Total Environment* 407, 3024–3032
- Allen E.B (1988) "The reconstruction of disturbed arid lands: An ecological approach. In: Glenn EP, Waugh WJ, Moore D, Mckee C, Nelson SG (2001). *Revegetation of an abandoned uranium millsite on the Colorado Plateau, Arizona.*

- Allison S.D., Martiny J.B.H. (2008) "Resistance, resilience, and redundancy in microbial communities" *Proceedings of the National Academy of Sciences of the United States of America* 105, 11512–11519
- Alloway B.J. (1990) "Heavy metals in soils" Glasgow & London: Blackie and Son Ltd
- Amann R.I., Ludwig W., Schleifer K.H. (1995) "Phylogenetic identification and in situ detection of individual microbial cells without cultivation" *Microbiol Rev*; 59, 143–169
- Amato M., Ladd J.N. (1988) "Assay for microbial biomass based on ninhydrin–reactive nitrogen in extracts of fumigated soils" *Soil Biol Biochem* 20,107–114
- Anderson T.A., Guthrie E.A., Walton B.T. (1993) "Bioremediation in the rhizosphere. Plant roots and associated microbes clean contaminated soil" *Environ Sci Technol* 27,2630–2636
- Anderson T.H. (2003) "Microbial eco–physiological indicators to assess soil quality" *Agriculture, Ecosystems and Environment* 98, 285–293
- Anderson T.H., Domsch K.H. (1990) "Application of eco–physiological quotients (qCO₂ and qD) on microbial biomasses from soils of different cropping histories" *Soil Biol Biochem*;22:251– 255
- Anderson T.H., Domsch K.H. (1993) "The metabolic quotient for CO₂ (qCO₂) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils" *Soil Biol Biochem*;25, 393–395
- AOAC (1990) "AOAC official methods of analysis" 15th ed. Association of official analytical chemists, Arlington, Virginia, 84–85
- Arshad M.A., Martin S. (2002) "Identifying critical limits for soil quality indicators in agro–ecosystems" *Agric. Ecosyst. Environ.* 88, 153–160.
- Axiom Environmental (2009) "Technical appendices for barite soil remediation guidelines" *Climate Change, Air, and Land Policy Branch Alberta Environment*, ISBN No. 978–0–7785–7693–8
- Bååth E (1989) "Effects of heavy metals in soil on microbial processes and populations (a review)" *Water Air Soil Pollut* 47,325–375
- Baker A.J.M. (1981) "Accumulators and excluders strategies in the response of plants to heavy metals" *J. Plant Nutr.* 3, 643–654
- Baker A.J.M., McGrath S.P., Reeves R.D., Smith J.A.C. (2000) "Metal hyperaccumulator plants: a review of the ecology and physiology of a biochemical resource for phytoremediation of metal–polluted soils" *In Phytoremediation of Contaminated Soil and Water*. Eds. N Terry, G Bañuelos and J Vangronsveld. 85–107, Lewis Publishers, Boca Raton, FL, USA
- Baldrian P. (2003) "Interaction of heavy metals with white–rot fungi." *Enzyme and Microbial Technology* 32, 78–91
- Bañuelos G.S. (2006) "Phyto–products may be essential for sustainability and implementation of phytoremediation." *Environ Pollut* 144, 19–23

- Barajas Aceves M., Grace C., Ansorena J., Dendooven L., Brookes P.C. (1999) "Soil microbial biomass and organic C in a gradient of zinc concentrations in soils around a mine spoil tip" *Soil Biol. Biochem.* 31, 867–876
- Barkay T., Schaefer J. (2001) "Metal and radionuclide bioremediation: issues, considerations and potentials" *Current Opinion in Microbiology* 4, 318–323
- Bar-Ness E., Hadar Y., Chen Y., Shanzer A., Libman J. (1992) "Iron uptake by plant from microbial siderophores" *Plant Physiol.*, 99(4), 1329–1335
- Bastida F., Zsolnay A., Hernández T., García C. (2008) "Past, present and future of soil quality indices: A biological perspective" *Geoderma* 147, 159–171
- Baudoin E., Benizri E., Guckert A. (2003) "Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere" *Soil Biol. Biochem.* 35, 1183–1192.
- Belimov A.A., Hontzas N., Safronova V.I., Demchinskaya S.V., Piluzza G., Bullitta S., Glick B.R. (2005) "Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.)" *Soil Biology & Biochemistry* 37, 241–250
- Belimov A.A., Kunakova A.M., Safronova V.I., Stepanok V.V., Yudkin L.Y., Alekseev Y.V., Kozhemyakov A.P. (2004) "Employment of rhizobacteria for the inoculation of barley plants cultivated in soil contaminated with lead and cadmium" *Microbiology (Moscow)*, 73(1), 99–106
- Bending G.D., Turner M.K., Jones J.E. (2002) "Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities" *Soil Biol. Biochem.* 34, 1073–1082
- Bending G.D., Turner M.K., Rayns F., Marx M.C., Wood M. (2004) "Microbial and biochemical soil quality indicators and their potential for differentiating areas under contrasting agricultural management regimes" *Soil Biol. Biochem.* 36, 1785–1792
- Benes P., Gjessing E. T., Steinnes E. (1976) "Interactions between humus and trace elements in fresh water" *Water Res.* 10, 711–716
- Bennett L.E., Burkhead J.L., Hale K.L., Terry N., Pilon M., Pilon-smits E.A.H. (2003) "Analysis of transgenic Indian Mustard plants for phytoremediation of metals-contaminated mine tailings" *J. Environ. Qual.* 32, 432–440
- Bennisse R., Labat M., Elaslí A., Brhada F., Chandad F., Liegbott P.P., Hibti M., Qatibi A.I. (2004) "Rhizosphere bacterial populations of metallophyte plants in heavy metal-contaminated soils from mining areas in semiarid climate" *World Journal of Microbiology and Biotechnology* 20, 759–766
- Bezdicsek D.F., Papendick R.I., Lal R. (1996) "Introduction: importance of soil quality to health and sustainable land management" *In* Doran JW, Jones AJ (ed), *Methods for assessing soil quality*. SSSA Special Publication 49, Soil Science Society of America, Madison, Wisconsin, 1–8

- Biebl, H., Pfennig N. (1981) "Isolation of members of the family Rhodospirillaceae, p. 267–273. In M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), *The prokaryotes, a handbook on habitats, isolation and identification of bacteria*, vol. 1. Springer–Verlag KG, Berlin.
- Bloem J., Hopkins D.W., Benedetti A., (2006) "Microbiological Methods for Assessing Soil Quality" CABI Publishing, Cambridge, 320
- Borch T., Kretzschmar R., Kappler A., Van Cappelen P., Ginder–Vogel M., Voegelin A., Campbell K. (2010) "Biogeochemical redox processes and their impact on contaminant dynamics" *Environ. Sci. & Technol.* 44, 15–23
- Bremer E., van Kessel C. (1990) "Extractibility of microbial 14C and 15N following addition of variable rates of labelled glucose and (NH₄)₂SO₄ to soil" *Soil Biology and Biochemistry* 22, 707–713
- Bremer E., Kuikman P. (1994) "Microbial utilization of 14C(U) glucose in soil is affected by the amount and timing of glucose additions" *Soil Biology and Biochemistry* 26, 511–517.
- Breure A.M., Mulder C., Römbke J., Ruf A. (2005) "Ecological classification and assessment concepts in soil protection" *Ecotoxicology and Environmental Safety*, 62, 211–229
- Broes P.C. (1995) "The use of microbial parameters in monitoring soil pollution by heavy metals" *Biol Fertil Soils*; 19, 269– 279
- Broes P.C., McGrath S.P. (1984) "Effects of metal toxicity on the size of the soil microbial biomass" *Soil Sci.* 35 341–346.
- Brookes P.C. (1995) "The use of microbial parameters in monitoring soil pollution by heavy metals" *Biol. Fertil. Soils* 19, 269–279
- Brooks R.R. (1998) "Geobotany and hyperaccumulators" *In* *Plants that Hyperaccumulate Heavy Metals*. Ed. Brooks R R. pp 55–94. CAB International, Wallingford, UK
- Brooks R.R., Lee J., Reeves R.D., Jaffre T. (1977) "Detection of nickeliferous rocks by analysis of herbarium species of indicator plants" *J. Geochem. Explor.* 7, 49–57
- Broos K., Warne M.ST.J., Heemsbergen D.A., Stevens D., Barnes M.B., Correll R.L., McLaughlin M.J. (2007) "Soil factors controlling the toxicity of copper and zinc to microbial processes in Australian soils" *Environ. Toxicol. Chem.* 26, 583–590
- Bruins M.R., Kapil S., Oehme F.W. (2000) "Microbial resistance to metals in the environment" *Ecotoxicology and Environmental Safety* 45, 198–207
- Brun L.A., Maillet J., Hinsinger P., Pépin M. (2001) "Evaluation of copper availability to plants in copper-contaminated vineyard soils" *Environ Pollut*, 111, 293–302
- Bundy J.G., Paton G.I., Campbell C.D. (2004) "Combined microbial community level and single species biosensor responses to monitor recovery of oil polluted soil" *Soil Biology & Biochemistry* 36, 1149–1159

- Burd G.I., Dixon D.G., Glick B.R. (2000) "Plant growth-promoting bacteria that decrease heavy metal toxicity in plants" *Can J Microbiol* 46, 237–245
- Burns R.G. (1978) "Enzyme activity in soil: some theoretical and practical considerations" In: Burns RG (ed) *Soil enzymes*. Academic Press, London, 295–340
- Burns R.G. (1982) "Enzyme activity in soil: location and a possible role in microbial ecology" *Soil Biol Biochem* 14,423–427
- Caldwell B.A. (2005) "Enzyme activities as a component of soil biodiversity: A review" *Pedobiologia* 49 637–644
- Callaham, M., Rhoades C. R., Heneghan L. (2008) "A striking profile: soils knowledge in restoration management and science" *Restoration Ecology* 16,604–607
- Campbell C.D., Chapman S.J., Cameron C.M., Davidson M.S., Potts J.M. (2003) "A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil" *Appl Environ Microbiol* 69, 3593–3599
- Cao A., Carucci A., Lai T., Bacchetta G., Casti M. (2009) "Use of native species and biodegradable chelating agents in the phytoremediation of abandoned mining areas" *J Chem Technol Biotechnol* 84, 884–889
- Caporali F. (2004) "Agriculture and health" *The challenge of Organic Agriculture* Editeam, Cento (FE), Italy
- Carter M.R. (1993) "Soil Sampling and Methods of Analysis" Canadian Society of Soil Science. Lewis Publishers, 1993
- Carter M.R., Moody P.W., Merry R., Naidu R., Nortcliff S., Gregorich E.G., Doran J.W. (1996) "Soil Quality is in the Hands of the Land Manager" In *Proceedings Advances in Soil Quality for Land Management: Science, Practice and Policy 17–19 April 1996* University of Ballarat, Victoria, Editors: Richard J. MacEwan, Martin R. Carter, ISBN 0 908026 77 3
- Cattani I., Fragoulis G., Boccelli R., Capri E. (2006) "Copper bioavailability in the rhizosphere of maize (*Zea mays* L.) grown in two Italian soils" *Chemosphere* 64, 1972–1979
- Chaignon V., Sánchez-Neira I., Herrmann P., Jaillard B., Hinsinger P. (2003) "Copper bioavailability and extractability as related to chemical properties of contaminated soils from a vine-growing area" *Environmental Pollution*, 123, 229–238
- Chander K., Broes P.C. (1991) "Plant inputs of carbon to metal-contaminated soil and effects on the soil microbial biomass" *Soil Biol. Biochem.* 23, 1169–1177.
- Chander K., Dyckmans J., Joergensen R.G., Meyer B., Raubuch M. (2001) "Different source of heavy metal and their long-term effects on soil microbial properties" *Biol. Fertil. Soil* 34, 241–247

- Chaney R.L., Malik M., Li Y.M., Brown S.L., Brewer E.P., Angle J.S., Baker A.J.M. (1997) "Phytoremediation of soil metals" *Curr Opin Biotechnol* 8, 279–284
- Chapman S.J., Campbell C.D., Artz R.R.E. (2007) "Assessing CLPPs Using MicroResp™ A Comparison with Biolog and multi-SIR" *J Soils Sediments* 7 6,406–410
- Chaudhry T.M., Hayes W.J., Khan A.G., Khoo C.S. (1998) "Phytoremediation—focusing on hyperaccumulator plants that remediate metal-contaminated soils" *Aust J Ecotoxicol*, 4, 37–51
- Chen Y.X., Lin Q., Luo Y.M., He Y.F., Zhen S.J., Yu Y.L., Tian G.M., Wong M.H. (2003) "The role of citric acid on the phytoremediation of heavy metal contaminated soil" *Chemosphere* 50, 807–811
- Chojnacka K., Chojnacki A., Górecka H., Górecki H. (2005) "Bioavailability of heavy metals from polluted soils to plants" *Sci Total Environ*, 337, 175–182
- Clemente R., Almela C., Bernal M.P. (2006) "A remediation strategy based on active phytoremediation followed by natural attenuation in a soil contaminated by pyrite waste" *Environ Pollut* 143, 397–406
- Collins Y.E., Stotzky G. (1989) "Factors affecting the toxicity of heavy metals to microbes" *In* Beveridge TJ, Doyle RJ (ed), *Metal ions and bacteria*. John Wiley and Sons, Toronto, 31–91
- Conesa H.M., Evangelou M.W.H., Robinson B.H., Schulin R. (2011) "A Critical View of Current State of Phytotechnologies to Remediate Soils: Still a Promising Tool?" *The Scientific World Journal*, doi:10.1100/2012/173829
- Cummings D.E., Snoeyenbos-West O.L., Newby D.T., Niggemyer A.M., Lovley D.R., Achenbach L.A. (2003) "Diversity of Geobacteraceae species inhabiting metalpolluted freshwater lake sediments ascertained by 16S rDNA analyses" *Microb Ecol*;46, 257–269
- Cunningham S.D., Berti W.R., (1993) "Remediation of contaminated soils with green plants: an overview" *Vitro Cell Dev Biol* 29,207–212
- Cunningham S.D., Berti W.R., Huang J.W. (1995) "Phytoremediation of contaminated soils" *Trends Biotechnol* 13, 393–397
- Cunningham S.D., Ow D.W. (1996) "Promises and prospects of phytoremediation" *Plant Physiol*, 110, 715– 719
- D'Amore J.J., Al-Abed S.R., Scheckel K.G., Ryan J.A. (2005). "Methods for Speciation of Metals in Soils: A Review" *J. Environ. Qual.* 34, 1707–1745
- Dai J., Becquer T., Rouiller J.H., Reversat G., Bernhard-Reversat F., Lavelle P. (2004) "Influence of heavy metals on C and N mineralisation and microbial biomass in Zn-, Pb-, Cu-, and Cd-contaminated soils" *Applied Soil Ecology* 25 99–109
- Dasappa S.M., Loehr R.C. (1991) "Toxicity reduction in contaminated soil remediation processes" *Water Res* 25, 1121–1130

- De Haan F.A.M., Bourg A.C.M., Broes P.C., Verstraete W., van Riemsdijk W.H., van der Zee S.E.A.T.M., Giraldez J.V., McGrath S.P. (1989) Soil quality assessment. State of the art report on soil quality” Final report to the C. E. E. Directorate–General XII, Science, Research and Development Directorate E, Environment and Non–Nuclear Energy Contract EV4A/0008/NL
- De la Paz Jimenez M., de la Horra A. M., Pruzzo L., Palma R. M. (2002) “Soil quality: a new index based on microbiological and biochemical parameters” *Biol Fertil Soils* 35, 302–306
- de Souza M.P., Huang C.P., Chee N., Terry N. (1999) “Rhizosphere bacteria enhance the accumulation of selenium and mercury in wetland plants” *Planta*, 209(2), 259–263
- de–Bashan, L.E., Hernandez Juan–Pablo, Bashana Y. “The potential contribution of plant growth–promoting bacteria to reduce environmental degradation – A comprehensive evaluation. *Appl. Soil Ecol.* (2011), doi:10.1016/j.apsoil.2011.09.003
- Degens B.P., Schipper L.A., Sparling G.P., Duncan L.C. (2001) “Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance?” *Soil Biology & Biochemistry* 33 1143–1153
- Degens B.P., Schipper L.A., Sparling G.P., Vojvodic–Vukovic M. (2000) “Decreases in organic C reserves in soils can reduce the catabolic diversity of soil microbial communities” *Soil Biology & Biochemistry* 32, 189–196
- Dejonghe W., Boon N., Seghers D., Top E.M., Verstraete W. (2001) “Bioaugmentation of soils by increasing microbial richness: missing links” *Environmental Microbiology* 3(10), 649–657
- Dick R.P. (1997) “Soil enzyme activities as integrative indicators of soil health” In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R. (Eds.), *Biological Indicators of Soil Health*. CAB International, Wallingford, 121–156
- Dilly O., Blume H.P., Sehy U., Jiménez M., Munich J.C. (2003) “Variation of stabilised, microbial and biologically active carbon and nitrogen soil under contrasting land use and agricultural management practices” *Chemosphere* 52, 557–569
- Dilly O., Munch J.C. (1998) “Ratios between estimates of microbial biomass content and microbial activity in soils” *Biol Fertil Soils* 27, 374–379
- Dobler R., Burri P., Gruiz K., Brandl H., Bachofen R. (2001) “Variability in microbial populations in soil highly polluted with heavy metals on the basis of substrate utilization pattern analysis” *J Soil Sediment* 1, 151–158
- Doran J.W., Parkin T.B. (1994) “Defining and assessing soil quality” *In* Doran JW, Coleman DC, Bezdiceck DF, Stewart BA (ed), *Defining soil quality for a sustainable environment*. SSSA Special Publication 35, Soil Science Society of America, Madison, 3–21
- Doran J.W., Parkin T.B., (1996) “Quantitative indicators of soil quality: a minimum data set” *In*: Doran JW, Jones AJ (eds) *Methods for assessing soil quality*. SSSA Special Publication 49, Soil Science Society of America, Madison, WI, 25–37

- Doran J.W., Zeiss M.R. (2000) "Soil health and sustainability: managing the biotic component of soil quality" *Applied Soil Ecology* 15, 3–11
- Dube A., Zbytniewski R., Kowalkowski T., Cukrowska E., Buszewski B. (2001) "Adsorption and Migration of Heavy Metals in soil" *Polish Journal of Environmental Studies* vol.10, No. 1, 1–10
- Dworkin M., Foster J.W. (1958) "Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* 75, 592–601.
- Ehers L.J., Luthy R.G. (2003) "Contaminant bioavailability in soil and sediment" *Environmental Science & Technology* 37, 295–302
- Eilers K.G., Lauber C. L., Knight R., Fierer N. (2010) "Shifts in bacterial community structure associated with inputs of low molecular weight carbon compounds to soil" *Soil Biology & Biochemistry* 42, 896 – 903
- El Fantroussi S., Agathos S.N. (2005) "Is bioaugmentation a feasible strategy for pollutant removal and site remediation?" *Current Opinion in Microbiology*, 8,268–275
- El-Latif Hesham A., Khan S., Liu X., Zhang Y., Wang Z., Yang M. (2006) "Application of PCR–DGGE to analyse the yeast population dynamics in slurry reactors during degradation of polycyclic aromatic hydrocarbons in weathered oil" *Yeast*;23, 879–887
- Elliott L.F., Lynch J.M. (1994) "Biodiversity and soil resilience" In: Greenland, D.J., Szabolcs, I. (Eds.). *Soil Resilience and Sustainable Land Use*. CAB International, Wallingford, UK, 353–364.
- EPA (2000) "Introduction to phytoremediation" National Risk Management Research Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati
- Epelde L., Becerril J.M., Hernández-Allica J., Barrutia O., Garbisu C. (2008) "Functional diversity as indicator of the recovery of soil health derived from *Thlaspi caerulescens* growth and metal phytoextraction" *Applied Soil Ecology* 39, 299–310
- Epelde L., Becerril J.M., Alkorta I., Garbisu C., (2009a) "Heavy Metal Phytoremediation: Microbial Indicators of Soil Health for the Assessment of Remediation Efficiency" In A. Singh et al. (eds.), *Advances in Applied Bioremediation*, *Soil Biology* 17, 299 DOI 10.1007/978-3-540-89621-0_16
- Epelde L., Mijangos I., Becerril J.M., Garbisu C., (2009b) "Soil microbial community as bioindicator of the recovery of soil functioning derived from metal phytoextraction with sorghum" *Soil Biology and Biochemistry* 41, 1788–1794
- Epelde L. (2010) "Evaluation of the efficiency of metal phytoremediation processes with microbiological indicators of soil health" Thesis doctoral, Universidad del País Vasco EHU
- Epelde L., Becerril J.M., Barrutia O., González-Oreja J.A., Garbisu C. (2010) "Interactions between plant and rhizosphere microbial communities in a metalliferous soil" *Environmental Pollution* 158, 1576–1583

- Ernst W.H.O. (1996) "Bioavailability of heavy metals and decontamination of soils by plants" *Applied Geochemistry*, Vol. 11,163–167
- European Environment Agency (EEA) (2010) "The European environment — state and outlook 2010: synthesis" European Environment Agency, Copenhagen
- Fedotov P.S., Mirò M. (2008) "Fractionation and mobility of trace elements in soils and sediments" In: A Violante, P.M. Huang, G.M. Gadd. (eds). *Biophysico-Chemical Processes of Heavy Metals and Metalloids in Soil Environments*. Wiley–Jupac Series, Vol 1 John Wiley & Sons, Hoboken, NY, 467–520
- Fein J.B., Martin A.M., Wightman P.G. (2001) "Metal adsorption onto bacterial surfaces: development of a predictive approach" *Geochim Cosmochim Acta* 65,4267–4273
- Feng M–H., Shan X.–Q., Zhang S., Wen B. (2005) "A comparison of the rhizosphere–based method with DTPA, EDTA, CaCl₂, and NaNO₃ extraction methods for prediction of bioavailability of metals in soil to barley" *Environmental Pollution* 137 231–240
- Fernández M.D., Milagrosa Vega M., Tarazona J.V. (2006) "Risk–based ecological soil quality criteria for the characterization of contaminated soils. Combination of chemical and biological tools" *Science of the Total Environment* 366, 466–484
- Fierer N., Breitbart M., Nulton J., Salamon P., Lozupone C., Jones R., Robeson M., Edwards R.A., Felts B., Rayhawk S., Knight R., Rohwer F., Jackson R.B. (2007) "Metagenomic and small–subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil" *Applied and Environmental Microbiology* 73, 7059– 7066
- Filgueiras A.V., Lavilla I., Bendicho C. (2002) "Chemical sequential extraction for metal partitioning in environmental solid samples" *J. Environ. Monit.* 4, 823–857
- Filip Z. (2002) "International approach to assessing soil quality by ecologically–related biological parameters" *Agriculture, Ecosystems and Environment* 88, 169–174
- Filser J., Koehler H., Ruf A., Römbke J., Prinzing A., Schaefer M. (2008) "Ecological theory meets soil ecotoxicology: Challenge and chance" *Basic and Applied Ecology* 9, 346–355
- Fischer S.G., Lerman L.S. (1979) "Length–independent separation of DNA restriction fragments in two–dimensional gel electrophoresis" *Cell*;16,191–200
- Fitz W.J., Wenzel W.W. (2002) "Arsenic transformations in the soil–rhizosphere–plant system: fundamentals and potential application to phytoremediation" *J Biotechnol* 99,259–278
- Fließbach A., Martens R., Reber H.H. (1994) "Soil microbial biomass and microbial activity in soils treated with heavy metal contaminated sewage sludge. *Soil Biol Biochem* 26, 1201–1205
- Frische T., Mebes K.–H., Filser J. (2003) "Assessing the bioavailability of contaminants in soils: Are view on recent concepts" Federal Environmental Agency (Umweltbundesamt) Report66/2003,Berlin

- Gadd G.M. (2000) “Bioremedial potential of microbial mechanisms of metal mobilization and immobilization” *Current Opinion in Biotechnology* 11, 271–279
- Gadd G.M. (2004) “Microbial influence on metal mobility and application for bioremediation” *Geoderma* 122, 109–119
- Gadd G.M. (2007) “Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation” *Mycological Research* 111, 3–49
- Gadd G.M., Griffiths A.J. (1978) “Microorganisms and Heavy Metal Toxicity” *Microbial Ecology* 4, 303–317
- Garbisu C., Alkorta I., (1997) “Bioremediation: principles and future” *J Clean Technol Environ Toxicol Occup Med* 6,351–366
- Garbisu C., Alkorta I., (2001) “Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment” *Biores Technol* 77,229–236
- Garbisu C., Hernández-Allica J., Barrutia O., Alkorta I., Becerril J.M. (2002) “Phytoremediation: a technology using green plants to remove contaminants from polluted areas” *Rev Environ Health* 17, 173–188
- Garbisu C., Alkorta I. (2003) “Basic concepts on heavy metal soil bioremediation” *Eur J Min Proc Environ Protect* 3, 58–66
- García C., Hernández T. (1997) “Biological and biochemical indicators in derelict soils subject to erosion” *Soil Biol. Biochem.* 29, 171–177.
- Garland J.L., Mills A.L. (1991) “Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology* 57, 2351–2359
- Gelsomino A., Badalucco L., Ambrosoli R., Crecchio C., Puglisi E., Meli S.M., (2006) “Changes in chemicals and biological soil properties as induced by anthropogenic disturbance: a case study of an agricultural soil under recurrent flooding by wastewaters” *Soil Biol. Biochem.* 38, 2069–2080
- Gianfreda L., Bollag J.M. (1996) “Influence of natural and anthropogenic factors on enzyme activity in soil. In: Stotzky, G., Bollag, J.M. (Eds.), *Soil Biochemistry*, vol. 9. Marcel Dekker, New York, 123–194
- Gianfreda L., Ruggiero P. (2006) “Enzyme Activities in Soil” In: *Soil Biology*, Vol.8, 12, *Nucleic Acids and Proteins in Soil*, P. Nannipieri, K. Smalla (Eds.) ©Springer-Verlag Berlin Heidelberg
- Giller K.E., Beare M.H., Lavelle P., Izac A.M.N., Swift M.J. (1997) “Agricultural intensification, soil biodiversity and agroecosystem function. *Applied Soil Ecology* 6, 3–16.
- Giller K.E., Witter E., McGrath S.P. (1998) “Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review”, *Soil Biol. Biochem.* 30, 1389–1414

- Giller K.E., Witter E., McGrath S.P. (1999) "Assessing risks of heavy metal toxicity in agricultural soils" *Human Ecol Risk Assess* 5,683–689
- Gil–Sotres F., Trasar–Cepeda C., Leirós M.C., Seoane S., (2005) "Different approaches to evaluating soil quality using biochemical properties" *Soil Biol. Biochem.* 37, 877–887
- GLASOD (2004) "Global Land Assessment of Degradation"
<http://www.fao.org/landandwater/agll/glasod/glasodmaps.jsp>
- Glass D.J. (1999) "Economic potential of phytoremediation" In *Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment*, eds. I. Raskin and B.D. Ensley, pp 15–31, John Wiley & Sons Inc, New York, NY.
- Gleysez C., Tellier S., Astruc M. (2002) "Fractionation studies of trace elements in contaminated soils and sediments: a review of sequential extraction procedures" *Trends Anal. Chem.* 21, 451–466
- Glick B.R. (1995) "The enhancement of plant growth by free–living bacteria" *Can. J. Microbiol.*, 41, 109–117
- Glick B.R. (2003) "Phytoremediation: synergistic use of plants and bacteria to clean up the environment" *Biotechnology Advances* 21, 383–393
- Glick B.R. (2010) "Using soil bacteria to facilitate phytoremediation" *Biotechnology Advances* 28, 367–374
- Glick B.R., Patten C.L., Holguin G., Penrose D.M. (1999) "Biochemical and genetic mechanisms used by plant growth–promoting bacteria" London, UK: Imperial College Press
- Glick B.R., Penrose D.M., Li J. (1998) "A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria" *J Theor Biol*;190,63–68
- Gordon S.A., Weber R. P. (1951) "Colorimetric estimation of indoleacetic acid" *Plant Physiol.* 26:192–195.
- Grayston S.J., Vaughan D., Jones D. (1996) "Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability" *Applied Soil Ecology* 5, 29–56
- Gregorich E.G., Carter M.R., Angers D.A., Monreal C., Ellert B.H. (1994) "Towards a minimum data set to assess soil organic matter quality in agricultural soils" *Can J Soil Sci* 74, 367–385
- Gremion F, Chatzinotas A., Kaufmann K., Von Sigler W., Harms H. (2004) "Impacts of heavy metal contamination and phytoremediation on a microbial community during a twelve–month microcosm experiment" *FEMS Microbiology Ecology* 48 273–283
- Griffiths B.S., Ritz K., Ebbelwhite N., Dobson G. (1999) "Soil microbial community structure: effects of substrate loading rates" *Soil Biology and Biochemistry* 31, 145–153

- Gryschko R., Kuhnle R., Terytze K., Breuer J., Stahr K. (2004) "Soil Extraction of Readily Soluble Heavy Metals and As with 1 M NH₄NO₃-Solution, Evaluation of DIN 19730" *JSS – J Soils & Sediments (OnlineFirst)*, 1–6
- Gupta S.K., Vollmer M.K., Krebs R. (1996) "The importance of mobile, mobilisable and pseudo total heavy metal fractions in soil for three-level risk assessment and risk management" *The Science of the Total Environment* 178, 11–20
- Hantke K. (2005) "Bacterial zinc uptake and regulators" *Current Opinion in Microbiology*, 8, 196–202
- Harmsen J. (2007) "Measuring Bioavailability: From a Scientific Approach to Standard Methods" *J. Environ. Qual.* 36, 1420–1428
- Harmsen J., Rulkens W., Eijsackers H. (2005) "Bioavailability: Concept for understanding or tool for predicting" *Land Contam. Reclam.* 13, 161–171
- Harris R.F., Bezdicsek D.F. (1994) "Descriptive aspects of soil quality/health" Pages 22–35 in J. W. Doran *et al.*, (eds.) *Defining Soil Quality For A Sustainable Environment*. Soil Science Society of America Special Publication no. 35, Madison, WI
- Hattori H., (1992) "Influence of heavy metals on soil microbial activities" *Soil Science and Plant Nutrition* 38, 93–100
- Heneghan L., Miller S.P., Baer S., Callahan M.A., Montgomery J., Pavao-Zuckerman M., Rhoades C. C., Richardson S., (2008) "Integrating Soil Ecological Knowledge into Restoration Management" *Restoration Ecology* 16, 4, 608–617
- Hietala K.A., Roane T.M. (2009) "Microbial Remediation of Metals in Soils" In A. Singh *et al.* (eds.), *Advances in Applied Bioremediation*, *Soil Biology* 17, 11, Springer-Verlag Berlin Heidelberg
- Hill G.T., Mitkowski N.A., Aldrich-Wolfe L., Emele L.R., Jurkonie D.D., Ficke A., Maldonado-Ramirez S., Lynch S.T., Nelson E.B. (2000) "Methods for assessing the composition and diversity of soil microbial communities" *Applied Soil Ecology* 15, 25–36
- Hinsinger P. (2001) "Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review" *Plant Soil* 237, 173–195
- Hinsinger P., Courchesne F. (2008) "Biogeochemistry of metals and metalloids at the soil-root interface" In: Violante A, Huang PM, Gadd GM (eds) *Biophysic-chemical processes of heavy metals and metalloids in soil environments*. Wiley, Hoboken, USA, 267–311
- Hlavay J., Prohaska T., Weisz M., Wenzel W.W., Stingeder G.J. (2004) "Determination of trace elements bound to soils and sediment fractions" *IUPAC Technical Report. Pure and Applied Chemistry* 76, 415–442
- Hodgson J. F. (1963) "Chemistry of the micronutrient elements in soils" *Adv. Agron.* 15, 119–159

- Hofman J., Bezchlebová J., Dušek L., Doležal L., Holoubek I., Anděl P., Ansorgova A., Maly S. (2003) "Novel approach to monitoring of the soil biological quality" *Environment International* 28 771–778
- Huang J.V., Cunningham S.D. (1996) "Lead phytoextraction: species variation in lead uptake and translocation" *New Phytol.* 134, 73–84
- Huang P.M., Gobran G.R. (2005) "Biogeochemistry of trace elements in the rhizosphere" Elsevier B.V. Amsterdam. In: Bollang J–M, Stotzky G (eds) *Soil Biochemistry*, vol 6. Dekker, New York, 293–355
- Hudson–Edwards K.A., Jamieson H.E., Lottermoser B.G. (2011) "Mine Wastes: Mine Wastes: Past, Present, Future" *Elements*, 7, 375–380
- Ibekwe A.M., Kennedy A.C., Frohne P.S., Papiernik S.K., Yang C.–H., Crowley D.E. (2002) "Microbial diversity along a transect of agronomic zones" *FEMS Microbiology Ecology* 39, 183–191
- Insam H., Domsch K.H. (1988) "Relationship between soil organic carbon and microbial biomass on chronsequences of reclamation sites" *Microb Ecol*;15:177–188
- ISO (2005) "ISO–11074: Soil quality–Vocabulary" ISO, Geneva, Switzerland
- Izquierdo I., Caravaca F., Alguacil M.M., Hernández G., Roldán A., (2005) "Use of microbiological indicators for evaluating success in soil restoration after revegetation of a mining area under subtropical conditions" *Applied Soil Ecology* 30, 3–10
- Jadia C.D., Fulekar M.H. (2009) "Phytoremediation of heavy metals: Recent techniques" *African Journal of Biotechnology*, 8 (6), 921–928
- Jing Yan–de, He Zhen–li, Yang Xiao–e (2007) "Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils" *J Zhejiang Univ Sci B* 8(3),192–207
- Joergensen R.G., Broes P.C., Jenkinson D.S. (1990) "Survival of the microbial biomass at elevated–temperatures" *Soil Biol. Biochem.* 22, 1129–1136
- Joergensen R.G., Mueller T. (1996) "The fumigation–extraction method to estimate soil microbial biomass: Calibration of the Kec value" *Soil Biol. Biochem.*, 28, 33–37
- Kabala C., Singh B.R. (2001) "Fractionation and Mobility of Copper, Lead, and Zinc in Soil Profiles in the Vicinity of a Copper Smelter", *J. Environ. Qual.* 30, 485–492
- Kabata–Pendias A. (2004) "Soil–plant transfer of trace elements— an environmental issue" *Geoderma*, 122, 143–149
- Kalis E.J.J. (2006) "Chemical speciation and bioavailability of heavy metals in soil and surface waters" Doctoral thesis, Wageningen University, The Netherlands
- Kamnev A.A., van der Lelie (2000) "Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation" *Biosci Rep*; 20(4), 239–258

- Kandeler E., Kampichler C., Horak O. (1996) "Influence of heavy metals on the functional diversity of soil microbial communities" *Biol Fertil Soils* 23,299–306
- Karlen, D. L., Andrews S. S., Doran J. W. (2001) "Soil quality: current concepts and applications" *Advances in Agronomy* 74, 1–40
- Karlen, D. L., Andrews S. S., Weinhold B. J., Doran J. W. (2003) "Soil quality: humankind's foundation for survival. *Journal of Soil and Water Conservation* 58,171–179
- Keepax R.E., Lesley N.M., Livens F.R. "Speciation of Heavy Metals and Radioisotopes" *Environmental and ecological chemistry, Vol. II –©Encyclopedia of Life Support Systems (EOLSS)*
- Kelly J. J., Tate R. L. (1998) "Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter" *J. Environ. Qual.* 27,609–617
- Kelly J.J., Haggblom M., Tate R.L. (1999) "Changes in soil microbial communities over time resulting from one time application of zinc: a laboratory microcosm study" *Soil Biol Biochem* 31, 1455–1465
- Khan A.G. (2005) "Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation" *Journal of Trace Elements in Medicine and Biology* 18, 355–364
- Khan F.I., Husain T., Hejazi R. (2004) "An overview and analysis of site remediation technologies" *J. Environ. Manage.* 11, 95–122
- Khan H.S., Joergensen R.G. (2009) "Simultaneous measurement of S macronutrients and heavy metals in the soil microbial biomass with CHCl₃ fumigation and NH₄NO₃ extraction" *Soil Biology & Biochemistry* 41, 309–314
- Khan M., Scullion J. (2000) "Effect of soil on microbial responses to metal contamination" *Environ Pollution* 110, 115–125
- Kidd P., Barceló J., Pilar Bernal M., Navari-Izzo F., Poschenrieder C., Shileve S., Clemente R. Monterroso C. (2009) "Trace element behaviour at the root–soil interface: Implications in phytoremediation" *Environmental and Experimental Botany* 67, 243–259
- Killham K. (1985) "A physiological determination of the impact of environmental stress on the activity of microbial biomass" *Environ Pollut* 38, 283–294
- Kirk J.L., Beaudette L.A., Hart M., Moutoglis P., Klironomos J.N., Lee H., Trevors J.T., 2004. "Methods of studying soil microbial diversity" *Journal of Microbiological Methods* 58, 169–188
- Klein D.A., Sorensen D.L., Redente E.F. (1985) "Soil enzymes: a predictor of reclamation potential and progress" in: Tate, R.L., Klein, D.A. (Eds.), *Soil Reclamation Processes. Microbiological Analyses and Applications*. Marcel Dekker, New York, 273–340

- Kloepper J.W., Lifshitz R., Zablotowicz R.M. (1989) "Free-living bacterial inocula for enhancing crop productivity" *Trends. Biotechnol.*, 7(2), 39–44
- Kozdrój J., van Elsas J.D. (2000) "Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches" *Soil Biology & Biochemistry* 32, 1405–1417
- Krishnamurti G.S.R., Cieslinski G., Huang P.M., Van Rees K.C.J. (1997) "Kinetics of cadmium release from soils as influenced by organic acids: implication in cadmium availability" *J. Environ. Qual.* 26, 271–277
- Krishnamurti G.S.R., Naidu R. (2002) "Solid solution speciation and phytoavailability of copper and zinc in soils" *Environ. Sci. Technol.* 36, 2645–2651
- Krishnamurti G.S.R., Pigna M., Arienzo M., Violante A. (2007) "Solid-Phase Speciation and Phytoavailability of Copper in a Few Representative Soils of Italy. *Chem. Spec. & Bioav.* 19, 57–67
- Kuiper I., Legendijk E.L., Bloemberg G.V., Lugtenberg B.J. (2004) "Rhizoremediation: a beneficial plant-microbe interaction *Mol Plant Microbe Interact.* 17(1), 6–15
- Kuiters A.T., Mulder W. (1993) "Water-soluble organic matter in forest soils" *Plant Soil* 152, 215–235
- Kumar P.A.B.A.N., Dushenkov V., Motto H., Raskin I. (1995) "Phytoextraction: the use of plants to remove heavy metals from soils" *Environ Sci Technol* 29, 1232–1239
- Kumpiene J., Guerri G., Landi L., Pietramellara G., Nannipieri P., Renella G. (2009) "Microbial biomass, respiration and enzyme activities after in situ aided phytostabilization of a Pb and Cu-contaminated soil. *Ecotoxicol Environ Saf* 72, 115–119
- Kuperman R.G., Carreiro M.M. (1997) "Soil heavy metal concentrations, microbial biomass and enzyme activities in a contaminated grassland ecosystem" *Soil Biol Biochem* 29, 179–190
- Lagomarsino A., Marinari S. (2008) "Microbial biomass and activity of soil under organic management" In: Marinari S., Caporali F. "Soil Carbon Sequestration under Organic Farming in the Mediterranean Environment" ISBN: 978-81-7895-327-4
- Lalor B.M., Coson W.R., Murphy D.V. (2007) "Comparison of two methods that assess soil community level physiological profiles in a forest ecosystem" *Soil Biology & Biochemistry* 39 454–462
- Larson W.E., Pierce F.J. (1991) "Conservation and enhancement of soil quality" 175–203. In: *Evaluation for Sustainable Land Management in the Developing World, Vol. 2: Technical papers.* Bangkok, Thailand: International Board for Research and Management, 1991. IBSRAM Proceedings No. 12(2)
- Lasat H.A. (2002) "Phytoextraction of toxic metals: a review of biological mechanisms" *J. Environ. Qual.*, 31(1), 109–120

- Lebeau T., Braud A., Jézéquel K. (2008) "Performance of bioaugmentation–assisted phytoextraction applied to metal contaminated soils: A review" *Environmental pollution* 153, 497–522
- Leckie S. E. (2005) "Methods of microbial community profiling and their application to forest soils" *Forest Ecology and Management* 220, 88–106
- Ledin M. (2000) "Accumulation of metals by microorganisms – processes and importance for soil systems" *Earth–Science Reviews* 51.1–31;
- LeDuc D.L., Norman T. (2005) "Phytoremediation of toxic trace elements in soil and water" *J Ind Microbiol Biotechnol*, 32, 514–520
- Leirós M.C., Trasar–Cepeda C., García–Fernández F., Gil–Sotres F. (1999) "Defining the validity of a biochemical index of soil quality" *Biol Fertil Soils*, 30, 140–146
- Leita L., De Nobili M., Muhlbachova G., Mondini C., Marchiol L., Zerbi G. (1995) "Bioavailability and effects of heavy metals on soil microbial biomass survival during laboratory incubation" *Biol. Fertil. Soils* 19, 103–118.
- Lewandowski I., Schmidt U., Londo M., Faaij A. (2006) "The economic value of the phytoremediation function—assessed by the example of cadmium remediation by willow (*Salix*)
- Liao Min, Xie Xiao M. (2007) "Effect of heavy metals on substrate utilization pattern, biomass, and activity of microbial communities in a reclaimed mining wasteland of red soil area" *Ecotoxicology and Environmental Safety* 66, 217–223
- Lindsay W.L., Norvell W.A. (1978) "Development of a DTPA test for zinc, iron, manganese, and copper" *Soil. Sci. Soc. Am. J.* 42,421–428.
- Lippmann B., Leinhos V., Bergmann H. (1995) "Influence of auxin producing rhizobacteria on root morphology and nutrient accumulation of crops. 1. Changes in root morphology and nutrient accumulation in maize (*Zea mays* L.) caused by inoculation with indol–3 acetic acid (IAA) producing *Pseudomonas* and *Acinetobacter* strains of IAA applied exogenously. *Angew Bot*;69:31–36
- Lloyd J.R., Lovley D.R. (2001) "Microbial detoxification of metals and radionuclides" *Current Opinion in Biotechnology* 12, 248–253
- Loisel P., Harmand J., Zemb O., Latrille E., Lobry C., Delgenes J.P., Godon J.J. (2006) "Denaturing gradient electrophoresis (DGE) and single–strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity" *Environmental Microbiology* 8, 720–731
- Lombi E., Zhao F.J., Dunham S.J., McGrath S.P. (2001) "Phytoremediation of heavy metal contaminated soils: natural hyperaccumulation versus chemically enhanced phytoextraction" *Journal of Environmental Quality* 30, 1919–1926

- Lone M.I., He Zhen-li, Stoffella P. J., Yang Xiao-e (2008) “Phytoremediation of heavy metal polluted soils and water: Progresses and perspectives” *J Zhejiang Univ Sci B* 9(3), 210–220
- Lovell R.D., Jarvis S.C., Bardgett R.D. (1995) “Soil microbial biomass and activity in long-term grassland: Effects of management changes. *Soil Biol Biochem* 27, 969–975
- Lovley D.R. (1995) “Bioremediation of organic and metal contaminants with dissimilatory metal reduction” *J. Ind. Microbiol.*, 14(2), 85–93
- Lovley D.R., Coates J.D. (1997) “Bioremediation of metal contamination” *Current Opinion in Biotechnology* 8, 285–289
- Lucy M., Reed E., Glick B.R. (2004) “Application of free living plant growth promoting rhizobacteria” *Antonie von Leeuwenhoek*;86, 1–25
- Ma Y., Prasad M.N.V., Rajkumar M., Freitas H. (2011) “Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils” *Biotechnology Advances* 29 248–258
- Magurran A.E. (1988) “Ecological Diversity and Its Measurement” Croom Helm, London.
- Maiz I., Esnaola MaN., Millfin E. (1997) “Evaluation of heavy metal availability in contaminated soils by a short sequential extraction procedure” *The Science of the Total Environment* 206, 107–115
- Malik A. (2004) “Metal bioremediation through growing cells” *Environment International* 30, 261–278.
- Malik S., Beer M., Megharaj M., Naidu R., (2008) “The use of molecular techniques to characterize the microbial communities in contaminated soil and water”. *Environ Int* 34,265–276
- Mantelin S., Touraine B. (2004) “Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake” *Journal of Experimental Botany*, Vol. 55, No. 394, 27–34
- Martens R. (1995) “Current methods for measuring microbial biomass C in soil: Potentials and limitations” *Biol Fertil Soils* 19,87–99
- Martínez-Sánchez M.J., Navarro M.C., Pérez-Sirvent C., Marimón J., Vidal J., García-Lorenzo M.L., Bech J. (2008) “Assessment of the mobility of metals in a mining-impacted coastal area (Spain, Western Mediterranean)” *Journal of Geochemical Exploration* 96, 171–182
- Marx M.C., Wood M., Jarvis S.C. (2001) “A microplate fluorimetric assay for the study of enzyme diversity in soils” *Soil Biol Biochem* 33, 1633–1640
- Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W. (2008) “How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environ Microbiol*; 10(6):1571–1581
- Masciandaro G., Ceccanti B., Gallardo-Lancho J.F. (1998) “Organic matter properties in cultivated versus set-aside arable soils” *Agric. Ecosyst. Environ.* 67, 267–274

- McGrath S.P. (1999) “Adverse effect of cadmium on soil microflora and fauna” In: McLaughlin, M.J., Singh, B.R. (Eds.), *Cadmium in Soils and Plants*. Kluwer Academic Publishers, Dordrecht, 199–218
- McGrath S.P., Dunham S.J., Correll R.L. (2000) “Potential for phytoextraction of zinc and cadmium from soils using hyperaccumulator plants” *In* *Phytoremediation of Contaminated Soil and Water*. Eds. N Terry and G Bañuelos, pp. 109–128. Lewis Publishers, Boca Raton, FL, USA
- McGrath S.P., Zhao F.J., Lombi E. (2001) “Plant and rhizosphere processes involved in phytoremediation of metal-contaminated soils” *Plant and Soil* 232, 207–214
- Meagher R.B., (2000) “Phytoremediation of toxic elemental and organic pollutants” *Curr Opin Plant Biol* 3,153–162
- Menzies N. W., Donn M. J., Kopittke P. M. (2007).”Evaluation of extractants for estimation of the phytoavailable trace metals in soils” *Environmental Pollution*, 145, 121–130
- Mertens J., Springael D., DeTroyer I., Cheyns K., Wattiau P., Smolders E. (2006) “Long term exposure to elevated zinc concentrations induced structural changes and zinc tolerance of the nitrifying community in soil” *Environ Microbiol* 8, 2170–2178
- Mirza M.S., Ahmad W., Latif F., Haurat J., Bally R., Normand P., Malik K.A. (2001) “Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro” *Plant and Soil* 237, 47–54
- Morel J.L. (1997) “Bioavailability of trace elements to terrestrial plants” In: Tarradellas, J., Bitton, G., Rossel, D. (Eds.), *Soil Ecotoxicology*. Lewis Publishers, Boca Raton, 141–178.
- Moscatelli M.C., Lagomarsino A., Marinari S., De Angelis P., Grego S. (2005) “Soil microbial indices as bioindicators of environmental changes in a poplar plantation” *Ecological Indicators* 5, 171–179
- Mulligan C.N., Yong R.N., Gibbs B.F. (2001) “Remediation technologies for metal-contaminated soils and groundwater: an evaluation” *Eng. Geol.* 60, 193–207.
- Muyzer G., Smalla K. (1998) “Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology” *Antonie Van Leeuwenhoek*;73,127–141
- Nannipieri P. (1994) “The potential use of soil enzymes as indicators of productivity, sustainability and pollution” In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) *Soil biota: management in sustainable farming systems*. CSIRO, Adelaide, 238–244
- Nannipieri P., Ceccanti B., Grego S. (1990) “Ecological significance of biological activity in soil, in: Bollag, J.M., Stotzky, G. (Eds.), *Soil Biochemistry*, vol. 6. Marcel Dekker, New York, pp. 293–355

- Nannipieri P., Kandeler E., Ruggiero P. (2002) "Enzyme activities and microbiological and biochemical processes in soil" In: Burns RG, Dick RP (eds) *Enzymes in the environment. Activity, ecology and applications*. Marcel Dekker, New York, 1–33
- Neubauer U., Furrer G., Schulin R. (2002) "Heavy metal sorption on soil minerals affected by the siderophore desferrioxamine B: the role of Fe(III) (hydr)oxides and dissolved Fe(III)" *Eur J Soil Sci* 53,45–55
- Nies D. H. (1999) "Microbial heavy–metal resistance" *Appl Microbiol Biotechnol* 51, 730–750
- Nortcliff S. (2002) "Standardisation of soil quality attributes" *Agric. Ecosyst. Environ.* 88, 161–168
- Norvell W.A. (1984) "Comparison of chelating agents as extractants for metals in diverse soil materials" *Soil Science Society of America Journal* 48, 1285–1292
- Nubel U., Garcia–Pichel F., Muyzer G. "PCR primers to amplify 16S rRNA genes from cyanobacteria" *Appl Environ Microbiol* 1997;63,3327–3332
- OECD (1993) "OECD Core set of indicators for environmental performance reviews– A synthesis report by the group on the state of the environment" *Environmental Monoigraphs*, n.83 OECD7GD(93)179, Organization for Economic Cooperation and Development, Paris
- Palma R.M., Arrigo N.M., Saubidet M.I., Conti M.E. (2000) "Chemical and biochemical properties as potential indicators of disturbances" *Biol Fertil Soils* 32, 381–384
- Pankhurst C.E. (1997) "Biodiversity of soil organisms as an indicator of soil health" In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R. (Eds.), *Biological indicators of soil health*, C.A.B. International, Wallingford, 297–324
- Parkin T.B., Doran J.W., Franco–Vizcaino E.(1996) "Field and laboratory tests of soil respiration. In: Doran JW, Jones AJ (eds) *Methods for assessing soil quality*. (Special publication no 49) SSSA, Madison, Wis., 231–245
- Patten C.L., Glick B.R. (1996) "Bacterial biosynthesis of indole–3–acetic acid" *Can J Microbiol*; 42,207– 220
- Peijnenburg W.J.G.M., Jager T. (2003) "Monitoring approaches to assess bioaccessibility and bioavailability of metals: Matrix issues" *Ecotoxicology and Environmental Safety* 56, 63–77
- Pérez de Mora A., Ortega–Calvo J.J., Cabrera F., Madejón E. (2005) "Changes in enzyme activities and microbial biomass after "in situ" remediation of heavy metal–contaminated soil" *Appl Soil Ecol* 28, 125–137
- Pérez–de–Mora A., Burgos P., Madejón E., Cabrera F., Jaeckel P., Schloter M. (2006) "Microbial community structure and function in a soil contaminated by heavy metals: effects of plant growth and different amendments" *Soil Biology & Biochemistry* 38, 327–341

Pickett S.T.A., Cadenasso M.L., Grove J.M., Nilon C.H., Pouyat R.V., Zipperer W.C., Costanza R. (2001) "Urban ecological systems: linking terrestrial, ecological, physical, and socioeconomic components of metropolitan areas" *Annual Review of Ecology and Systematics* 21,127–157

Pikovskaya R.I. (1948) "Mobilization of phosphorus in soil in connection with vital activity of some microbial species" *Microbiology* 17: 362–370.

Pilon–Smits E. (2005) "Phytoremediation" *Annu Rev Plant Biol* 56, 15–39

Pinto V., Chiusolo F., Cremisini C. (2010) "Proposal of a simple screening method for a rapid preliminary evaluation of "heavy metals" mobility in soils of contaminated sites" *J Soils Sediments*, 10, 1115–1122

Pinzari F., Trinchera A., Benedetti A., Sequi P. (1999) "Use of biochemical indices in the Mediterranean environment: comparison among soils under different forest vegetation" *J. Microbiol. Meth.* 36, 21–28

Piotrowska–Seget Z., Cycon M., Kozdroj J. (2005) "Metal–tolerant bacteria occurring in heavily polluted soil and mine spoil" *Applied Soil Ecology*. 28, 237–246

Powlson D.S., Jenkinson D.S. (1981) "A comparison of the organic matter, biomass, adenosine–triphosphate, and mineralizable nitrogen contents of ploughed and direct drilled soils" *J. Agric. Sci.* 97, 713–721

Preston–Mafham J., Boddy L., Randerson P.F. (2002) "Analysis of microbial community functional diversity using sole–carbon utilization profiles – A critique" *FEMS Microbiol Ecol* 42, 1–14

Priha O., Grayston, S. J., Pennanen, T., and Smolander, A. (1999) "Microbial activities related to C and N cycling and microbial community structure in the rhizospheres of *Pinus sylvestris*, *Picea abies* and *Betula pendula* seedlings in an organic and mineral soil" *FEMS Microbiol. Ecol.* 30, 187–199

Prop Z., Cupr P., Zlevorova–Zlamalikova V., Komarek J., Dusek L., Holoubek I. (2003) "Mobility, bioavailability, and toxic effects of cadmium in soil samples" *Environmental Research* 91, 119–126

Pueyo M., López–Sánchez J.F., Rauret G. (2004) "Assessment of CaCl₂, NaNO₃ and NH₄NO₃ extraction procedures for the study of Cd, Cu, Pb and Zn extractability in contaminated soils" *Analytica Chimica Acta* 504, 217–226

Puglisi E., Del Re A.A.M., Rao M.A., Gianfreda L. (2006) "Development and validation of numerical indices integrating enzyme activities of soils" *Soil Biol. Biochem.* 38, 1673–1681

Qian J, Shari Xiao–quan, Wang Zi–jian, Qiang Tu (1996) "Distribution and plant availability of heavy metals in different particle–size fractions of soil" *The Science of the Total Environment* 187,131–141

Quevauviller Ph. (1998) "Operationally defined extraction procedures for soil and sediment analysis" I. Standardization, *Trends in analytical Chemistrey*, vol. 17,n.5

- Rao C.R.M., Sahuquillo A., Lopez Sanchez J.F. (2008) "A Review of the Different Methods Applied in Environmental Geochemistry For Single and Sequential Extraction of Trace Elements in Soils and Related Materials" *Water Air Soil Pollut* 189, 291–333
- Raskin I., Kumar P.B.A.N., Dushenkov S., Salt D.E. (1994) "Bioconcentration of heavy metals by plants" *Curr Opin Biotechnol* 5, 285–290
- Raskin I., Smith R.D., Salt D.E., (1997) "Phytoremediation of metals: using plants to remove pollutants from the environment" *Curr Opin Biotechnol* 8,221–226
- Reboreda R., Caçador I. (2008) "Enzymatic activity in the rhizosphere of *Spartina maritima*: Potential contribution for phytoremediation of metals" *Marine Environmental Research* 65, 77–84
- Reichle D.E. (1997) "The role of soil invertebrates in nutrient cycling" In: Lohm V, Persson T (eds) *Soil organisms as components of ecosystems*. Swedish Natural Science Research Council, Stockholm, 145–156
- Reichman S.M. (2002) "The Responses of Plants to Metal Toxicity: A review focusing on Copper, Manganese and Zinc" Australian Minerals & Energy Environment Foundation, published as Occasional Paper No.14, ISBN 1–876205–13–X
- Roane T.M., Kellogg S.T. (1996) "Characterization of bacterial communities in heavy metal contaminated soils" *Can J Microbiol* 42, 593–603
- Roane T.M., Pepper I.L. (2000) "Microorganisms and metal pollutants" In: Maier RM, Pepper IL, Gerba CP (eds) *Environmental Microbiology*. Academic, San Diego, 403–423
- Robinson B., Fernández J.E., Madejón P., Marañón T., Murillo J.M., Green S., Clothier B., (2003) "Phytoextraction: an assessment of biogeochemical and economic viability" *Plant Soil* 249,117–125
- Rossello–Mora R., Amann R. (2001) "The species concept for praryotes" *FEMS Microbiol Rev*;25,39–67
- Rost U., Joergensen R.G., Chander K. (2001) "Effects of Zn enriched sewage sludge on microbial activities and biomass in soil" *Soil Biol. Biochem.* 33, 633–638
- Sabienė N., Brazauskienė D. M. (2004) "Determination of heavy metal mobile forms by different extraction methods" *EKOLOGIJA*, 1, 36–41
- Salmaki Y., Zarre S., Esser H.J., Heubl G. (2011) "Seed and gland morphology in *Euphorbia* (Euphorbiaceae) with focus on their systematic and phylogenetic importance, a case study in Iranian highlands" *Flora* 206, 957– 973
- Salt D.E., Blaylock M., Kumar N.P.B.A., Dushenkov V., Ensley B.D., Chet I., Raskin I. (1995) "Phytoremediation: a novel strategy for removal of toxic metals from the environment using plants. *Biotechnol* 13, 468–474
- Sarma H. (2011) "Metal hyperaccumulation in plants: a review focusing on phytoremediation technology" *Journal of Environmental Science and Technology*, 4 (2), 118–138

- Sauvé S., Hendershot W., Allen H. (2000) "Solid–Solution Partitioning of Metals in Contaminated Soils: Dependence on pH, Total Metal Burden, and Organic Matter" *Environmental Science and Technology*, 34,7
- Sauvé S., McBride M.B., Norvell W.A., Hendershot W.H. (1996) "Copper solubility and speciation of in situ contaminated soils: effects of copper level, pH and organic matter" *Water Air Soil Pollut.* 100, 133–149
- Sauvé S., Norvell W.A., McBride M., Hendershot W. (2000) "Speciation and complexation of cadmium in extracted soil solutions" *Environmental Science & Technology* 34, 291–296
- Schimel J.P. (1995) "Ecosystem consequences of microbial diversity and community structure" In: Chapin, F.S., Korner, C. (Eds.), *Arctic and Alpine Biodiversity: Patterns, Causes, and Ecosystem Consequences*. Springer–Verlag, Berlin, 239–244
- Schmeisser C., Steele H., Streit W.R. (2007) "Metagenomics, biotechnology with non culturable microbes" *Appl. Microbiol. Biotechnol.* 75, 955–962
- Schnoor J. (1997) "Phytoremediation: Groundwater Remediation Technologies Analysis Center Technology Evaluation" Report TE–98–01, 37
- Schwitzguebel J.P., van der Lelie D., Baker A., Glass D.J., Vangronsveld J. (2002) "Phytoremediation: European and American Trends, Successes, O b s t a c l e s and Needs" *J Soils & Sediments* 2 (2) 91 99
- Schwyn, B., Neilands J. B. (1987) "Universal chemical assay for the detection and determination of siderophores" *Anal. Biochem.* 160,47–56.
- Semple K.T., Doick K.J., Jones K.C., Burauel P., Craven A., Harms H. (2004) "Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated" *Environ. Sci. Technol.* 38,228A–231A
- Shen Z.G., Zhao F.J., McGrath S.P. (1997) "Uptake and transport of zinc in the hyperaccumulator *Thlaspi caerulescens* and the nonhyperaccumulator *Thlaspi ochroleucum*". *Plant Cell Environ.* 20, 898–906
- Shepherd S.C., Gaudet G., Shepherd M.I., Cureton P.M., Wong, M.P. (1992) "The development of assessment and remediation guidelines for contaminated soil, a review of the science" *Canadian Journal of Soil Science.* 72,359–395
- Shuman L.M. (1991) " Chemical forms of micronutrients in soils" In *Micronutrients in Agriculture 2nd* (ed.Mortvedt J.J., Cox F.R., Shuman L.M., Welch R.M.), Soil Science Society of America, Madison, 113–144
- Sikora R.A., Hoffmann–Hergarten S. (1992) "Importance of plant health–promoting rhizobacteria for the control of soil–borne fungal diseases and plant parasitic nematodes" *Arab. J. Plant Prot.*, 10(4):53–58

- Singer A.C., van der Gast C.J., Thompson I.P. (2005) “Perspectives and vision for strain selection in bioaugmentation” *TRENDS in Biotechnology*, 23,2
- Singh, B.R. (1997) “Soil pollution and contamination” p. 279–299. In R. Lal (ed.) *Methods of Lal (ed.) Methods for assessment of soil degradation*. CRC Press, Boca Raton, FL.
- Sitaula B.K., Almas A., Bakken L.R., Singh B.R. (1999) “Assessment of heavy metals associated with bacteria in soil” *Soil Biol. Biochem.*, 31(2),315–316
- Skujins J.J. (1978) “History of abiotic soil enzyme research” In: Burns RG (ed) *Soil enzymes*. *Soil Biol Biochem* 14,423–427
- Sojka R.E., Upchurch R.R. (1999) “Reservation regarding the soil quality concept” *Soil Sci. Soc. Am. J.* 63, 1039–1054
- Soriano–Disla J.M., Speir T.W., Gómez I., Clucas L.M., McLaren R.G., José Navarro–Pedreño (2010) “Evaluation of Different Extraction Methods for the Assessment of Heavy Metal Bioavailability in Various Soils” *Water Air Soil Pollut* 213, 471–483
- Speir T.W., Ross D.J. (2002) “Hydrolytic enzyme activities to assess soil degradation and recovery” In: Burns, R.G., Dick, R.P. (Eds.), *Enzymes in the Environment: Activity, Ecology and Applications*. Marcel Dekker, New York, 407–431
- Sprocati A.R., Alisi C., Segre L., Tasso F., Galletti M., Cremisini C. (2006) “Investigating heavy metal resistance, bioaccumulation and metabolic profile of a metallophile microbial consortium native to an abandoned mine” *Science of the Total Environment* 366, 649–658
- Sprocati A.R., Alisi C., Tasso F., Marconi P., Sciullo A., Pinto V., Chiavarini S., Ubaldi C., Cremisini C. (2011) “Effectiveness of a microbial formula, as a bioaugmentation agent, tailored for bioremediation of diesel oil and heavy metal co-contaminated soil” *Process Biochem* doi:10.1016/j.procbio.2011.10.001
- Stevenson B.A., Sparling G.P., Schipper L.A., Degens B.P., Duncan L.C. (2004) “Pasture and forest soil microbial communities show distinct patterns in their catabolic respiration responses at a landscape scale” *Soil Biology & Biochemistry* 36, 49–55
- Stevenson F.J., Cole M.A. (1999) “Cycles of Soil: Carbon, Nitrogen, Phosphorus, Sulfur, Micronutrients. (2nd edn), Wiley
- Strasser H., Burgstaller W., Schinner F. (1994) “High yield production of oxalic acid for metal leaching purposes by *Aspergillus niger*” *FEMS Microbiol Lett* 119, 365–370
- Tabatabai M.A. (1994) “Soil enzymes”. In: Weaver RW, Angel JS, Bottomley PS (eds) *Methods of soil analysis, part 2. Microbiological and biochemical properties*. *Soil Sci Soc Am Bo Series n° 5*, SSSA, Madison, WI, 775–833
- Tack F.M.G., Verloo M.G. (1995) “Chemical speciation and fractionation in soil and sediment heavy metal analysis: A review” *International Journal of Environmental Analytical Chemistry*, 59, 225–238

Tebo B.M., Ghiorse W.C., van Waasbergen L.G., Siering P.L., Caspi R. (1997) "Bacterially-mediated mineral formation: insights into manganese(II) oxidation from molecular genetic and biochemical studies" *Rev Mineral* 35, 225–266

Tessier A., Campbell P.G.C., Bisson M. (1979) "Sequential extraction procedure for the speciation of particulate trace metals" *Anal Chem* 51, 844–851

Thompson I.P., van der Gast C.J., Ciric L., Singer A.C. (2005) "Bioaugmentation for bioremediation: the challenge of strain selection" *Environmental Microbiology* 7, 909–915

Torsvik V., Ovreas L. (2002) "Microbial diversity and function in soil: from genes to ecosystems" *Curr. Opin. Microbiol.* 5, 240–245

Trasar-Cepeda C., Leirós C., Gil-Sotres F., Seoane S. (1998) "Towards a biochemical quality index for soils: an expression relating several biological and biochemical properties" *Biol Fertil Soils* 26, 100–106

Trasar-Cepeda C., Leirós M.C., Seoane S., Gil-Sotres F. (2000) "Limitations of soil enzymes as indicators of soil pollution" *Soil Biology & Biochemistry* 32, 1867–1875

Treeby M., Marschner H., Roemheld V. (1989) "Mobilization of iron and other micronutrient cations from a calcareous soil by plant-borne, microbial, and synthetic metal chelators" *Plant Soil* 114, 217–226

Turpeinen R. (2002) "Interactions between metals, microbes and plants – Bioremediation of arsenic and lead contaminated soils" Academic dissertation in environmental ecology, Department of Ecological and Environmental Sciences, University of Helsinki

Tyler G. (1980) "Metals in sporophores of basidiomycetes" *Transactions of the British Mycological Society* 74, 41–49

Tyler G., Balsberg-Pahlsson A.M., Bengtsson G., Bååth E., Tranvik L. (1989) "Heavy-metal ecology of terrestrial plants, microorganisms and invertebrates: a review" *WaterAirSoilPollut.* 47, 189–215

Valsecchi G., Gigliotti C., Farini A. (1995) "Microbial biomass, activity, and organic matter accumulation in soils contaminated with heavy metals" *Biol Fertil Soils* 20, 253–259

Van Camp L., Bujarrabal B., Gentile A.R., Jones R.J.A., Montanarella L., Olazabal C., Selvaradjou S.-K. (2004) "Reports of the technical working groups established under the thematic strategy for soil protection" EUR 21319 EN/4. 872 Office for Official Publ. of the European Communities, Luxembourg

van der Lelie D. (1998) "Biological interactions: the role of soil bacteria in the bioremediation of heavy metal-polluted soils" In: *Metal-Contaminated Soils: in situ Inactivation and Phytoremediation* (Vangronsveld, J., and Cunningham, S. D., eds.) Springer-Verlag and R. G. Landes Co., Austin, TX, Chapter 3, 31–50

Van Veen J.A., Van Overbeek L.S., Van Elsas J.D. (1997) "Fate and Activity of Microorganisms Introduced into Soil" *Microbiology and molecular biology Reviews* 61, 2, 121–135,

Vance E.D., Broes P.C., Jenkinson D.S. (1987) "An extraction method for measuring soil microbial biomass C" *Soil Biol. Biochem.* 6. 703–707

Vásquez–Murrieta M.S., Migueles–Garduño I., Franco–Hernández O., Govaerts B., Dendooven L. (2006) "C and N mineralization and microbial biomass in heavy–metal contaminated soil" *European Journal of Soil Biology* 42, 89–98

Violante A., Cozzolino V., Perelomov L., Caporale A.G., Pigna M. (2010) "Mobility and bioavailability of heavy metals and metalloids in the soil environments" *J. Soil. Sci. Plant Nutr.* 10 (3), 268–292

Visser S., Parkinson D. (1992) "Soil biological criteria as indicators of soil quality: soil microorganisms" *American Journal of Alternative Agriculture* 7, 33–37

Waldrop M.P., Balser T.C., Firestone M.K. (2000) "Linking microbial community composition to function in a tropical soil" *Soil Biology & Biochemistry* 32, 1837–1846

Wallace A., Wallace G.A., Cha J.W. (1992) "Some modifications in trace elements toxicities and deficiencies in plants resulting from interactions with other elements and chelating agents. The special case of iron. *J. Plant. Nutr.*, 15(2), 1589–1598

Wallander H., Mahmood S., Hagerberg D., Johansson L., Pallon J. (2003) "Elemental composition of ectomycorrhizal mycelia identified by PCR–RFLP analysis and grown in contact with apatite or wood ash in forest soil. *FEMS Microbiology Ecology* 44, 57–65

Wang F., Yao J., Si Y., Chen H., Russel M., Chen K., Qian, Y., Zaray G., Bramanti E. (2010) "Short–time effect of heavy metals upon microbial community activity" *Journal of Hazardous Materials* 173, 510–516

Wang Y.P., Shi J.Y., Wang H., Lin Q., Chen X.C., Chen Y.X., (2007) "The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter" *Ecotox. Environ. Safe.* 67, 75–81

Wang Y.T., Shen H. (1995) "Bacterial reduction of hexavalent chromium" *J. Ind. Microbiol.*, 14(2), 159–163

Wang Z., Shan X.Q., Zhang S. (2002) "Comparison between fractionation and bioavailability of trace elements in rhizosphere and bulk soils" *Chemosphere*, 46, 1163–1171

Wardle D.A., Ghani A., (1995) "A critique of the microbial metabolic quotient qCO_2 as a bioindicator of disturbance and ecosystem development. *Soil Biol. Biochem.* 27, 1601–1610

Wardle, D.A., Giller, K.E., Barker, G.M. (1999) "The regulation and functional significance of soil biodiversity in agro–ecosystems. In: Wood, D., Lenne, J.M. (Eds.). *Agrobiodiversity: Characterisation, Utilisation and Management*. CABI, London, pp. 87–121.

- Watanabe K. (2001) "Microorganisms relevant to bioremediation" *Curr Opin Biotechnol*;12,237–241
- Weast R.C. (1984) "CRC handbook of chemistry and physics" 64 edn.CRC, Boca Raton, Fla
- Weber J.B. (1991) "Fate and behavior of herbicides in soils" *Applied Plant Sci.* 5, 28–41
- Wenzel W.W. (2009) "Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils" *Plant Soil* 321, 385–408
- Wenzel W.W., Adriano D.C., Salt D., Smith R. (1999) "Phytoremediation: a plant-microbe-based remediation system" In: Adriano DC, et al. editors. *Bioremediation of Contaminated Soils. Agronomy Monographs 37.* Madison, WI:ASA, CSSA and SSSA, 457–508
- White C., Tardif J.C., Adkins A., Staniforth R. (2005) "Functional diversity of microbial communities in the mixed boreal plain forest of central Canada" *Soil Biology & Biochemistry*, 37 1359–1372
- Winding A., Hendriksen N.B. (2007) "Comparison of CLPP and Enzyme Activity Assay for Functional Characterization of Bacterial Soil Communities" *J Soils Sediments* 7 (6) 411 – 417
- Woese C.R. (1987) "Bacterial evolution" *Microbiol Rev*;51, 221–271
- Wong M.H. (2003) "Ecological restoration of degraded soils with emphasis on metal contaminated soils" *Chemosphere* 50, 775–780
- Wu S.C., Luo Y.M., Cheung K.C., Wong M.H. (2006) "Influence of bacteria on Pb and Zn speciation, mobility and bioavailability in soil: A laboratory study" *Environmental Pollution* 144 765–773
- Yakovchenko V.I., Sikora L.J., Rauffman D.D. (1996) "A biologically based indicator of soil quality" *Biology and Fertility of Soils* 21, 245–251
- Yang X., Feng Y., He Z., Stoffella P.J. (2005) "Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation" *Journal of Trace Elements in Medicine and Biology* 18, 339–353
- Yao H., Xu J., Huang C. (2003) "Substrate utilization pattern, biomass and activity of microbial communities in a sequence of heavy metal-polluted paddy soils" *Geoderma* 115,139– 148
- Zaidi S., Musarrat J. (2004) "Characterisation and nickel sorption kinetics of a new metal hyper-accumulator *Bacillus* sp." *J Environ Sci Health A* 39, 681–691
- Zak J.C., Willig M.R., Moorhead D.L., Wildman H.G. (1994) "Functional diversity of microbial communities: A quantitative approach" *Soil Biology and Biochemistry*, 26, 9, 1101–1108
- Zehnder G., Kloepper J., Yao C., Wei G. (1997) "Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth promoting rhizobacteria" *J. Econ. Entomol.*, 90(2), 391–396

