DIPARTIMENTO PER LA INNOVAZIONE NEI SISTEMI BIOLOGICI AGROALIMENTARI E FORESTALI - DIBAF

Università degli Studi della Tuscia

Via S. Camillo de Lellis , 01100 Viterbo

TESI SPERIMENTALE

CORSO DI LAUREA MAGISTRALE IN

"SCIENZE FORESTALI E AMBIENTALI" (CLASSE LM-73)

"ARSENIC AVAILABILITY AND MICROBIAL FUNCTIONAL DIVERSITY AS INFLUENCED BY DIFFERENT PLANT COVERS IN CALCAREOUS SOILS OF CENTRAL ITALY"

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ANNO ACCADEMICO 2014/2015

Riassunto

Arsenic Availability And Microbial Functional Diversity As Influenced By Different Plant Covers In Calcareous Soils Of Central Italy

L'area che circonda la città di Viterbo è caratterizzata da una contaminazione naturale da arsenico (As). La presenza di arsenico nel suolo e nelle acque sotterranee del distretto vulcanico Cimino-Vicano è dovuta in parte ad un arricchimento primario dovuto alla natura vulcanica delle rocce cui si aggiunge un arricchimento secondario dovuto alla risalita di fluidi profondi derivanti dall'attività idrotermale della zona. Con il presente studio si è voluto caratterizzare il suolo in prossimità della sorgente solfurea Bullicame (Controllo) confrontandolo con quello dell'Orto Botanico dell'Università della Tuscia (suoli prelevati sotto *Pinus halepensis*, Macchia mediterranea, Bosco di querce, Sito non interessato da copertura arborea) sorto su medesimo substrato pedologico nel 1985. L'obiettivo è stato quello di: i) determinare la distribuzione di arsenico nelle varie frazioni di suolo; ii) valutare un eventuale effetto sulla sua distribuzione dovuto alla presenza di diverse coperture vegetali; iii) valutare l'effetto dell'As sul pool microbico del suolo in termini di diversità funzionale. Il suolo di controllo è caratterizzato dal contenuto più elevato di As pari a circa 500 ppm. La frazione carbonatica del suolo trattiene una quantità considerevole di arsenico, (in alcuni casi fino al 70% del totale), frazione che si può definire potenzialmente biodisponibile in seguito a cambiamenti ambientali (pH, potenziale redox). Si è osservato un significativo effetto delle piante sulla distribuzione dell'As nel suolo con una notevole riduzione nel sito a copertura *Pinus halepensis* suggerendo meccanismi indotti dalla lettiera della conifera che meritano un approfondimento. Il pool microbico del suolo ha beneficiato degli input organici da parte delle piante ed ha aumentato la sua attività metabolica, attività enzimatica e diversità funzionale.

Il sito offre un interessante ambiente in cui studiare e monitorare le complesse interazioni tra il metalloide (As), le peculiarità fisico-chimiche proprie dei suoli calcarei, il sistema pianta-microrganismi.

Parole chiave: *Arsenico, suoli calcarei, biodisponibilità, diversità funzionale, microrganismi*

Summary

Arsenic Availability And Microbial Functional Diversity As Influenced By Different Plant Covers In Calcareous Soils Of Central Italy

Viterbo area is naturally polluted with arsenic (As) as a result of geochemical mobilization of this metalloid through hydrothermal processes that lead to the up-flow of thermal waters. The area is characterised by thermal springs with arsenic concentrations ranging from 180 to 370 μgL^{-1} and the soils are calcareous. Soil samples were taken in the area surrounding Bullicame hot springs and inside the nearby University Botanical Gardens with the aim to assess: i) the distribution and bioavailability of arsenic in the soil, ii) the effect of plant cover on its distribution and iii) microbial functional diversity. The sampling points were then the Control (near the thermal springs), *Pinus halepensis, Quercus spp*., Mediterranean maquis and anot-planted area all found within theBotanical gardens. The results reflected a considerable amount of arsenic associated to the $CaCO_3$ fraction which may become unstable under changing environmental conditions thus potentially hazardous. The presence of different plant covers affects the arsenic content in the soil differently as was evident in the drastic reduction of As content under conifer plants (*Pinus halepensis*) cover. The presence of plant products favours a higher functional diversity of microbial processes as opposed to the drastic reduction in microbial functional diversity observed in the control site where the $CaCO_3$ content is elevated and no vegetation is present.

These preliminary results offer an interesting opportunity to study the complex interactions of this toxic metalloid in a soil-plant system in a naturally polluted environment.

Keywords: *Arsenic, calcareous soils, bio-availability, plant uptake, functional diversity, microorganisms*

ACKNOWLEDGEMENTS

My sincere appreciation goes to the European Union Education and Culture Agency through the Erasmus Mundus Master Grants, the Instituto Superior de Agronomia of the University of Lisbon (Portugal), University of Valladolid (Spain) and the University of Tuscia (Italy) for hosting me and making this program a success.

I am so much grateful for the supervision and support from Professor Maria Cristina Moscatelli who was there to guide and support me throughout the entire research. I am also very thankful for the valuable and irreplaceable contributions from Prof. Paulo De Angelis, Prof. Maurizio Sabatti and Prof. Sara Marinari.

I will like to thank Dr. Rosita Marabottini and Ruxandra Papp who were always there at anytime to help and guide me throughout my time and beyond at the laboratory of soil biochemistry of the University of Tiscia. All physical, chemical and biochemical laboratory analyses would not have been possible without their constant assistance. My sincere appreciations go to Dr. Silvia Rita Stazi for the support, collaboration and supervision.

Thanks to my parents and siblings for all the support and love.

To God be the glory for the protection and the possibility to thrive all this while.

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1. INTRODUCTION

1.1. Heavy Metals Pollution In The Environment

Heavy metals generally refer to metals and metalloids having densities greater than 5 g/cm^3. Examples include lead (Pb), zinc (Zn), cadmium (Cd), mercury (Hg) and chromium (Cr) (Oves et al., 2012). Usually metalloids such as arsenic (As) and Thallium (Tl) often fall into the heavy metal category due to similarities in chemical properties and environmental behaviour (Chen et al., 1999). Heavy metals are introduced into the soils naturally through the weathering of the parent materials, and also from a variety of human activities such as mining, smelting, and other industrial processes that have metal residues in their waste streams. Indeed, numerous studies have shown that pollution sources of heavy metals in the environment mainly come from these anthropogenic sources (Wei and Yang, 2010). Heavy metals are very toxic, bio-accumulative, and resistant to biochemical degradation and their excessive addition and input of other synthetic chemicals into soils may lead to the deterioration of the soil biology and function, changes in the soil physico-chemical properties, and other environmental problems (Papa et al., 2010).

Heavy metal pollution is known to be concealed, persistent and irreversible (Wang et al., 2001). The mechanism of pollution has long term degrading effects on the quality of the atmosphere, water bodies, and food crops. It is also a threat to the health and well-being of animals and human beings through the food chain. For example, chronic exposure to Cadmium (Cd) can have adverse effects such as lung cancer, pulmonary adenocarcinomas, prostatic proliferative lesions, bone fractures, kidney dysfunction, and hypertension, while some of the chronic effects of Arsenic (As) consist of dermal lesions, peripheral neuropathy, skin cancer, and peripheral vascular disease (Żukowska and Biziuk, 2008). Moreover, Lead (Pb) is a non-essential element to the human body and its excessive intake can damage the nervous, skeletal, circulatory, enzymatic, endocrine, and immune systems of those exposed to it (Zhang et al., 2012).

Cadmium, Chromium, Arsenic, Mercury, Lead, Copper, Zinc and Nickel have been classified by the United States Environmental Protection Agency (USEPA) as priority

control pollutants because of their potential toxic, persistent and irreversible characteristic (Rodrigues et al., 2013).

1.2. Arsenic in the environment: natural and anthropogenic pollution

Arsenic (As) is a metalloid situated in the 33rd spot on the periodic table. It has an atomic weight of 74.92 and exists in the solid phase. It has a rhombohedral crystal structure and a density of 5.727 g/cm^3. It is known to have been discovered by a Roman Bishop called Albertus Magnus in the year 1250. It played a major role in the Bronze Age, as it was added to bronze as a strengthener (Lechtman, 1996).

Arsenic is a naturally occurring element and is widely distributed in the environment. It is the 20th most occurring trace element in the earth's crust (NRC, 1977). Arsenic mainly occurs in the oxidation states of arsenate (As^{V}), arsenite (As^{III}), arsenic (As^{0}), and arsine (As^{-III}) and its solubility depends on the pH and ionic environment. Arsenate (As^{V}) is the most stable (Gupta et al., 2011). The trivalent arsenic (AS^{3+}) and the pentavalent arsenic (AS^{5+}) are abundantly present in natural waters (Feng et al., 2001). In oxidized environment Arsenic appears mostly as oxyanion (Cutter, 1992). Arsenite ($NaAsO_2$) and Arsenate (Na_2HAsO_4) predominantly exist as inorganic arsenic and all of these two forms are toxic to humans and plants. However, studies from Schat et al. (2002) reveal that arsenite is more toxic than arsenate. Inorganic arsenic is always considered a potent human carcinogen as it has been found to be associated with increased risk of cancer of the lungs, cancer of the urinary bladder, liver cancer and kidney cancer (NRC, 1999). Arsenates are stable in aerobic or oxidizing conditions while arsenites are stable in anaerobic or reducing conditions.

Arsenic in the environment occurs in chemical forms such as monomethylarsonic acid [MMA; $CH_3AsO(OH)_2$], dimethylarsinic acid [DMA; $(CH_3)_2AsOOH$], trimethylarsine oxide [TMAO; $(CH_3)_3AsO$], arsenobetaine [AsB; $(CH_3)_3As+CH_2COOH$], arsenocholine[AsC], arsenosugars [AsS], arsenolipids etc. (Tangahu et al., 2011). MMA, DMA and TMAO are methylated arsenic compounds and are sometimes found as a minor component in the soil, but can reach high concentrations. MMA and DMA are widely used as pesticides and herbicides meanwhile DMA also serves as a cotton defoliant (Huang and Matzner, 2006).

Table 1.1 shows soil As concentrations from different arsenic-affected countries.

Country	**Region**	**Soil As concentration in mg/kg**
Bangladesh	Noakhali	3.6–26 mg/kg (Meghna River)
Brazil	MinasGerais (Southeastern Brazil)	200–860 mg/kg
Chile	Esquiña	Up to 489 mg/kg (Río Caritaya region)
India	Uttar Pradesh	16–417 mg/kg (Central India) 5.40–15.43 ppm (Uttar Pradesh)
Mexico	Lagunera	2215–2675 mg/g (Highly polluted area)
Poland	Lower Silesia, (Southwestern Poland)	Up to 18,100 mg/kg (Highly polluted area)
Spain	Duero Cenozoic Basin	23 mg/kg (Mean)
Turkey	Simav plain (Kutahya)	Up to 660 mg/kg (Highly polluted area)
United Kingdom	Cornwall	2–17 mg/kg (Bioaccessible)
USA	Tulare lake	average 280 mg/kg (Hawaii)

Table 1.1. Concentrations of arsenic in soil of the arsenic-affected countries (Singh et al., 2015)

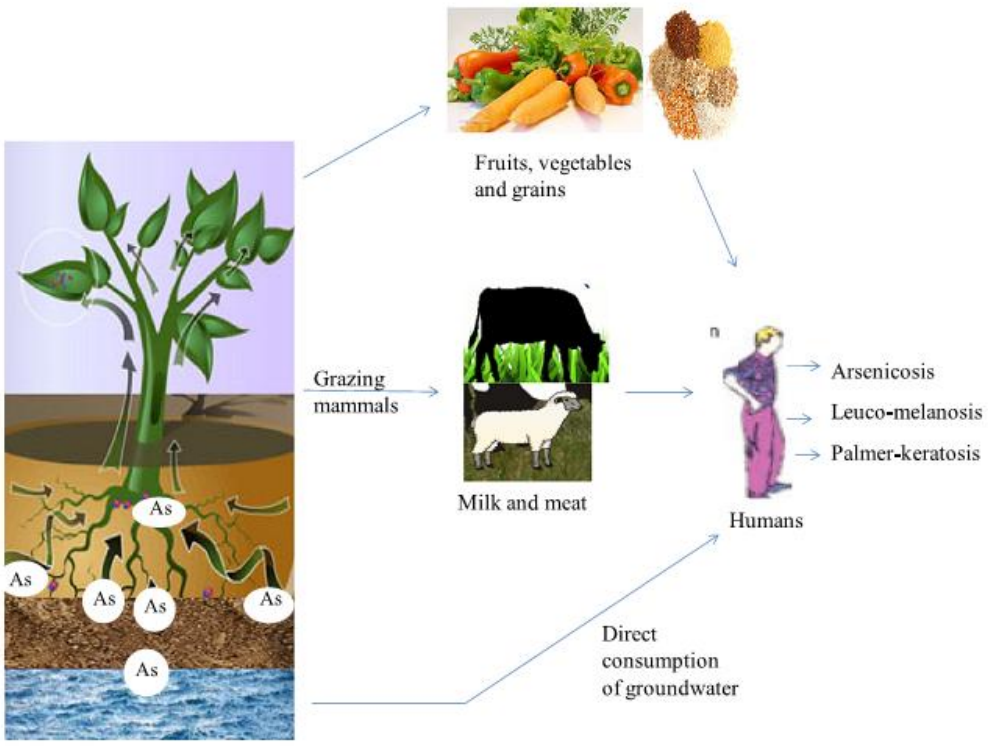

Fig. 1.1 Schematic diagram showing transfer of arsenic from soil and water to human beings through food chains. Intake of arsenic by human beings causes several diseases (Singh et al., 2015).

1.2.1. Natural sources and occurrence of arsenic in the environment

The release of Arsenic from arsenic-enriched minerals represents the main primary source of arsenic in the environment. In the earth's crust, Arsenic can be found concentrated in fine grained argillaceous sediments and phosphorites (Mandal and Suzuki, 2002). Arsenic can also be found co-precipitated with iron hydroxides and sulfides in sedimentary rocks, iron deposits, sedimentary iron ores and manganese nodules. It is also reported to be found concentrated in some reducing marine sediment, which might contain up to 3000 mg kg^{-1} (Mandal and Suzuki, 2002). There are over 200 different mineral forms of naturally occurring Arsenic and amongst these, approximately 60% are arsenates, 20% sulfides and sulfosalts and the remaining 20% includes arsenides, arsenites, oxides, silicates and elemental arsenic (Onishi et al., 1969). The most common arsenic mineral is called arsenopyrite.

There is vast documentation on the occurrence of As in groundwater in Italy and its presence could be attributed to different sources such as mineral deposits in Tuscany and Sardinia, highly reducing environments like the alluvial plains of Veneto, Emilia Romagna and Lombardy, and from volcanic areas of Tuscany, Latium and Campania. The presence of As in groundwater circulating in volcanic rocks is a widespread phenomenon and has mainly natural origins. Usually, As in groundwater is related to the presence of the element as a minor constituent of volcanic gases and geothermal fluids or to the leaching of ore deposits containing the element as a major or minor constituent (Baiocchi et al., 2011)**.**

In the soil, the level of arsenic concentration of various countries varies considerably amongst different geographical regions. Studies have shown that the concentration of arsenic is higher in soils than in rocks (Peterson et al., 1981). Sandy soils have the lowest concentration of arsenic while alluvial and organic soils have higher concentrations. The base concentration of arsenic in uncontaminated soils is between 1–40 mg kg^{-1} (Kabata and Pendias, 1984). Arsenic owes its presence in the soil to parent rock and human activities and these are the driving forces behind it growing to larger concentrations. Other factors that also influence the level of arsenic in the soils are climate, the organic and inorganic components of the soils and redox potential.

In natural waters, studies report a relatively low level of arsenic. The maximum permissible concentration of arsenic in drinking water as stated by the United States Environmental Protection agency (EPA) and the World Health Organization (WHO) is 50 g l^{-1} and the recommended value is 10 g l^{-1} (WHO, 2001; EPA, 1975). The average concentration of arsenic in unpolluted fresh waters typically ranges from 1–10g l^{-1}, rising to 100–5000g l^{-1} in areas of sulfide mineralization and mining (Smedley et al., 1996). Studies have reported the presence of complex organic forms of arsenic such as tetramethylarsonium salts, arsenocholine, arsenobetaine, dimethyl(ribosyl)arsine oxides and arsenic containing lipids in marine environment (Irgolic et al., 1995). However, only a small fraction of the arsenic in the marine environment remains in solution because the majority is adsorbed on to particulate material in suspension. Normal ground water has also been reported not to have methylated forms of arsenic but ponds and lakes have methylated forms, arsenite and arsenate (Mandal and Suzuki, 2002). Arsenic contamination in fresh water systems has

been attributed to surface recharge, base flow, and the bedrock lithology. Geothermal inputs, evaporation, and groundwater contamination are the principal cause of high concentrations of arsenic in rivers with basic concentrations that range between 0.1 to 2.1 mg/L with an average of 0.8 mg/L. Generally, the variation of arsenic concentration in rivers and lakes is in the range of 0.15 –0.45 μg/L (Bissen and Frimmel, 2003). Other high concentrations of arsenic in rivers have been attributed to mining activity like in the case of mining and processing of arsenopyrite ores (Ashley and Lottermoser, 1999).

Arsenic in the air is usually present as a mixture of the inorganic forms arsenite and arsenate and is predominantly absorbed to the surface of suspended particulate matter. The organic forms are of negligible importance except in areas of arsenic pesticide application or biotic activity (Davidson et al., 1985). There is a reported low risk of human exposure to atmospheric arsenic through inhalation because the concentration in the atmosphere is really low.

Arsenic is cumulative on human and animal tissue and the concentrations vary depending on the amount ingested from different areas. Some marine animals like shellfish may contain over 100g g^{-1} of arsenic. On the basis of total wet weight, the average arsenic content in freshwater fish was found in one study to be 0.54 g g^{-1} (Whitacre and Pearse, 1974). Meanwhile in mammals, arsenic accumulates in certain areas of the ectodermic tissue, primarily the hair and nails. Human beings have a total arsenic content between 3 and 4 mg and this amount seem to increases with age with most body tissues containing less than 0.3–147 g g^{-1} (dry weight) except for the hair, nails and teeth (WHO, 2001). Studies show that inorganic arsenic has a high affinity for hair and other keratin-rich tissues and the concentrations over the length of the hair strand shows the degree of exposure over a period of time. Normal amounts of arsenic in the human hair lies within the range of about 0.08–0.25g g^{-1} with 1.0g g^{-1} being indication of the presence of excess arsenic and poisoning (Pearson and Pounds, 1971).

1.2.2. Anthropogenic sources and occurrence of arsenic in the environment

Anthropogenic activities especially the utilization of natural resources have been known to significantly release arsenic into the soil, air and water. Previous studies show that these amounts from human activities far exceed the amounts that get into the environment

naturally and have significant effects on the levels found in animals and plants. Main activities that release arsenic into the soil are through the disposal of industrial and animal wastes, use of arsenic-containing pesticides, application of fertilizers and from dust from the burning of fossil fuels (Piver, 1983). In the 1970s, about 80% of the consumption of arsenic was for agricultural purposes. This amount has been declining over the years. Approximately 97% of the arsenic produced enters end product manufacture in the form of white arsenic and the remaining 3% as metal for metallurgic additives, in special lead and copper alloys (Mandal and Suzuki, 2002).

Arsenic was widely used in the past for the preparation of insecticides and pesticides. Most of it was in the form of pesticides, such as lead arsenate, calcium arsenate, copper acetoarsenite, Paris-Green (copper acetoarsenite), Arsenic acid, MSMA (monosodium methanearsonate), DSMA (disodium methanearsonate) and cacodylic acid are used in cotton production as pesticides (Thompson, 1973). Meanwhile inorganic arsenic compounds especially sodium arsenite are widely used as weed killers (pesticides), particularly as non-selective soil sterilants (Mandal and Suzuki, 2002) and this forms a major source of anthropogenic release of arsenic into the environment.

Desiccants and wood preservatives form another major pathway of entry of arsenic into the soil, air and water. Arsenic acid (H_3AsO_4) is extensively used as a cotton desiccant in many countries. Wood preservatives such as Fluor–Chrome–Arsenic–Phenol (FCAP), Chromated Copper Arsenate (CCA) and Ammonical Copper Arsenate (ACA) were used in 99% of the arsenical wood preservatives that were introduced in the USA and are still widely used today (Perker, 1981). Other commonly used arsenic based wood preservatives include Wolman salts and Osmosalts, zinc and chromium (Lansche, 1965)

The rate of arsenic release from sulphide minerals can be sped up by mining activities, which expose the minerals to weathering processes during excavation. Arsenic dust is produced during copper and gold smelting, and coal combustion.

Another anthropogenic activity that releases arsenic into the environment is the use of feed additives such as arsenic acid, 3-nitro-4-hydroxy phenylarsonic acid, 4-nitrophenylarsonic acidetc (Mandal and Suzuki, 2002). They eventually find their way into the soil and water bodies.

A study from Marabottini et al., (2013) on the mobility and distribution of arsenic in contaminated mine soils in the Piemonte region of northern Italy found the total content of As in the soil to range between 4062 to 4358 mg kg^{-1} with the highest fraction bound to amorphous oxides of Fe and Al. The importance of the sequential fractions of arsenic is worth mentioning because it determines the potential bio-available fraction which eventually is an environmental concern because of its ability to be mobile in the case of changes in pH or redox conditions.

1.2.3. Arsenic in Calcareous soils

Calcareous soils are those that have free calcium carbonate ($CaCO_3$) in the profile. Some soils that develop from calcareous parent materials will be calcareous throughout their profile. They are formed largely from the weathering of calcareous rocks and fossil shell beds. Different varieties usually contain chalk and limestone and frequently a large amount of phosphates. Calcareous soils have a 100% base saturation, and the exchange complex is dominated by calcium. The pH of calcareous soils is from 7.0 to 8.2 due to the hydrolysis of calcium carbonate which produces the strong base, calcium hydroxide, and the weak acid, carbonic acid.

The chemistry of Arsenic is complicated and may be affected by the changes in redox conditions of soils (Sadiq, 1997). A study from Sadiq et al. (1997) indicated that Manganese (II) Arsenate ($Mn_3(AsO_4)_2$) and calcium arsenate ($Ca_3(AsO_4)_2$) are the most probable arsenic minerals which could form and control arsenic solubility in the calcareous soils. Calcite has been known for its possible role in the retention and solubility of arsenic in calcareous soils and various other environments with abundance of carbonates. Average diameter of $CaCO_3$ is 2 - 8µm and surface area of 5 to 10 m^2/g. Arsenate sorption on calcite increases from pH 6 to 10, peaks between pH 10 and 12, and decreases above pH 12 (Goldberg and Glaubig, 1988). In natural systems, arsenic gets incorporated into the lattice structure of calcite as arsenite under alkaline pH. This hinders the mobility of the metalloid when iron and/or manganese oxyhydroxides lose their adsorbing effectiveness (Di Benedetto et al., 2006). There is evidence that arsenite retention mechanisms on calcite will change from adsorption to coprecipitation with an increasing As (III) concentration (Roman-Ross et al., 2006). There is generally a great affinity for calcite surface sites

exhibited by arsenate anions at pH 8.3, forming an inner-sphere complex at the calcite surface whereby AsO_4 tetrahedral units link through corner-sharing to Calcium (Alexandratos et al., 2007). Studies have shown that the non-specifically sorbed (easily exchangeable, outer sphere complexes) fraction of arsenate is dominant than the inner-sphere surface bound complexes of arsenate in the carbonate soil fraction, indicating high bioavailability and transport for arsenate in the carbonate-rich soils of which Fe and Al oxyhydroxide fractions are limited (Yolcubal and Akyol, 2008).

1.3. Plant responses to heavy metals

Heavy metals are thought to be one of the most detrimental stressors that exist in the environment. They are all toxic to plants at elevated levels, whether or not they are required for the growth of the plant (Gasic and Korban, 2006). Uptake and accumulation of these heavy metals at higher concentrations are bound to cause structural and ultra-structural changes. These changes can span from the cellular level to the whole-plant level like for example the inhibition of root elongation by some metal ions, inhibition of photosynthesis, enzyme activity, and cause oxidative damage to membranes (Shaw et al., 2004). The plants therefore become susceptible to other environmental stresses like drought conditions in the face of the effects of these elevated levels of heavy metals due to reduced water uptake capacity of the smaller root system, and due to decreased water use efficiency (Ryser and Emerson, 2007).

Metals like zinc, iron and copper are essential micronutrients required for a wide range of physiological processes in all plant organs for the activities of various metal-dependent enzymes and proteins but can however be toxic at elevated levels. Meanwhile metals like arsenic, mercury, cadmium and lead are non-essential and potentially highly toxic (Zhao and Chengcai, 2011).

1.3.1. Toxic effects of metals on plants and their responses

Plants exposed to high levels of Cadmium suffer reduction in photosynthesis, water uptake, and nutrient uptake. Plants grown in soil containing high levels of Cadmium show clearly visible symptoms of injury reflected in terms of chlorosis, growth inhibition, browning of root tips, and finally death (Mohanpuria et al., 2007).

When released into the soil, Mercury (Hg) mainly remains in the solid phase through adsorption onto sulfides, clay particles and organic matter. Studies indicate that Hg^{2+} can readily accumulate in higher and aquatic plants (Israr et al., 2006) capable of causing phytotoxicity to plant cells (Zhou et al., 2007).

Chromium (Cr) is a heavy metal and a serious environmental contaminant in soils, sediments, and groundwater (Shanker et al., 2005). Excess of Chromium causes plant growth inhibition, chlorosis in young leaves, nutrient imbalance, wilting of tops, and root injury (Scoccianti et al., 2006).

Zinc (Zn) in soil causes the inhibition of many plant metabolic functions, such as plant growth retardation and senescence. Zinc toxicity causes limited growth of roots and shoots and also causes chlorosis in the younger leaves, which can extend to older leaves after prolonged exposure to high soil Zn levels (Ebbs and Kochian, 1997).

Copper (Cu) is a micronutrient for plants and plays an important role in CO_2 assimilation and the synthesis of ATP. Excess of Copper in soil plays a cytotoxic role that induces stress and causes injury to plants causing leaf chlorosis and retarded growth (Lewis et al., 2001)

Lead (Pb) is most widely distributed and one of the most abundant toxic elements in the soil. It is very detrimental to the morphology, growth and photosynthetic processes of plants. Elevated levels of lead cause enzyme activity inhibition, water imbalance, and alterations in membrane permeability and disturbs mineral nutrition (Sharma and Dubey, 2005).

Cobalt (Co) occurs naturally in the earth's crust as cobaltite [CoAsS], erythrite [$Co_3(AsO_4)_2$] and smaltite [$CoAs_2$]. Plants have the ability to accumulate small amounts of cobalt from the soil. Elevated levels of Cobalt affect the translocation of P, S, Mn, Zn and Cu from roots to tops in cauliflower (Chatterjee and Chatterjee, 2000).

Nickel (Ni) exists in trace concentrations in the soil except in ultramafic or serpentinic soils. Excess of nickel ion Ni^{2+} in soil causes many physiological alterations and diverse toxicity symptoms such as chlorosis and necrosis in different plant species (Rahman et al., 2005).

Arsenic (As) is an analog of phosphate (P) and competes for the same uptake carriers in the root plasmalemma of plants (Meharg and Macnair, 1992). Arsenic tolerance has been identified in a number of plant species and in grasses it was discovered that it results from suppression of a high-affinity phosphorus/arsenic uptake system (Meharg and Macnair, 1992). This suppression reduces arsenic influx to a level at which the plant can easily detoxify it, presumably by constitutive mechanisms (Meharg, 1994). Despite the mechanism of arsenic tolerance that can lead to decrease uptake, plants growing in arsenic polluted environments can accumulate large concentrations over the years. Arsenic is also known to undergo transformations within the plant cells to other less phytotoxic arsenic species (Meharg, 1994). In previous studies, terrestrial plants have been documented only for the presence of arsenate and arsenite (Van den Broeck et al., 1998). However, a later study on a range of terrestrial plants has also reported low concentrations of methylated arsenic species such as MMA and DMA (Koch et al., 2000).

Plants have developed a complex network of highly effective homeostatic mechanisms that serve to control the uptake, accumulation, trafficking, and detoxification of metals. Some of these mechanisms include metal transporters in charge of metal uptake and vacuolar transport, chelators involved in the detoxification of metal ions by buffering their concentrations, and chaperones helping in the delivery and trafficking of metal ions (Clemens, 2001)

The solubility of metal ions in the soil is strongly dependent on the pH of the soil since heavy metal bioavailability to plants is dependent on the chemical and physiological conditions in the rhizosphere (Broadley et al., 2007). In acidic conditions, bivalent cations become available for plant roots and decrease in alkaline conditions during which they are precipitated out as insoluble hydroxides (McGrath et al., 1988). Plant growth is also known to significantly reduce when the rhizospheric pH is less than 5.0 i.e. in acidic conditions. To counter this effect, the plants increase the pH of the rhizosphere which greatly increases the extent of metal ion precipitation and complexation around the roots. This helps to prevent the availability and uptake of heavy metals, thus reducing the impact of heavy metal toxicity (Reichman, 2002). The plant's ability to perform this buffering effect in the rhizosphere depends on the soil organic matter, the forms of nitrogen present in the

rhizosphere and the availability of iron and phosphorus. This is because these three factors have significant effects on the accessibility and uptake of zinc, cadmium and other heavy metal ions (Broadley et al., 2007).

Another method of plant response to heavy metal pollution is through complexation at the cell wall/plasma membrane interface. A significant proportion of heavy metals accumulate at the cell wall-plasma membrane interface and studies have proposed that this could be the site of metal tolerance (Reichman, 2002). In a study, *Minuartiaverna ssp*. was planted on heavy metal contaminated medieval mine dumps and was found to have high concentrations of Fe, Cu, Zn and Pb associated with Si contained in the cell walls (Neumann et al., 1997). In comparison, no accumulation of heavy metals was detected in the cytoplasm suggesting strong use of exclusion by the metal adapted subspecies. However, the use of glutaraldehyde fixation techniques has been shown to result in the loss of up to 70% of the 27 metals present in the plant (Neumann et al., 1997). Thus the metal distribution pattern may largely be an artifact of the fixation process.

Plants can also achieve heavy metal tolerance by sequestering the heavy metals away in places within the cell where the metals cannot react with metabolically active cellular substances. Compartmentation in the vacuole is regularly put forward as the most probable site. It has been demonstrated that grasses can actively pump Zn into vacuoles with the more tolerant clones being able to continue the process at higher external Zn levels (Reichman, 2002).

Plants could also use metallothioneins for complexation as a mechanism to respond to stresses induced by heavy metal pollution. Metallothioneins are a group of low molecular mass, cysteine-rich, metal-binding proteins (Robinson and Jackson, 1986). Studies suggest that they function in the regulation of essential metals and in the detoxification of all metals (Steffens, 1990). Phytochelatins are cysteine-rich non-protein metal-binding peptides produced by plants (Zenk, 1996). It has been suggested that they may act in a similar way to that proposed for metallothioneins in the plant tolerance to metals.

Besides metal complexation, other metabolic changes also have a role in plant metal tolerance. The mechanism occurs in 2 ways: Firstly, metal-sensitive metabolic processes

could be avoided by the activation of alternative pathways and secondly, the sensitivity of enzyme activities to metals could be counteracted by increasing the production of enzymes. However, doubt still persists on this mechanism as there has not been substantial evidence to give it credibility (Reichman, 2002).

1.4 The soil system: microbial biomass

Soil is a multifunctional system and because of this, it lacks a universally accepted definition, same as any other natural entity whose definition depends on its use. In the case of soils, it has been noted that land use has conditioned the way different people perceive its definition. Historically, the popular concept of soil was that of agronomists; their perception of soil was that of a medium for plant growth, while for geologists soil was just a rather short phase in the long global cycle of rocks, and for engineers it was perceived as an unconsolidated earthy material that can be moved by machinery. Russian Vasilij V. Dokuchaev who is considered the father of pedology, (from the Greek pedon meaning "soil" and logos meaning "knowledge"), came up with a definition for soils in the late 1880s. He referred to the soil as *a tridimensional entity located at the earth's surface with morphology and unique physical, chemical and biological properties acquired by the interaction, through time, among living and dead organisms, rock, and climate on a given topographic position.* Recent definitions for soil like those of the World Reference Base for Soil Resources defines Soil as any material located within two meters from the Earth's surface that is in contact with the atmosphere, with the exclusion of living organisms, areas with continuous ice not covered by other material, and water bodies deeper than two meters (WRBSR, 2006). It can thus be deduced from the above definitions of soil that it is a complex system with various socio-economic and ecological functions.

The capacity of soil to provide socio-economic and ecological functions is made possible through a multiphase complex interaction between components of the soil's physical (texture, structure, porosity, density and temperature), chemical (pH, Cation Exchange Capacity, macro elements, salinity, conductivity etc) and biological properties like microbial populations and turnover. The multiphase system of the soil is made up of a solid phase which includes organic and inorganic constituents; a liquid phase made up of water and dissolved organic and inorganic compounds; and a gaseous phase which includes a

mixture of gases that have a close resemblance to the concentration of the gases in the atmosphere except for the fact that the soil has a higher carbon dioxide (CO_2) concentration.

Generally, soil is made up of pools (soil organic matter and microrganisms) and processes (Mineralization activity and soil enzyme activity) whose interactions determine soil biological fertility which can be described as an expression of microbial life and activity in the soil. Climate, organisms, relief, parent material and time are the main key factors in the formation of soil (Jenny, 1941).

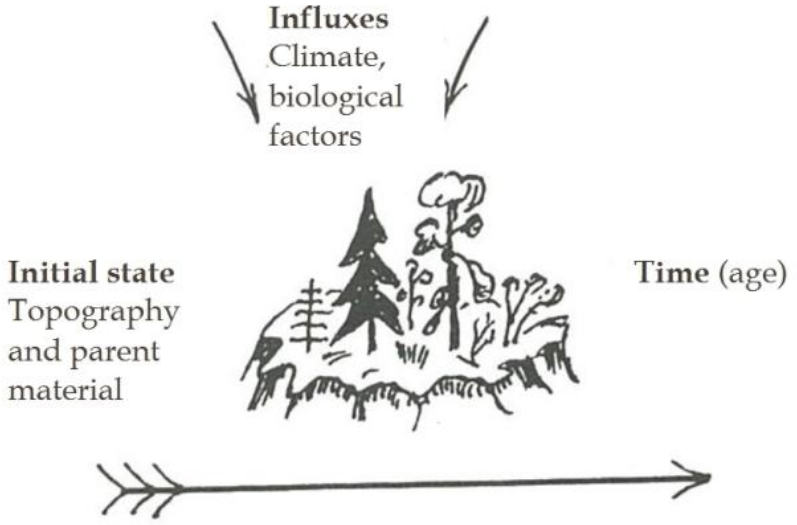

Fig 1.2. Factors of soil formation

The most important and relevant ecological biogeochemical processes in soils are mediated by micro organisms. There exist an enormous amount of microbial cells (i.e. 10^7 - 10^{12}) in one gram soil (Watt et al., 2006). Despite this huge population of microorganisms, they are localized and concentrated on very small microhabitats that comprise far less than 1% of the total soil volume (Young et al., 2008) and globally occupying just 10^{-6} % of the entire soil surface area (Young and Crawford, 2004). Microbial Biomass thus represents the living component of soil organic matter with size < 5000μm^3 and forms 1-5% (w/w) of Total Organic Carbon (Jenkinson& Ladd, 1981). This however is with the exclusion of macrofauna, mesofauna, microfauna and plant roots. Microbial biomass serves as a sensitive indicator and predictor of early changes in processes of soil organic matter.

Microbial biomass has been found to be correlated with several functional microorganisms, such as ammonifiers and nitrifiers, microbial diversity (Nogueira et al., 2006), populations of bacteria in the root nodules of leguminous plants (Pereira et al., 2007) and the activities of enzymes in the soil (Balota et al., 2004). There are a lot of other diverse processes in the soil that involve microbial biomass, like nutrient cycling, decomposition of organic residues, degradation of pollutants and xenobiotics, nutrient solubilization (especially phosphates), storage of organic matter, soils structuring and the biological control and suppression of soil pathogens. These functional criteria have always classified soil microbial biomass as an important component in the maintenance of soil quality and plant productivity (Nogueira et al., 2006).

Within the scope of sustainability in agriculture, soil microbial biomass has inspired lots of studies together with related parameters like soil chemical and physical characteristics, biodiversity and crop productivity as sensitive indicators of soil quality. Soil quality has been defined as the continuous capacity of the soil to function as a vital living system, within ecosystems and land-use boundaries, to sustain biological productivity, promote air and water quality, and maintain plant, animal and human health (Seybold et al., 1999). Soil microbial biomass is seen as one of the most reliable indicators of soil quality because of its prompt response to environmental changes which are often much earlier than the physical and chemical parameters, including total soil organic carbon (TOC) and even crop productivity. This sensitivity in response is maintained consistently over seasonal fluctuations due to climatic conditions (Hungria et al., 2009).

In terms of resilience and resistance, soil microbial biomass (Microbial carbon), total soil organic carbon (TOC) and the metabolic quotient (qCO_2) may be used as indicators to the vulnerability to disturbance of the soil (Seybold et al., 1999). It has been noted that the soil has low resistance if microbial biomass carbon significantly reduces maybe after a disturbance, but high resilience if the microbial biomass carbon, soil total organic carbon and the microbial quotient are barely affected (Glaciela et al., 2010). There is a greater advantage in high resilience because after a disturbance in the soil, the microbial biomass carbon and the related quality of the soil will recover eventually. Microbial biomass carbon is vulnerable, depending on pedogenetic and climatic conditions and often varies

significantly from one climate to another (Glaciela et al., 2010), and because of this, measurements of soil microbial biomass carbon of a given soil under several circumstances is however necessary before defining the thresholds of microbial biomass carbon for optimal soil quality.

Before the studies of Jenkinson and Powlson (1976), microbial biomass estimation measurements were done mainly by microscopic observations by trained personnel using sophisticated equipment. Then there was the fumigation – incubation (FI) approach in 1976 by Jenkinson and Powlson. This approach was a less subjective method in the evaluation of microbial biomass carbon based on fumigation, re-inoculation with live microbial biomass and later incubated under controlled conditions. They then measured the differences in fluxes of CO_2 between the fumigated and the non-fumigated soils. Vance et al. (1987) later proposed a modification to this method which was based on extraction immediately after fumigation. This was known as the fumigation extraction method and is still widely used today in the determination of microbial biomass carbon. Other measurement methods include substrate induced respiration (Anderson and Domsch, 1978), and arginine ammonification (Alef and Kleiner, 1986).

1.5. Microbial structural and functional diversity: tools for measurement

Soil biodiversity and ecosystem functioning have in the recent times maintained a central stage in global concern because of the fact that soil is one of the major biodiversity reservoirs in the world, and the loss of soil biodiversity makes soils more vulnerable to other soil degradation processes. Soil micro organisms are principal drivers of soil organic matter turnover. Diversity within soil microorganisms play an important role in governing a large number of essential soil processes including soil nutrient biochemical cycling (Cruz-Martínez et al., 2009), the decomposition of surface and sub-surface litter (Allison et al., 2013) and the formation of stable soil organic matter (Cotrufo et al., 2013), mineralization and humification of organic compounds in soils (Bauhus and Khanna, 1999). The rate of soil organic matter mineralization depends on the composition of microbial communities and their ability to metabolize different organic compounds (Garcia-Pausas and Paterson, 2011).

Soil microbial structural diversity refers to biological diversity at three levels: within species (genetic), species number (species) and community (ecological) diversity (Harpole, 2010). Diversity within species usually refers to two components which are the total number of microbial species present (also known as species richness which describes the quantitative variation among species) and the distribution of individuals among these species also known as evenness. Despite the fact that there are several thousands of microbial species that have not yet been described, microorganisms exist in high numbers within the soil. One gram of soil may harbor up to 10 billion microorganisms of possibly thousands of different species (Roselló-Mora and Amann, 2001). Knowledge on soil microbial diversity is limited because of the inability to study soil microorganisms and due to the fact that only a very limited amount of soil microorganisms can be cultured in the laboratory. Approximately 1% of the soil bacterial population can be cultured by standard laboratory practices and it is not known if this 1% is representative of the bacterial population (Torsvik et al., 1998). About 5000 bacterial species have been described (Pace, 1999) and an estimated 1,500,000 species of fungi exist in the world (Giller et al., 1997). Recently, molecular methods have been used to study soil bacterial communities and very little research has been undertaken for soil fungi. Meanwhile unlike bacteria, many fungi cannot be cultured by current standard laboratory methods (van Elsas et al., 2000).

There are problems associated with studying soil microbial diversity that arise from methodological limitations and from lack of taxonomic knowledge (Kirk et al., 2004). It is difficult to study the diversity of a group of microorganisms when it is not understood how to categorize or identify the species present. Some of these limitations are:

- Spatial heterogeneity
- Inability to culture soil microorganisms
- Limitations of molecular-based methods
- Taxonomic ambiguity of microbes

Methods to measure microbial diversity in soil can be categorized into two groups: biochemical-based techniques and molecular-based techniques. They have been summarized below.

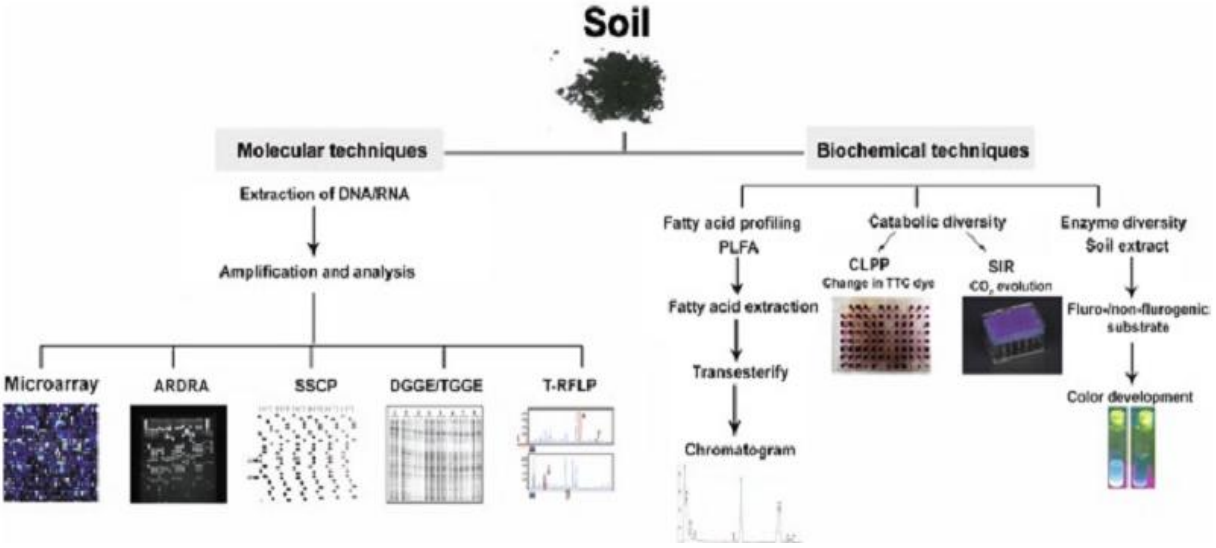

Fig 1.2. An overview of techniques used for soil microbial community structure and diversity (Sharma et al., 2011)

Method	Advantage	Disadvantage
Plate counts	• Fast • inexpensive	• Unculturable microorganisms not detected • Bias towards fast growing individuals • Bias towards fungal species that produce large quantities of spores
Community Level Physiological Profiling (CLPP)	• Fast • Highly reproducible • Relatively inexpensive • Differentiate between microbial communities • Generates large amount of data • Option of using bacterial, fungal plate or site specific carbon sources (Biolog)	• Only represents culturable portion of community • Favours only fast growing organisms • Only represents organisms capable of utilizing available carbon sources • Potential metabolic diversity, not in situ diversity
Fatty acid methyl ester analysis (FAME)	• No culturing of micro organisms, direct extraction from soil • Follow specific organisms or communities	• If using fungal spores, a lot of material is needed • Can be influenced by external factors • Possibility that results can be confounded by other micro organisms

Table 1.2. Advantages and disadvantages of biochemical-based methods to study soil microbial diversity (Kirk et al., 2004).

Studies have shown that a high plant diversity enhances net primary productivity, leading to an increase in the carbon input into the soil owing to a faster turnover of plant biomass and larger root exudation and may therefore positively affect carbon limited microbial communities in the soil (Bartelt-Ryser et al., 2005). In this light, the structure and diversity of soil microbial communities are therefore thought to be affected by composition and diversity of plant cover (Ladygina and Hedlund, 2010). A high diversity of the plant cover may support a greater diversity of decomposers due to high diversity of litter and root exudates. However, the exact relationships between plant communities and soil micro organisms remain unclear (Porazinska et al., 2003) and a positive relationship between plant and microbial diversity has not always been observed (Kielak et al., 2008). Gaining a more detailed understanding of the microbial community in soils is imperative for the evaluation of the stability and resilience of the soil. However, microbial biomass is a relatively small pool of nutrients and soil organic matter (SOM) can act as a pathway for the incorporation of organic matter into the soil, a mediator to transform nutrients between organic and inorganic components and also a short term sink for soil nutrients (Zogg et al., 2000).

The functional diversity of soil microbial communities represents the sum of all the ecological processes carried out by the organisms of a particular community and is a result of the genetic diversity of that community, environmental effects on gene expression and ecological interactions among taxa (Pignataro et al., 2012). The genetic diversity represents all the different species present in a soil while the functional diversity indicates the capacity of soil microorganisms to use a wide array of substrates and the capacity to perform many different functions (metabolic processes).

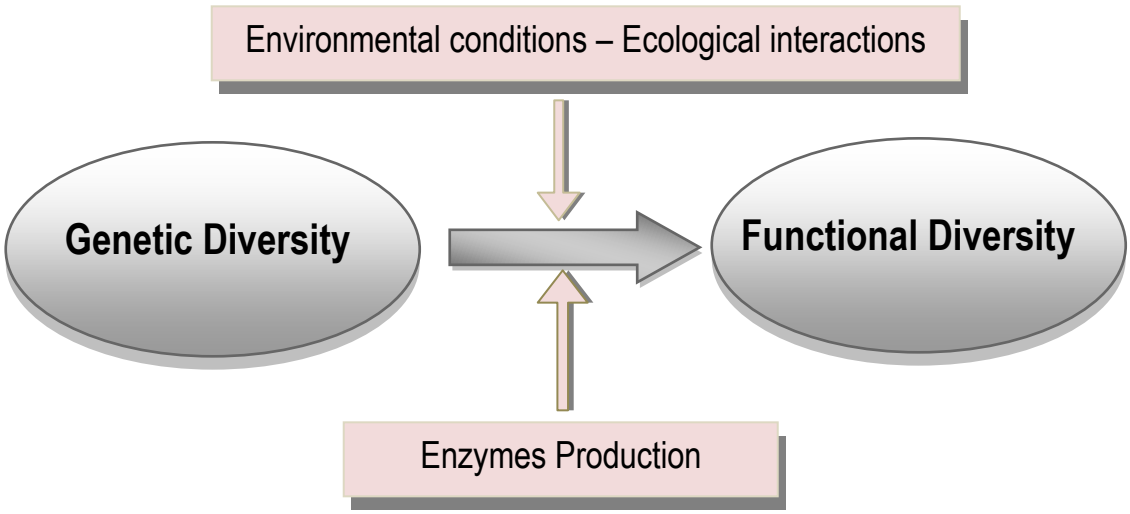

Fig 1.3. Relationship between microbial Genetic and Functional diversity.

Microbial functional diversity describes the activity of the microbial population in the soil, thus when methods of assessing the health and activity of the microbial population are to be considered, one basic and reasonable method to employ will be to measure the amount of carbondioxide (CO_2) being respired by microorganisms in the course of them decomposing organic substrates within the soil (Anderson, 1982). Another assessment possibility is to measure the substrate induced respiration (SIR) which involves measuring the amount of CO_2 evolved before and after the addition of an organic substrate like glucose (Anderson, 1978). The advantage of this technique is that it makes provision for additional information regarding the size of the microbial population and how different soils containing them might react in the face of stress like in the case of addition of a pollutant or an organic matter.

Individual species within the soil microbial population possess different abilities to respire different substrates, so we can obtain a catabolic finger print of the community (Degens and Harris, 1997) or otherwise known as Community Level Physiological Profile (CLPP) (Lehmanet al., 1995) by assessing their respiration after the addition of different substrates. Measuring the metabolic activity of soil microbial communities is one of the prominent methods for assessing microbial functional diversity. Note should however be taken that

this analytical method measures the potential rather than the real activities of microorganisms in the soil (Nannipieri et al., 2003). A very useful tool in the assessment of microbial functional diversity is the MicroResp™ method developed by Campbell et al. (2003). The MicroResp™ is a Community Level Physiological Profile (CLPP) technique based on the addition of several carbon substrates directly to soil and measuring the microbial response of CO_2 evolution. This method can be used to simultaneously test the utilization of 95 different substrates as sole carbon sources (Campbell et al. 2003). This method has also been employed in the recent past to compare physiological profiles of natural and rehabilitated mine soils (Banning et al., 2012), tropical and arable soils (Brackin et al.,2013) as well as different geomorphological units in a semi-stable sand-dune ecosystem (Yu and Steinberger, 2012).

1.6 Soil Enzymes

Enzymes are macromolecular biological catalysts and protein in nature. They accelerate, or catalyze biochemical reactions. They are either endocellular or exo-cellular (biontic and abiontic). Soil enzymes are a large group of enzymes and are of microbial origin. They function in soil organic matter decomposition and transformation, release of nutrients, N_2 fixation, nitrification, de-nitrification, detoxification etc and are sensitive soil bio-indicators. A bioindicator is defined as an organism, part of an organism, the product of an organism (e.g., enzyme), collection of organisms or biological process which can be used to obtain information on the quality of all or part of the environment (Killham, 2002). A number of bioindicators have been suggested for monitoring soil health, some of which include: soil microbial biomass, carbon and nutrient cycling, community structure and biodiversity, soil animals, plants, and soil enzymes (Killham, 2002). These bioindicators are thus very important for resource managers to understand the ecological changes that occur within the soil system.

Activity measurements of several soil enzymes has been used to establish soil fertility indices and the bio-geochemical cycling of carbon, nitrogen, phosphorus, sulfur and other nutrients (Caldwell, 2005). Plant residues are the main source of soil enzymes (Polacco, 1997) both as intracellular and extracellular enzymes. Some soil enzymes are involved in the transformation of carbon (e.g. β-glucosidase and β-galactosidase), nitrogen (e.g. urease)

phosphorus (e.g. Acid phosphatase) and sulphur (sulphatase) (Belyaeva, 2005). Owing to the practical importance soil enzymes, it is easy nowadays to measure the influence of agro-chemicals, industrial waste, heavy metals, as well as soil fertility management practices. Therefore, an integrative biochemical assessment of soil function and condition can be provided by information on enzyme activities and they are useful as indicators of soil functional diversity. Enzyme activity profiles reflect an essential part of the functional diversity in soils, which is driven by the genetic diversity of soil micro-organisms, plants and soil animals in close relation to environmental effects and ecological interactions (Nannipieri et al., 2002). Various pools of enzyme activities (intracellular, free extracellular, clay- and humus- adsorbed enzymes, etc.) contribute to the overall enzyme activity measured, therefore the understanding of fundamental soil biochemical processes due to changes in enzyme activity profiles is limited. The immediate agents of organic matter decomposition are the extracellular enzymes (Burns et al., 2013).

Soil enzyme assessment in the past has been done by the use of a colorimetric method based on p-nitrophenyl substrates (Jean et al., 2012). This method is based on a colorimetric determination of p-nitro-phenol (PNP) that is released by enzyme reactions when a soil sample is incubated in an optimum buffer solution containing substrate-conjugated PNP at optimum temperature (Tabatabai and Bremner, 1969). These assays are based on a spectroscopic detection. Nowadays, a microplatefluorimetry assay that uses fluorescent compound 4-methylumbelliferone (MUB) that was developed by Marx et al. (2001) is used (German et al., 2011). This technology is rapid, sensitive, and has the possibility of simultaneous analysis. It is also known for taking measurements using standard conditions with the ability of automatic calculation of reaction rates (Marx et al., 2001). However, the microplateflourimetric assay technique measures the potential rather than the real enzyme activity within the soils. This is because it uses optimal conditions and synthetic substrates that measure enzyme activities which are not in situ (German et al., 2011).

Soil enzymes are known to be the mediators of all biological processes in the soil. Soil enzyme activities are often closely related to soil organic matter, soil physical properties and microbial activity or biomass. Soil enzymes also are very sensitive to small changes

within the soil, thus providing early indications of changes in soil health (Dick et al.1996). In addition, soil enzyme activities can be used as measures of microbial activity, soil productivity, and inhibiting effects of pollutants (Tate 1995). There are well-documented assays available for a greater number of soil enzyme activities (Dick et al. 1996) some of which include dehydrogenase, glucosidases, urease, amidases, phosphatases, arylsulphatase, cellulases, and phenol oxidases.

Soil enzyme	**Enzyme reaction**	**Indicator of microbial activity**
Dehydrogenase	Electron transport system	C-cycling
β-glucosidase	Cellobiose hydrolysis	C-cycling
Cellulase	Cellulose hydrolysis	C-cycling
Phenol oxidase	Lignin hydrolysis	C-cycling
Urease	Urea hydrolysis	N-cycling
Amidase	N-mineralization	N-cycling
Phosphatase	Release of PO_4^-	P-cycling
Arylsulphatase	Release of SO_4^-	S-cycling

Table 1.3. Soil enzymes as indicators of soil health (Shukla and Varma, 2011).

1.7 Microbial Physiological Profile By Means of Microresp™ Technique

Microbial functional diversity is sometimes preferred to species diversity in microbial ecological studies because the functional diversity relates to the soil activity and provides information about those groups of soil microorganism involved in specific processes being performed at the moment of the analysis. Meanwhile microbial species diversity is difficult to be assessed by classical methods (cultured) because new molecular methods are expensive and require special equipment and qualification and, mainly assess a potential diversity which could not be expressed (Vidican et al., 2013). Studies by Garland and Mills (1991) showed that sole source of carbon utilizations patterns can provide information on the differences in community compositions (Garland and Mills, 1991). They then

highlighted the advantages of this approach after they used this idea to assess differences between habitats and samples. This approach was then called Community Level Physiological Profile (CLPP) and has since then been used intensely in the description and assessment of soil microbial functional diversity. The soil microbial population is considered to have a vast functional diversity if they are capable of efficiently using a wide range of different carbon substrates. This also provides information about the size of the microbial biomass and how different soils might respond to different disturbing factors (Chapman et al., 2007). Low manpower requirements and the reliance on metabolic traits that could lead to functionally relevant characterization of change in microbial community have been identified as the major strengths and advantage of the CLPP approach to measuring microbial functional diversity (Gardland, 1997). Different technologies have been developed in order to process CLPP. The most popular and widely used are Biolog plates (Bilog Inc., USA) and MicroResp™ techniques. For the purpose of this study, we shall focus on explaining in detail the setup and functional mechanism of the MicroResp™ technique.

The MicroResp™ technique was developed by Campbell et al. (2003) and it uses the capacity of the microbial community to metabolize simple organic compounds to assess functional microbial diversity of soil microbial community. This technique has the advantage that the whole soil sample is used and results are obtained in a short time of 4 - 6 hours (Campbell et al., 2003). The MicroResp™ technique uses 15 carbon sources and the response time is obtained after 6 hours of incubation. Up to 96 soil samples (or replicates) can be simultaneously analysed and test a range of carbon sources in a small compact space.

2. AIM OF THE STUDY

The present research activity was funded by the Italian Ministry of University and Research (PRIN project "Health of agro-ecosystems: chemical, biochemical and biological processes that regulate the mobility of As in the soil-water-plant compartments "code 2010JBNLJ7_006)

In particular, the O.U. University of Tuscia, coordinated by prof. Maurizio Petruccioli, is performing a preliminary study on the distribution of arsenic and its effects on microbial biomass in calcareous soils in Central Italy under different vegetation cover.

Specific objectives of this thesis:

- To assess the distribution and availability of arsenic in the soil and in different soil fractions
- To assess the effect of plant cover on its distribution
- To assess microbial biomass size, activity and functional diversity in response to arsenic.

3. MATERIALS AND METHODS

3.1. The study area

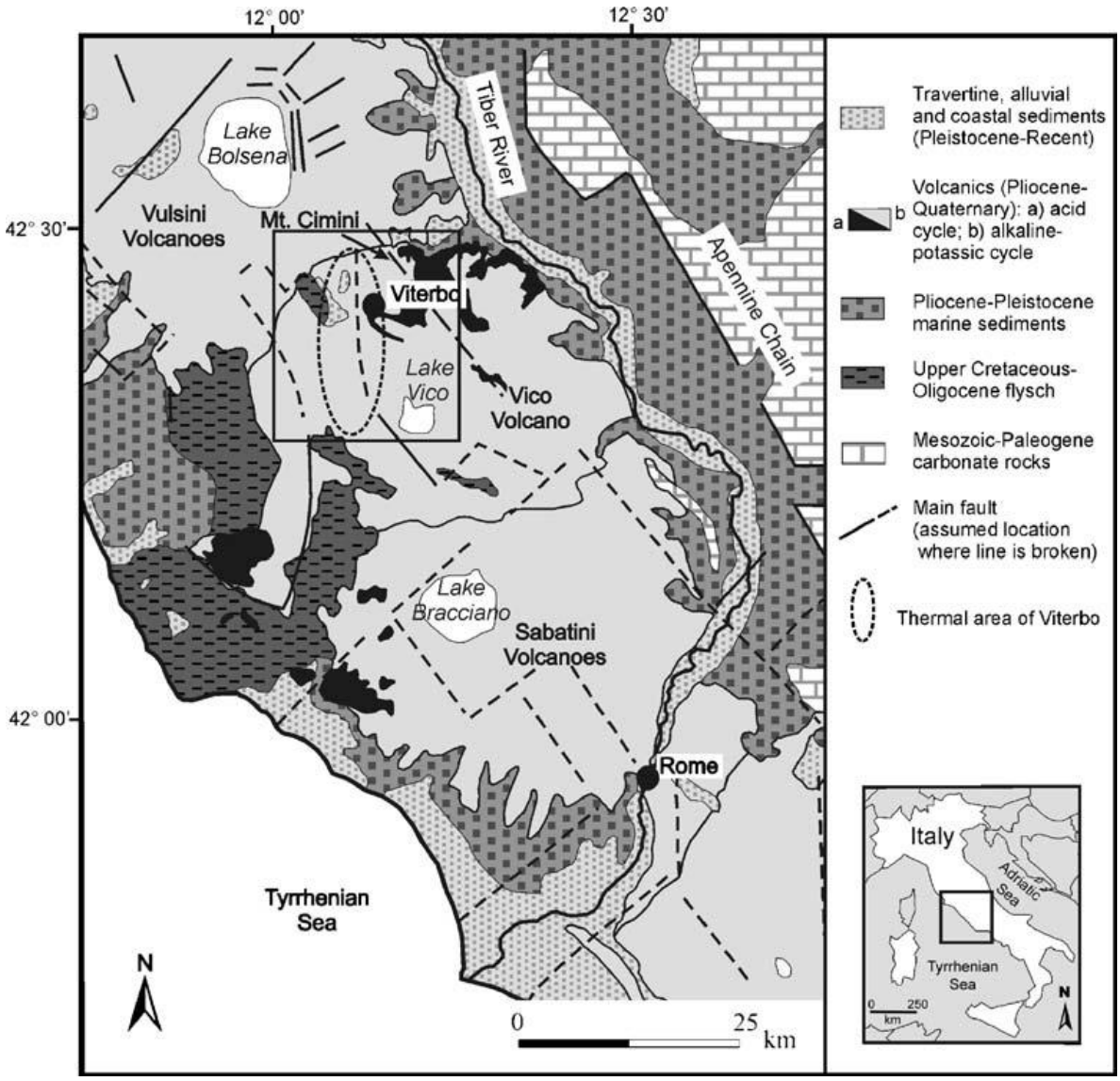

Fig. 3.1 Viterbo and its geothermal area (from Piscopo et al., 2006)

The study area is located in Italy, Lazio region, Viterbo province (Figg. 3.1).

Viterbo area, in northern Latium, hosts several thermal springs (30°- 60°C) which were known since the Roman times for the therapeutic properties of their waters. The Bullicame Spring, the most famous, is mentioned by Dante Alighieri in his 'Divine Comedy' (Inferno,

Canto XIV, verse 76–84) (Fig. 3.2). The vertical up flows of thermal waters of the sulphate-chloride-alkaline-earthtype with higher gas contents, are due to the locally uplifted carbonate reservoir, the reduced thickness of the semi-confining layer and the high local geothermal gradient. (Piscopo et al., 2006).

The geochemical mobilization of arsenic is due to hydrothermal processes causing the up flow of thermal waters. The concentration of As in the thermal waters at the springs ranges from 180 to 370 $\mu g\ L^{-1}$ (Fig.3.2).

Fig 3.2: Bullicame hot-springs

3.1.1. The University of Tuscia Botanical Gardens

The Botanical Gardens of the University of Tuscia were established in 1985 over an area of approximately 6 hectars, 300 m above sea level, in the western side of Viterbo city and in the vicinity of the Bullicame hot springs. The internal spatial organization of the Gardens follows specific taxonomic and phytogeographical criteria. Different natural environments (conifer and broadleaves woods, Mediterranean maquis, desert, Australian ecosystem,

wetlands, tropical etc.) have been created in relation to the climatic and pedological characteristics of the area.

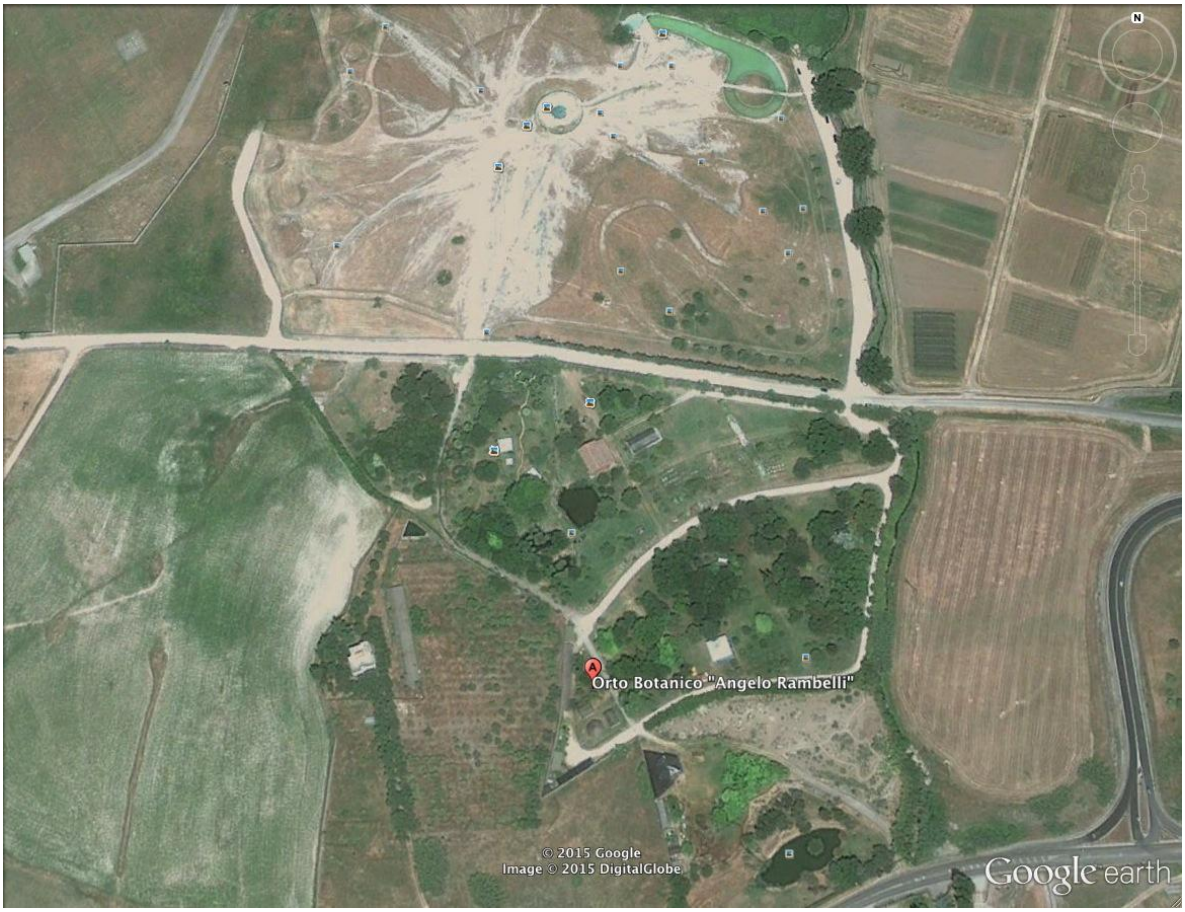

Figure 3.3: aerial view of the Botanical Gardens and the nearby Bullicame hot springs

3.2. Experiment Description

4 soil cores were sampled at 0-20 cm and 20-40 cm, in May 2014 at the hot springs area (Control) and in four plots within the Botanical Gardens under different vegetation covers (*Pinus halepensis*, P; Mediterranean Maquis, M; *Quercus spp*., Q and in a not planted area of the Gardens covered only by a herbaceous layer, N.P.) (Figure 3.5). The sampling sites within the Botanical Gardens area were chosen where no additions of external soil and/or specific amendements were made before trees planting. Soils were immediately sieved (2mm mesh) and kept at 4°C. Prior to biochemical analyses, soil moisture was adjusted to

60% of the water holding capacity (WHC) and left to incubate at room temperature for 24hrs.

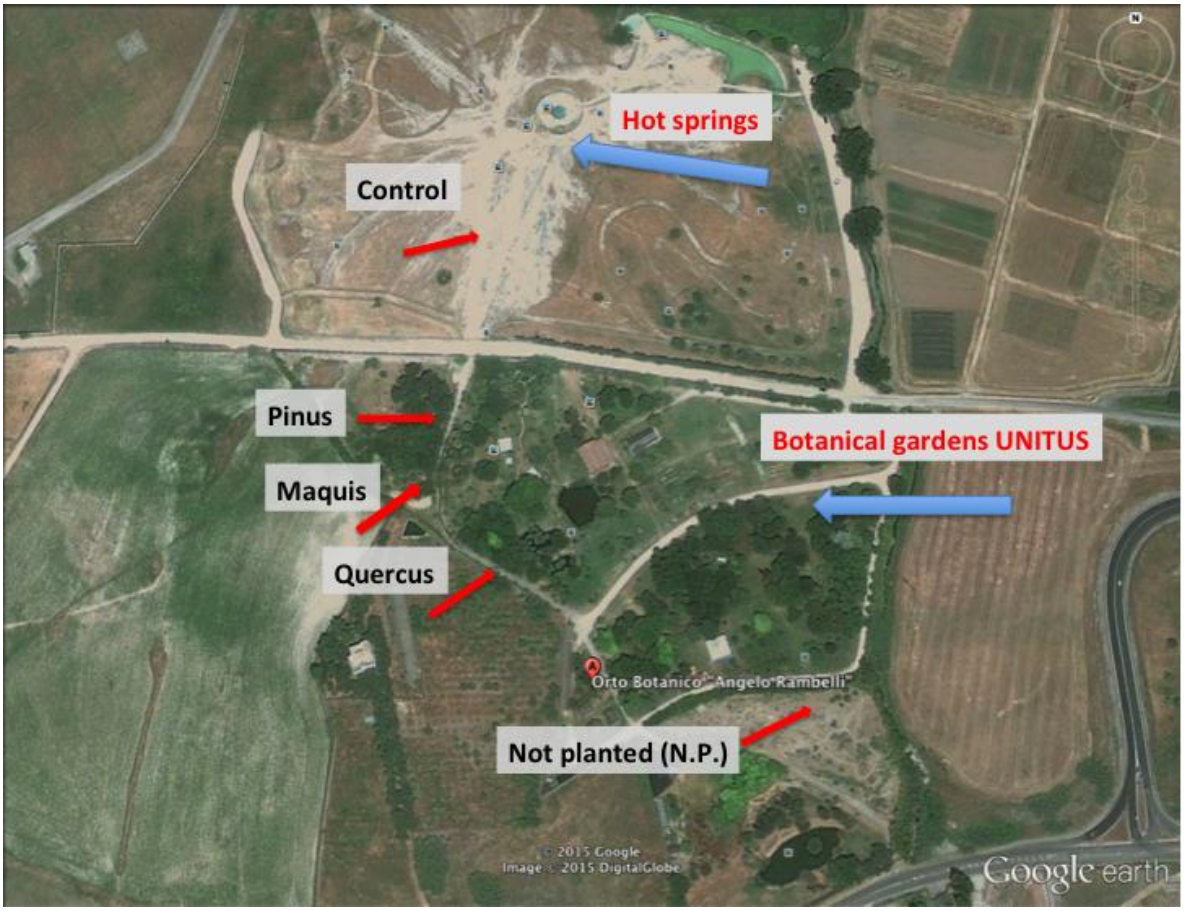

Fig 3.4: Map of study area showing sites of sample collection

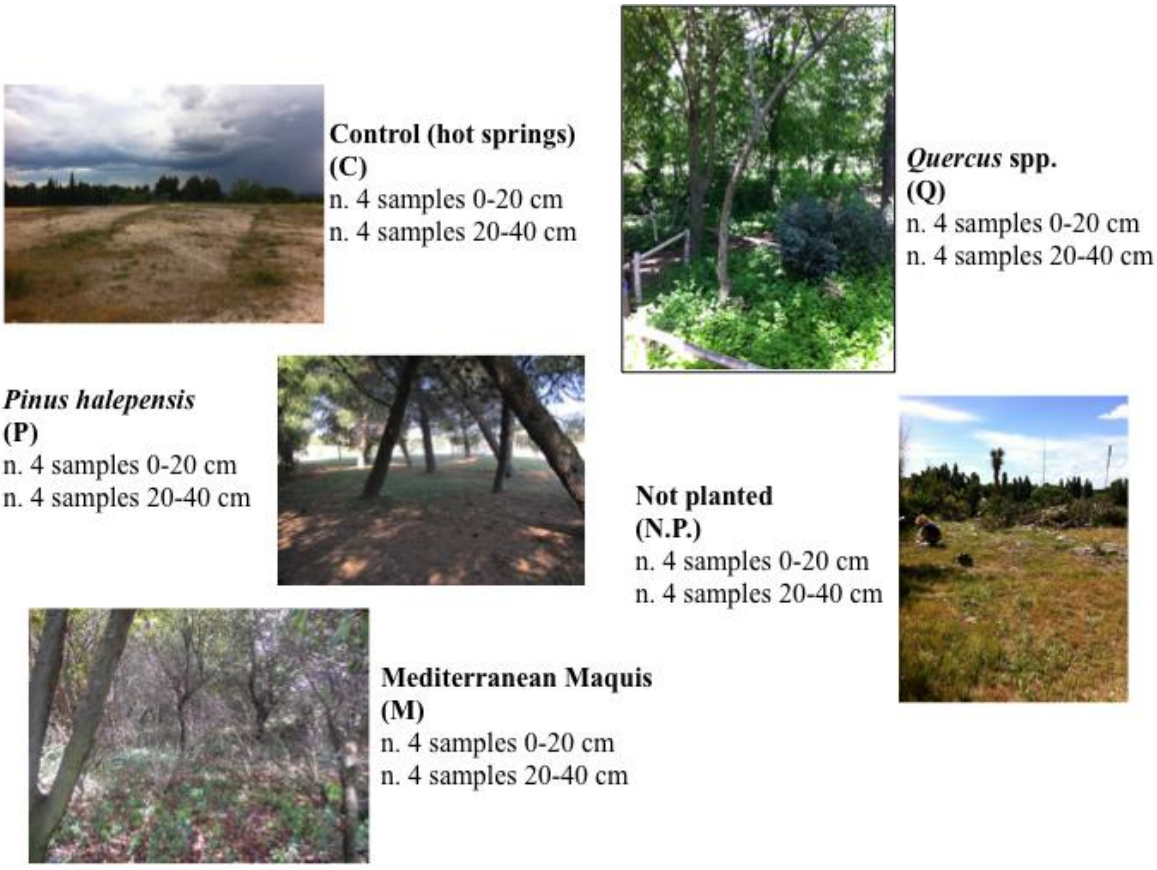

Figure 3.5 Soil sampling

This present work refers to the results of 0-20 cm soil layer.

3.3. Physical analyses

3.3.1. Soil texture

Soil texture was measured using the Pipette method and the main laboratory equipment used was the Andreasen levigator. The purpose of this method is to determine the quantity of the soil's sand, silt and clay with each of them having the following diameters:

- clay less than 0.002 mm
- silt 0.02-0.05 mm
- fine sand 0.2- 0.05 mm
- course sand 2.0-0.2 mm

This method is based on Stoke's Law which affirms that larger particles sediment faster than smaller particles when suspended in a liquid. Before the actual analysis, the samples were treated with hexametaphosphate and oxygenated water so as to destroy the organic/inorganic cements between the soil particles thereby dispersing and desegregating them.

10g of soil was weighed per sample and then added to the oxygenated water (30%) and hexametaphosphate (5%) and left to agitate for 2 hours. The samples were then poured in the levigator and shaken after which a turbid soil suspension was obtained. Two samplings were done; one after 9' and 36'', sedimentation time of clay and another at 16 h, sedimentation time of silt. 10ml of the turbid suspension was taken both times and placed in a weighted crucible and then dried at 105°C. For the coarse sand particles, the mixture was passed through a 0.2mm sieve and dried. At the end of the method, the sample's dry net weight was obtained after 9', 36'', 16h and the sand's dry weight. The percentages of soil particles according to texture were calculated as follows:

a) % silt = (dry net weight at 9'36''- dry net weight at 16h) x levigator volume

b) % clay = (dry net weight at 16h- 2,5 x 10/levigator volume)x levigator volume

c) % coarse sand = (sand dry weight/soil sample) x 100

d) % fine sand= 100-(%silt + %clay + %coarse sand)

3.4. Chemical Analyses

3.4.1. Soil pH

The pH of the soils was measured in a 1:2.5 (w/v) soil/water mixture and 1:2.5 (w/v) soil/Potassium chloride mixture composed of 5g of soils and 12.5 ml of de-ionized water and the replicates with 12.5 ml KCl. The samples were stirred for 30 minutes and then centrifuged for another 15 minutes. The pH was measured on a Mettler Toledo Seven-Multi pH meter with an InLab Routine Pro combination electrode, calibrated to pH buffers of 4 and 7

3.4.2. Total Organic Carbon (TOC) and Total Nitrogen

Before total organic carbon analysis, samples were subjected to an acid treatment with HCl 0.1 N with the aim to remove the inorganic carbon fraction. Then 500mg of the< 2 mm fraction of dried and disaggregated soil samples were weighed and air-dried and heated to high temperatures of above 1000^{o}C within a resistance or induction furnace of an infrared elementalanalyzer in a stream of oxygen to convert all forms of Carbon into CO_2. The evolved CO_2 was detected and quantified using thermal conductivity detection. 500mg of each soil sample was also analyzed for total Nitrogen by Dry combustion in an elemental analyzer.

3.4.3. Total Iron Content

The Iron content of the soils was determined by treating 10 g of air-dried soil with 20 ml of DTPA (Diethylenetriamine-pentaacetic acid) extracting solution. After shaking for two hours, the sample was filtered and the extract analyzed by an inductively coupled plasma atomic emission spectrophotometer (ICP-AES)

3.4.4. Available phosphorus

Available phosphorus content was measured following Bray & Kurtz (1945). The phosphorus was extracted by a solution consisting of 0.5 N HCl and 1 N NH_4F, referred to as Bray-1 extractant. A 1 gram scoop of air-dried soil and 10 ml of extractant were agitated for 5 minutes. The amount of phosphorus extracted was determined by measuring the intensity of the blue colour developed in the filtrate when treated with ammonium paramolybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$] reagent. They were agitated in the test tubes for 20 seconds to ensure a homogeneous mixture. The colour was measured by a UV-VIS mini 1240 Spectrophotometer at 660 nm.

3.4.5. Calcium Carbonate ($CaCO_3$) content

Total carbonate content was measured by the Calcimetry method using a De Astis calcimeter with a solution of 10% hydrochloric acid (HCl).

3.4.6. Reactive carbonates

Active $CaCO_3$ defined as the carbonate capable of reacting with neutral NH_4-oxalate was determined with a 0.1 M NH_4-oxalate, by using a 1:25 soil:solution ratio (2g of sample in 50ml of extractant) and shaking for 2hours at 250 opm in a reciprocating shaker (Drouineau, 1942). To prevent a drastic decrease in the concentration of the oxalate solution during extraction, a soil:solution ratio of 1:50 was used for those samples having a high (>150g/kg) Active CaCO3 content.

3.4.7. Determination of Total Arsenic In Soils

Total arsenic in the soil samples was determined after a microwave assisted acid digestion. 10 ml of concentrated HNO_3 was added to 0.5 g of each sample placed in a 100 ml PFA HP-500 Plus digestion vessel. Samples and reagents were then mixed, sealed, and digested in a CEM MARS Plus microwave oven, working at a temperature of 165°C (2') - 175°C (10'). After cooling to room temperature, the digested samples were then filtered through ash-free filter papers (Whatman 40) and transferred to 50 ml volumetric flasks, and brought to volume with deionized water. Total arsenic determination was carried out using the Hydride Generation with inductively coupled plasma-optical emission spectroscopy (HG-ICP-OES) technique (Perkin-Elmer ICP-OES 8000 DV Perkin-Elmer Corp., Norwalk, CT, USA). A 0.2% (m/v) sodium tetrahydroborate solution (Aldrich Chemical Co., >98%) was prepared in 0.05% (m/v) sodium hydroxide solution and then filtered through a Whatman GF/A filter to remove undissolved solids. This solution was prepared daily. A standard solution of 1000 mg/l As_2O_3 (mono-element , calibration standard, CaPurAn) A 5.0% (m/v) ascorbic acid (Fluka), 5,0% KI (Fluka) in Ultra pure water (18 MΩ cm) solution and HCl 10% was used for pre-reduction of As(V). Ultra pure water (18 MΩ cm) was obtained from Millipore. All reagents were of analytical-reagent grade and the presence of arsenic was not detected in the working range (close to 0.1–25 μg/l). Measurements of total As concentration was performed by means of Hydride Generation with inductively coupled plasma-optical emission spectroscopy (HG-ICP-OES) using an Optima 8000 DV spectrometer (Perkin Elmer) with yttrium as an internal standard. In all analytical determinations, blanks and triplicate samples were used to ensure the quality and reproducibility of the results.

3.4.7.1. As sequential extraction

The sequential extraction of arsenic was carried out following a scheme as was established by Tessier et al. (1979) and Shuman (1985). This was an already established scheme for elements giving anionic species, such as arsenic, in the same environment (contaminated soils) by Gleyzes et al. (2001). 1 g each of the dried soil samples was continuously agitated for the appropriate time in centrifuge tubes. Separation was made by centrifugation between each successive extraction (30 min at 4000 rd min^{-1}). The supernatant was removed after centrifugation and the residue was washed with 16 ml of deionized water. Each extract and the rinse waters associated were pooled. The sequential extraction experiments were performed in triplicates. The sequential extraction scheme used was as follows:

- **F0**: *As water soluble*: 1g of soil was agitated at room temperature for 16 h.

- **F1:** *As soluble in $MgCl_2$*: the solid residue were agitated at room temperature for 1 h with 16 ml $MgCl_2$ (1 mol l^{-1}) at pH 7,

- **F2:** *As bound to carbonates*: the solid residue from F1 was agitated with 16 ml of 1 mol l^{-1} sodium acetate/acetic acid buffer at pH 4.5 for 15 hours at room temperature,

- **F3-Mn**: *As bound to Mn-oxides*: the solid residue from F2 was extracted in weak reducing conditions with 40 ml of 0.04 mol l^{-1} of hydroxylamine hydrochloride in 25% (v/v) acetic acid at 96±5 °C in a water bath during 5.5 h.

- **F3-Fe,a**: *As bound to amorphous Fe oxides*: the solid residue from F3-Mn was agitated with 100 ml of 0.2 mol l^{-1} oxalate/0.2 mol l^{-1} oxalic acid for 4 h in the dark.

- **F3-Fe,c**: *As bound to crystalline Fe oxides*: the solid residue from F3-Fe,a was extracted with 100 ml of 0.2 mol l^{-1} oxalate/oxalic acid/0.1 mol l^{-1} ascorbic acid, in a boiling water bath for 30 min.

- **F4**: *As bound to organic matter and sulphides*: the solid residue from F3-Fe,c was extracted with 6 ml of 0.02 nitric acid and 10 ml of 30% (v/v) hydrogen peroxide. The mixture was heated to 85±5 °C in a water bath for 2 h. A second aliquot of 6 ml of 30% H_2O_2 was then added, and the mixture was heated at the same temperature for 3 h. After cooling, 10 ml of 3.2 mol l^{-1} ammonium acetate in 20% (v/v) nitric acid was added. The sample was then diluted to 40 ml and agitated continuously for 30 min.

- **F5**: *Residual As*: the final residue was treated through a microwave digestion with HNO_3

The potentially bioavailable fraction was considered as the sum of F0, F1 and F2 while the contamination factor (Cf) was calculated as the ratio of the bioavailable fraction to the residual fraction (Li et al., 2015)

3.5. Biochemical analyses

3.5.1. Enzyme activities

Soil enzyme activity was determined by the Micro-Plate Fluorometric Enzyme Assay analytical method (Marx et al., 2001). 2g each of fresh soil was homogenised with 50 ml of pure water using Ultra Turrax for 3 min. 50 µg of soil suspension together with 50 µl of acetate buffer solution were pipetted in three replicas into the 96 multi-well microplate. One column on the microplate was titrated only with the buffer solution so as to test for the chemical hydrolyses. 100 µg of substrate for each enzyme was added and incubated at 30°C for 3 hours. The table below shows the enzymes and their different substrates.

Enzyme name	**Substrate used**
Cellulase	MUF-cellobioside
β-glucosidase	MUF-β-D-glucoside
Acid Phosphatase	MUF-phosphate
Arylsulphatase	MUF-sulfate
Xylanase	MUF-xyloside
Esterase-butyrate	MUF-butyrate

Chitinase	N-acetyl-β-D-glucosaminide
Esterase-aceate	MUF-acetate

Table 3.1. : List of enzymes and their respective substrates

The first reading was done after 30 minutes (t_0) using a Labtech LT-4000 microplate reader. Subsequent readings were taken 30 minutes later (t_{30}), 60 minutes later (t_{60}), 120 minutes later (t_{120}), and 180 minutes later (t_{180}).The calibration curve was done by using 10 µM of MUF solution in amounts of 0.5, 10, 20, 40, 60, 80 and 100µl.Results are expressed as nmoles MUF g^{-1} h^{-1}.

3.5.2. CLPP-MicroResp™

The determination of the community level and physiological profile (CLPP) was proposed by Campbell et al., (2003). It uses the capacity of the microbial community to metabolize simple organic compounds to assess functional microbial diversity of soil microbial community. This technique has the advantage that the whole soil sample is used and results are obtained in a short time of 4 - 6 hours (Campbell et al., 2003). The MicroResp™ technique uses 15 carbon sources and the response time is obtained after 6 hours of incubation. Up to 96 soil samples (or replicates) can be simultaneously analyzed and test a range of carbon sources in a small compact space.

The MicroResp™ Soil Respiration System consists of the following components:

- A 96-well 1.2ml deep well microplate that holds the soil samples and carbon sources,
- A 96-well microplate also called the detection plate that holds the carbon dioxide detection indicator gel. The indicator gel is a mixture of cresol red, potassium chloride, sodium bicarbonate and noble agar
- The MicroResp™ seal
- A filling device
- A metal clamp.

In the colourimetric method of MicroReso™, during incubation the CO_2 formed in deep well plates passes through the silicone rubber to the detection plate and causes a change of color in the gel. After the incubation period the change in color development was read at 570 nm with a spectrophotometer. The indicator dye changes colour with the change in pH when CO_2 reacts with bicarbonate as seen in the equation below:

$$CO_{2(gas)} + H_2O + HCO_3^- \leftrightarrow 2CO_3^{2-} + 3H^+$$

The cresol red changes from pink to yellow as the pH decreases. Potassium Chloride (KCl) is present to reduce the effect of ionic strength on pH (Rowell, 1995).

Each soil sample was conditioned to be between 30-60% of its water holding capacity to ensure a good reaction between soil and carbon substrate. In the preparation of the detection plates, agarose and an indicator solution of red cresol, KCl and $NaHCO_3$ at a 1:2 ratio was used. Aliquots of 150 µl per well were pipetted and then stored for 6 days in a dark room. 16 carbon based substrates (ascorbic acid, oxalic acid, N-acetyl-Glucosamine, L-arginine, citric acid, G-amino-butyric acid, vanilic acid, galactose, fructose, siringic acid, glucose, L-aspartic acid, L-arabinose, glycine and L-leucine) were used with 30mg of substrate/gram of H_20 in the soil samples. They were then left to incubate for 4 hours to at 25ºC. After the incubation period, the detection plate was read with a spectrophotometer (Labtech LT-4000 microplate reader) at a 570nm wavelength specifying that two readings were done one at T0 (before the reaction) and T4 (after the reaction).

Data from the MicroResp™ technique was elaborated using excel. The absorbance values (At) ware normalized as follows:

0hr data= (At0/At0) x Average (At0)

4hr data= (At4/At0) x Average (At0)

The At4 data is converted to $\%CO_2$ using the following formula:

%CO2= A+B / (1+D x At4) where A = -0.3409, B = -1.4606, D = -6.771

Finally the percentage of CO_2 produced in 4 hrs was converted to µg/g/h CO_2 using the following formula:

$$\mu g\ CO_2 g^{-1} h^{-1} = \frac{[(\%CO2/100)\ x\ vol\ x\ (44/22.4)\ x\ (12/44)\ x\ (273/(273+T))]}{soilfwt\ x\ \left(soil\%dwt/100\right)}$$

where:

T= temperature of incubation

Vol= headspace volume in the well (normally 945µl)

Fwt= fresh weight of soil per well

Dwt= soil sample % dry weight

The rate of CO_2 production measured in the wells without any C source has been considered as microbial basal respiration.

3.6. Calculation of diversity indexes

From the results of the enzymatic activity and MicroResp, soil's functional diversity was calculated using the Shannon's index (H') which estimates the degradative potential of the microbial community.

$$H' = -\sum pi\ log_2 pi$$

Where *pi* is the ratio of the enzyme's activity to the sum of all enzymatic activities or the respiration rate of each single C-substrate for MicroResp

3.7. Statistical analysis

All analyses were performed in triplicate for each sample. Comparisons were made using a one-way analysis of variance (ANOVA) with a Tukey post hoc test to reveal significance between groups; differences resulting in $P < 0.05$ were considered statistically significant.

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

4. RESULTS AND DISCUSSIONS

4.1. Chemical Analyses

4.1.1. Texture, pH, total organic carbon and total nitrogen

Table 4.1 reports the physico-chemical characterization of the soils.

	Texture	pH (H_2O)	pH (KCl)	TOC (%)	TN (%)	Available P (ppm)
Control	Sandy-loam	7,9±0,05 ^{a}	7,7±0,05 ^{a}	0,60± 0,11^{b}	0,19± 0,01^{a}	3,16± 0,58^{b}
Pinus		7,9±0,02 ^{a}	7,5±0,06^{bc}	1,26± 0,11 ^{a}	0,27± 0,02^{b}	5,05± 1,00^{b}
Maquis		8,1±0,03 ^{a}	7,7±0,02 ^{ab}	1,35± 0,13 ^{a}	0,34± 0,11^{b}	1,99± 0,53^{b}
Quercus		8,1±0,04 ^{a}	7,7±0,02 ^{a}	1,59± 0,08 ^{a}	0,26± 0,06^{b}	2,71± 0,95^{b}
N.P.		7,7±0,02^{b}	7,5±0,02 ^{a}	1,43± 0,09 ^{a}	0,20± 0,03 ^{ab}	10,61± 1,30 ^{a}

Table 4.1: Physico-chemical properties measured in Control, *Pinus*, Maquis, *Quercus* and Not Planted (N.P.) soils. Error bars are reported. Different letters within columns mean significant differences (P<0,05). TOC: total organic carbon, TN: Total nitrogen, P: phosphorus, pH (H_2O): active acidity, pH (KCl): exchangeable acidity.

All soils can be classified as slightly/moderately alkaline. No significant differences were observed among samples except for a slight decrease for N.P. soil. Exchangeable acidity is significantly lower only under Pinus.

Total organic C content is very low ranging from 0,6 for control soil to 1,5 for *Quercus* soil. The control site is located around the hot springs where there was little or no vegetation combined to elevated levels of $CaCO_3$. The vegetation in this area is made up of early successional species like lichens and mosses. The lack of vegetation in this area accounts for the low level of TOC measured in the control. With the creation of the Botanical gardens, the organic carbon from the plant residue gradually accumulated over the years and accounts for the significantly higher values measured in the other samples. Guo and Gifford (2002), reported significant positive trends of soil organic matter accumulation following changes in land use from cropland to pasture and planted forests.

The availability of inorganic P in these soils is extremely low as compared to the general trend of inorganic phosphorus content in soils which is between 20 – 50 ppm. The low values in the sample sites that have vegetation could be accounted for by the fact that plants used up a greater fraction of it for their nutrition thereby keeping it low as opposed to the relatively high available phosphorus levels recorded in the unplanted area. Furthermore, another possible reason for the low phosphorus levels is because of adsorption and precipitation which are major mechanisms of phosphorus retention in calcareous soils thereby depressing its availability. Available phosphorus is negatively correlated to the amount of lime in soil, but not to Fe, clay content, or CEC (Afif et al., 1993). In contrast, other studies indicate that Phosphorus retention increases with the ratio of Fe oxides to $CaCO_3$ (Carreira and Lajtha, 1997). Amongst the planted areas, *Pinus halepensis* still recorded the highest levels of available phosphorus. This correlates with the low levels of As content recorded for samples from *Pinus* because Phosphorus acts as a chemical analogue to As (Adriano, 2001) and competes with As in plant uptake (Meharg and Macnair, 1992).

4.1.2. Total calcium carbonate ($CaCO_3$)

Figure 4.1 shows the distribution of total and reactive $CaCO_3$. The values of total $CaCO_3$ are extremely elevated with no significant difference between the samples except for the *Pinus halepensis* and the unplanted area that have significant lower content. The high level of total carbonates classifies these soils as extremely calcareous, while the reactive $CaCO_3$ fraction ranges from moderate for N.P. soil to extremely elevated for *Quercus* soil.

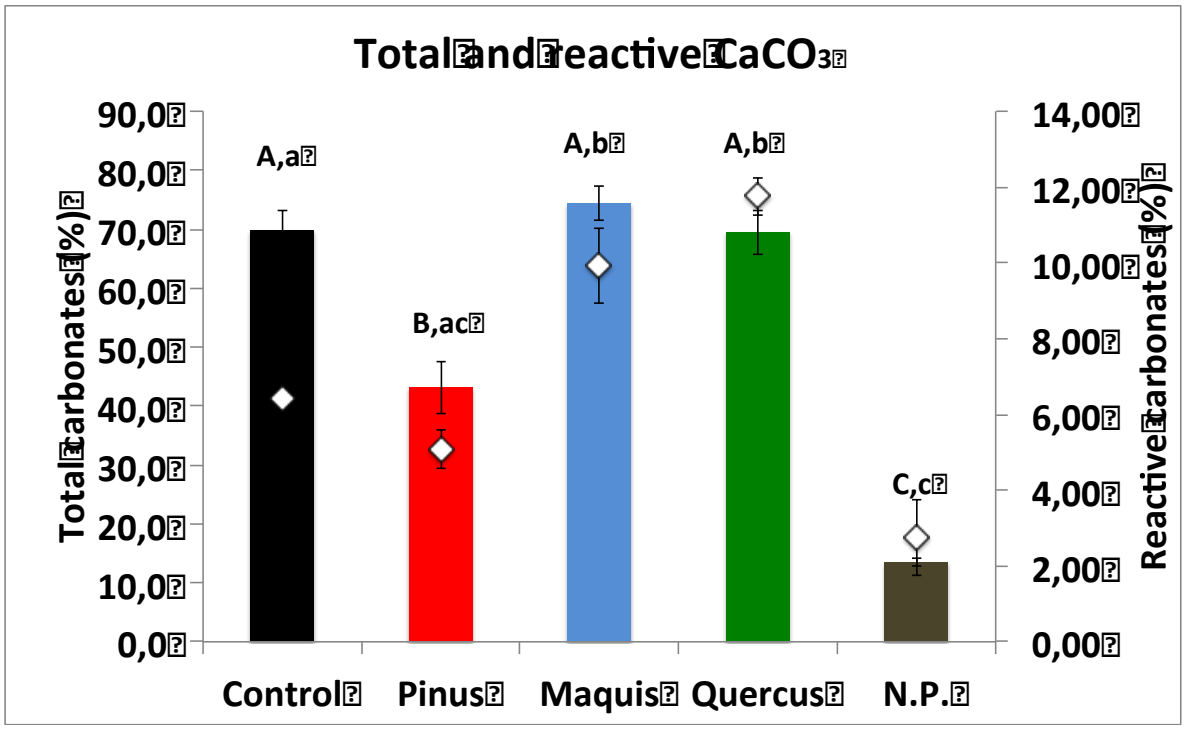

Fig 4.1: Total (bars) and reactive (diamonds) calcium carbonate ($CaCO_3$) content measured in Control, *Pinus*, Maquis, *Quercus* and Not Planted sites. Error bars are reported, different letters mean significant difference, $P<0,05$. Uppercase letters for total carbonates, lowercase letters for reactive carbonates.

The low $CaCO_3$ content found under *Pinus halepensis* can be explained by the following likely mechanism: Pine litter and rhizodepositions are known to promote soil acidification processes (Skyllberg et al., 2001). However no decrease of soil pH was measured under *Pinus*. Therefore we hypothesize that carbonates present in the soil acted as buffer to counterbalance soil acidity thus breaking down $CaCO_3$ structure.

The reactive carbonates represent the finest fraction which influences the availability of important ions such as phosphorus and favouring, thus, an immobilization process. This interaction can occur also with arsenic, given the high similarity between both anions. The amount of reactive carbonates is higher under Maquis and *Quercus* suggesting structural modification on carbonates structure probably induced by plant products. In the same soils we found the lowest amount of available phosphorus; this was confirmedby the inverse relationship found ($r=-0,769$; $p<0,01$) as also reported by Violante et al. (2006, 2007).

4.1.3. Total Arsenic and sequential fractionation

Fig 4.2 and 4.3 show the distribution of total arsenic in the soil and in the different soil fractions respectively. The different fractions are presented from the most stable such as the residual (upper part of the bars) to the most labile such as the water soluble (lower part of the bars)

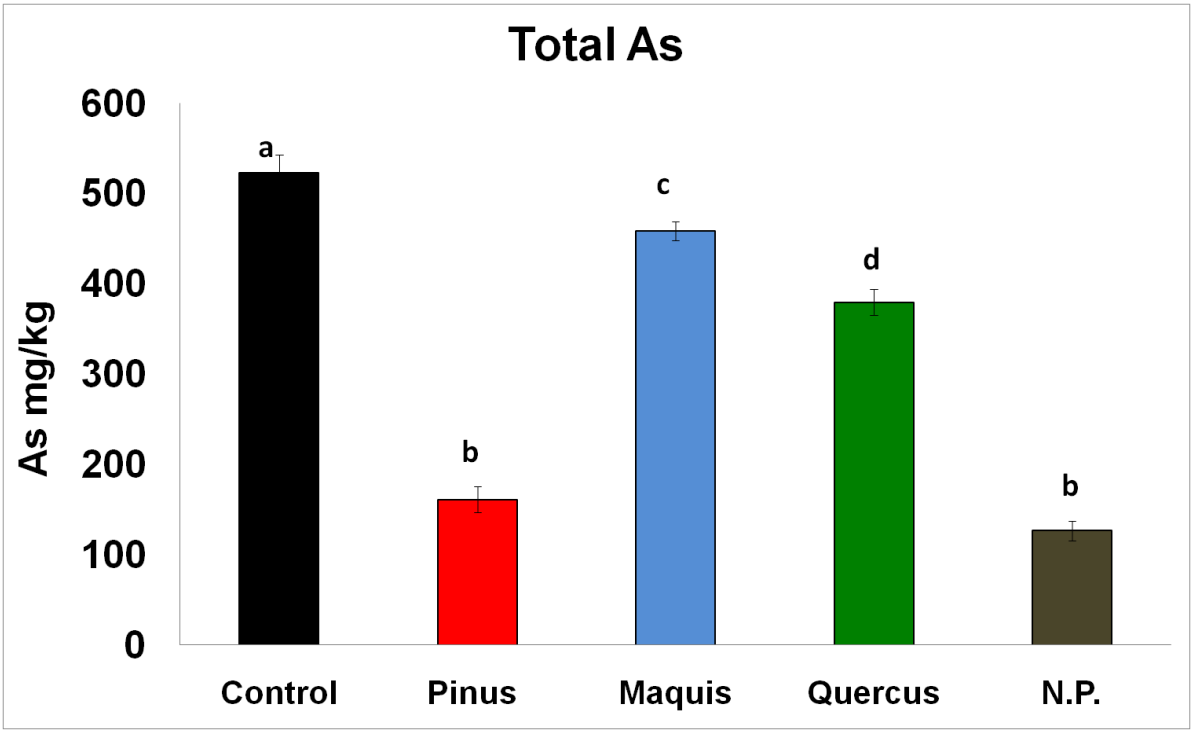

Fig 4.2: Total arsenic content measured in Control, *Pinus*, Maquis, *Quercus* and Not Planted sites. Error bars are reported, different letters mean significant difference, P<0,05.

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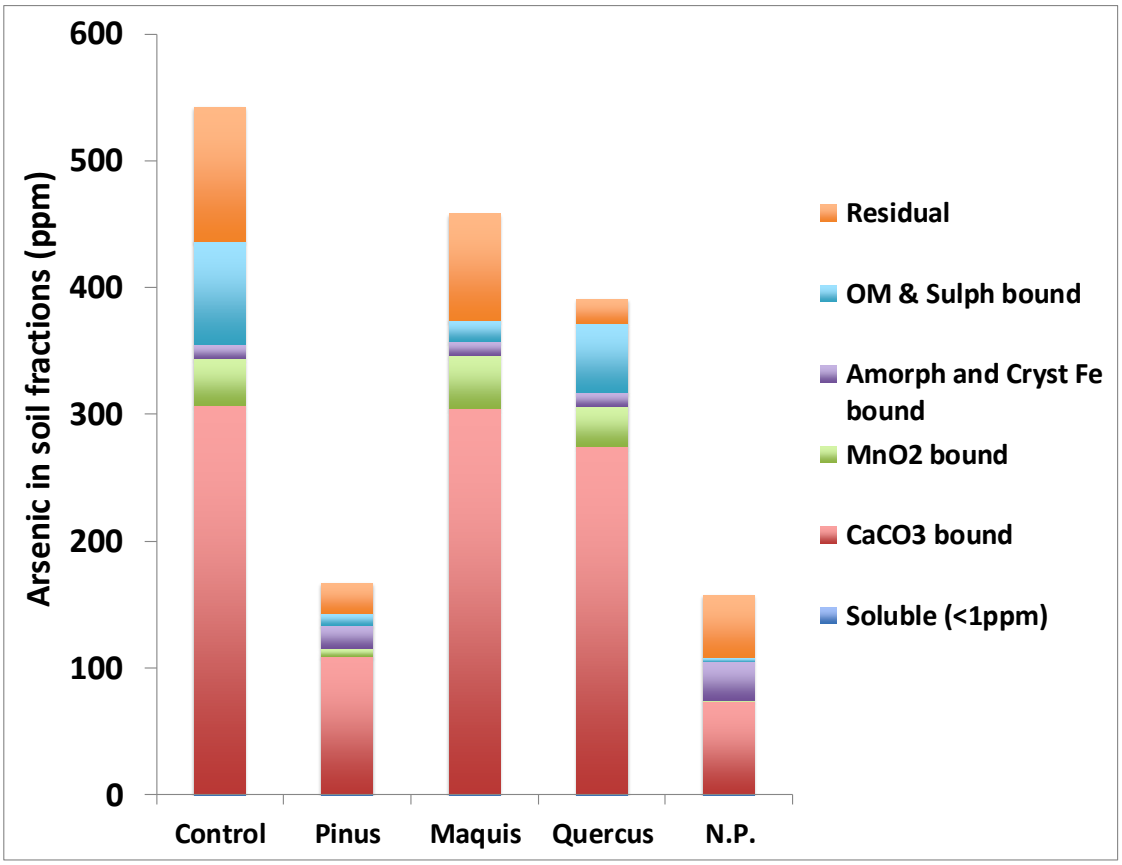

Fig 4.3. Arsenic content in soil fractions obtained through the sequential fractionation extraction. The different fractions were the following: water soluble, $CaCO_3$ bound, MnO_2 bound, Amorphous and crystalline Fe bound, Organic matter and Sulphides bound, Residual.

Before discussing the above results it is worth to emphasize that the arsenic concentration in the hot springs water is 240 ppb meanwhile that of the irrigation water used in the Botanical garden is 31 ppb.

The results show a general declining gradient of soil total As from the control to the unplanted area as reflected by the significant differences in all five sample points. This gradient of decline however is interrupted by *Pinus halepensis* soil where a drastic drop in As content is observed. A negative correlation of total As and available P ($r = -0{,}707$; $p < 0{,}01$) was found confirming the competion reported between these two anions.

The distribution of As in the different soil fractions shows a remarkable amount bound to soil carbonates ranging from 72% for *Quercus* to 46% for N.P. site. The other relevant

fractions are the organic matter/sulphides bound, particularly high in control and Maquis sites followed by the residual, the MnO_2 and the Fe minerals bound. The different distribution of these fractions in the sampling sites reflects the relative abundance of the matrix to which As is specifically associated. Sulphurous minerals, in fact, characterize the parent material in the area of the hot springs explaining the sulphurous properties of thermal waters, while Mn content is particularly elevated under *Pinus* and in the N.P. site (Fig. 4.8).

The ecological implications of a diverse association of Arsenic to the different soil fractions should be discussed in terms of its potential toxicity and environmental hazard. The carbonate fraction is thermodynamically unstable being highly pH and redox conditions dependent (Fuentes et al., 2008). So the As associated to this fraction can be easily be released and be potentially available in the environment.

Low As content in the soil under *Pinus halepensis* cover suggests two possible consequent processes: i) the As associated to the carbonates fraction is released by the acidification process induced by conifer litter and rhizodepositions, ii) the released As is absorbed by *Pinus halepensis* root system. On this purpose Nissen and Benson, (1982) showed that considerable amounts of arsenite were found in seedlings of *Pinus halepensis*, *Pinus pinea*, and *Pinus radiata* following plant uptake of arsenate. Also Párraga-Aguado et al., (2013) reported a positive adaptative behaviour of *Pinus halepensis* in a phytostabilization experiment on As polluted mining soils in Spain.

Additionally the higher level of available phosphorus found in *Pinus* soil could confirm the competition with As for plant root uptake as reported by Meharg & Macnair (1994). Phosphorus and arsenic are chemically analogous, belonging to group V elements and thus have similar electron configuration and chemical properties.

Figure 4.4 shows the bio-available fraction of As (the sum of water soluble As, $CaCO_3$ and MnO_2 bound) and the contamination factor (Cf) calculated as the ratio of the bio-available to the residual fraction. The general trend shows a high bio-available fraction greater than 50% for all sites except *Pinus*. On the other hand the Cf, which takes into account the

residual fraction, i.e. the most stable one, points to *Quercus* soil with a lower retention time and potentially high environmental mobility.

From the above preliminary results it emerges that, in this peculiar environment, the presence of a different vegetation cover can exert a specific influence in terms of root system, rhizodepositions, litter quality and preferential uptake on As dynamics, chemical behavior and interactions etc.

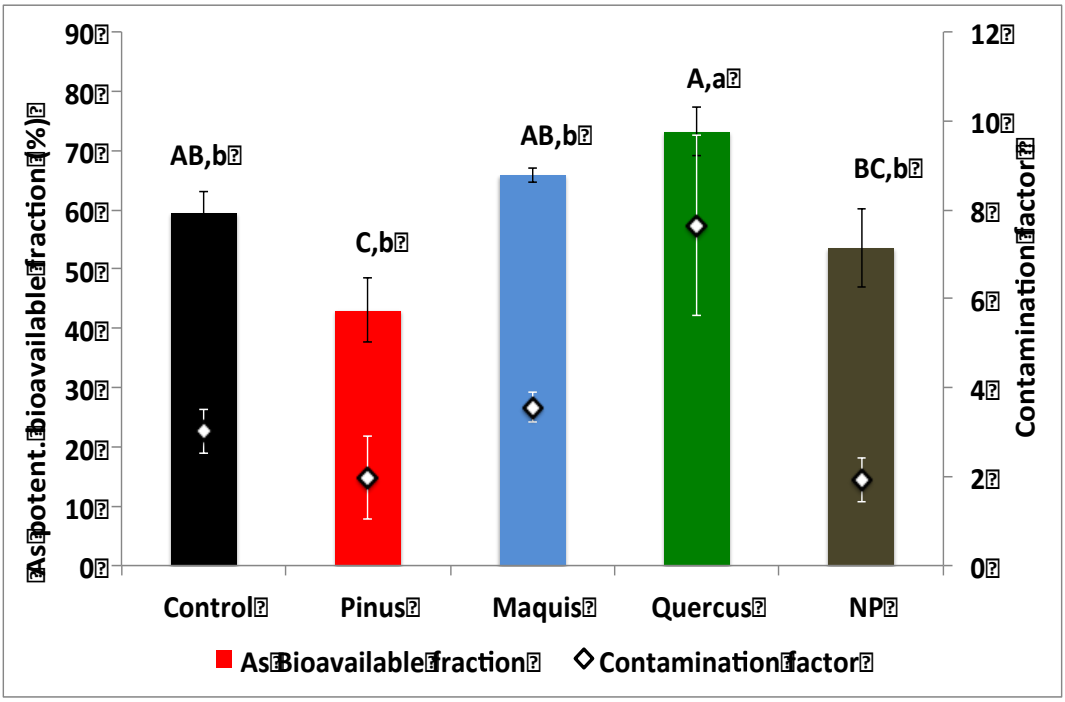

Fig 4.4: Potentially bioavailable fraction of Arsenic (bars) and the Contamination factor (Cf, diamonds) in Control, *Pinus*, Maquis, *Quercus* and Not Planted sites. Bars: As bio-available fraction; Diamonds: Contamination factor. Error bars are reported, different letters mean significant difference, P<0,05 (uppercase letters for As bio-available fraction, lowercase letters for Cf).

Fig 4.5 shows the distribution of total aluminium (Al), iron (Fe), manganese (Mn) and copper (Cu) content. The Al content is generally low as compared to the standard average amount of 88000ppm that is present in soils (Staley and Haupin 1992). There are no significant differences in the Al content except for the *Pinus halepensis* samples and those from the unplanted area that show higher contents of Al. The Fe content in the *Pinus* and

unplanted samples lie within the range of the normal soil iron content of 20,000 to 550,000 ppm (Bodek et al., 1988) and show significant differences while there is a generally lower trend below the normal soil Fe content in the rest of the samples and show no significant differences amongst them. Mn content is generally higher than the average soils concentration, between 40 to 900ppm (Barceloux, 1999). There are no significant differences except for the samples of the unplanted area and *Pinus halepensis*. Cu content is generally higher than the normal soil content of 5 to 70ppm and there are no reported significant differences in all samples.

In conclusion, under *Pinus* and in N.P. soil, a significantly higher content of Fe, Al and Mn is observed. Arsenic in the soil solution is controlled by reactions of retention and release along the surfaces of Fe, Mn, and Al oxides and hydroxides (de Brouwere et al., 2004). The addition of Fe to the soil is in fact capable of immobilizing arsenic. This may explain the lower Cf factor found in these soils indicating that the lower amount of As found under *Pinus* and N.P. soil is associated to Fe, Al and Mn oxides through stronger interaction processes.

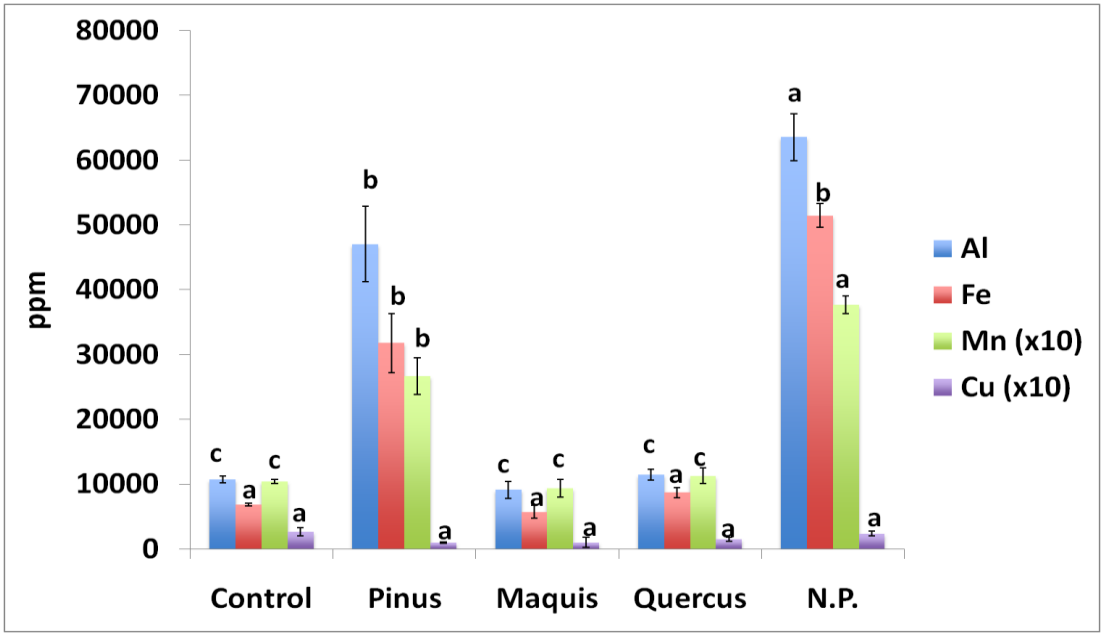

Fig 4.5: Total Al, Fe, Mn and Cu contents measured in Control, *Pinus*, Maquis, *Quercus* and Not Planted sites. Mn and Cu original values are 1 order of magnitude lower. Error bars are reported, different letters mean significant difference, P<0,05.

4.2. Biochemical analyses

4.2.1. Microbial biomass

Figure 4.6 shows soil microbial biomass content expressed as microbial biomass C.

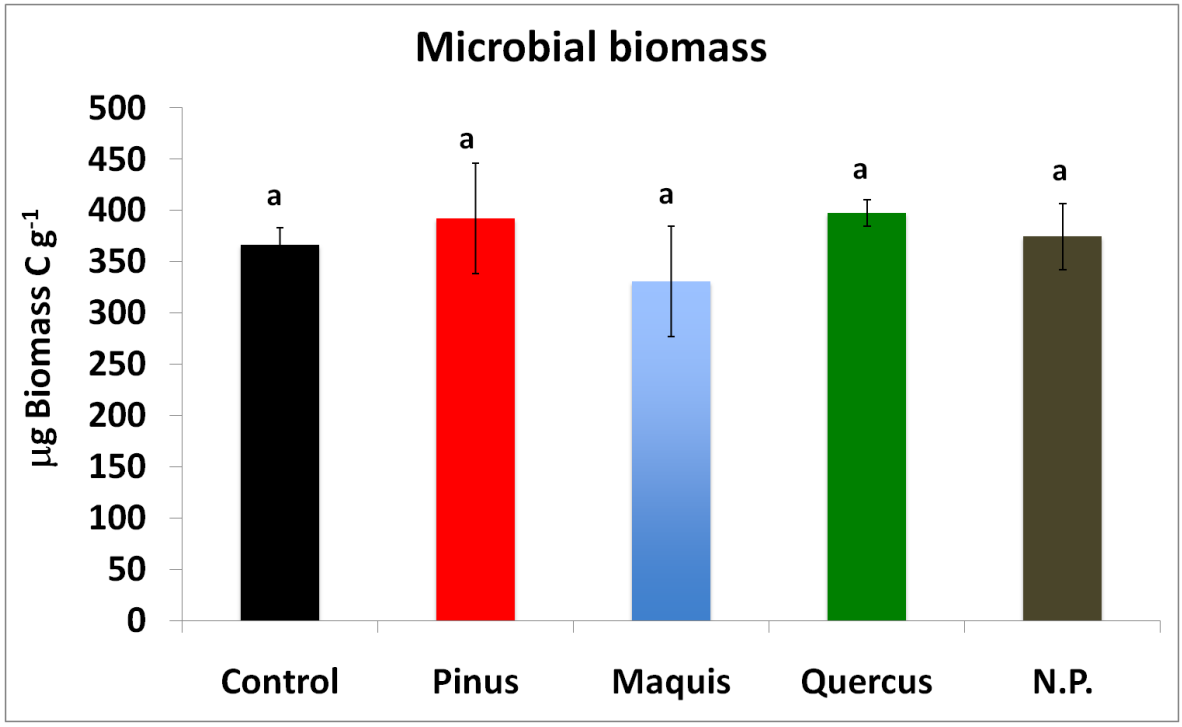

Fig 4.6: Microbial biomass C content measured in Control, *Pinus*, Maquis, *Quercus* and Not Planted sites. Error bars are reported, different letters mean significant difference, P<0,05

Microbial biomass size did not show any significant change among all samples. However, microbial community may react to environmental changes and or stress conditions with shifts within its structural and genetic diversity leaving its overall size unaltered. In this experiment, preliminary results obtained by other partners of the project indicate significant changes within fungal biomass pointing to this fraction of soil microbes as being more sensitive to arsenic pollution (Crognale, personal communication, data not shown)

4.2.2. Microbial Basal Respiration

Figure 4.7 shows microbial basal respiration reported as $\mu gCO_2\ g^{-1}\ h^{-1}$. A general increasing trend is observed in the soils of the Botanical Gardens, where the presence of vegetation, promotes soil microbes metabolism. In particular under *Pinus* and N.P. soils the highest rates are recorded.

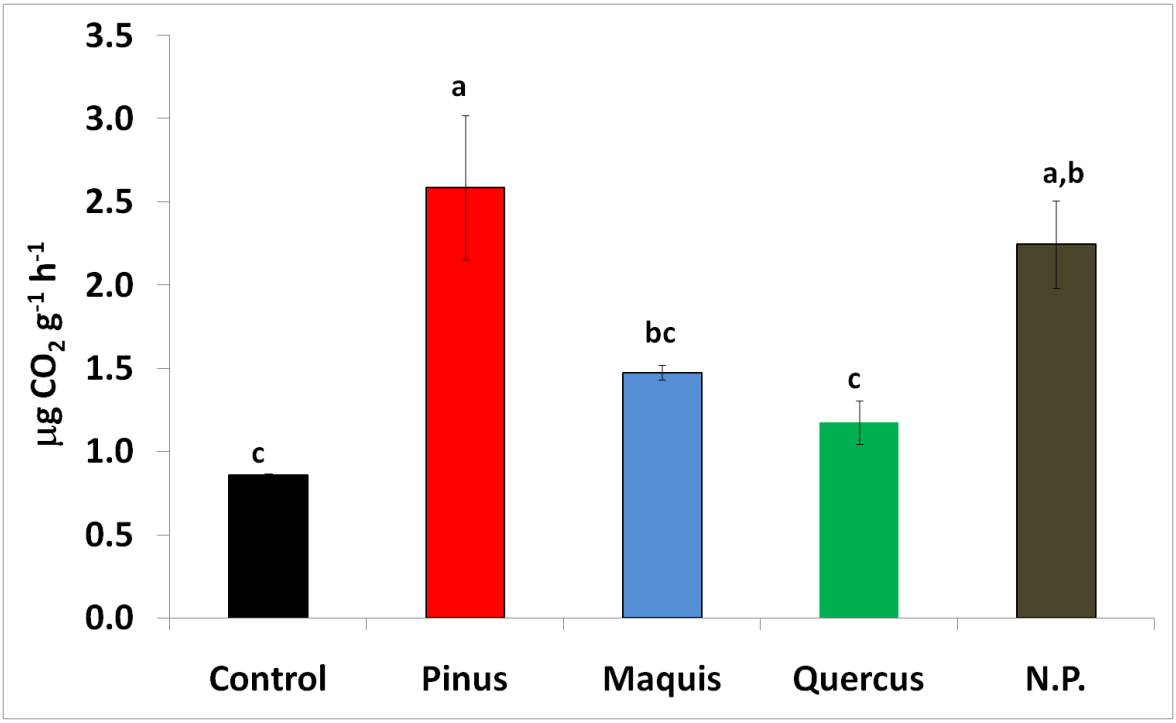

Fig 4.7: Microbial Respiration measured in Control, *Pinus*, Maquis, *Quercus* and Not Planted sites. Error bars are reported, different letters mean significant difference, P<0,05.

It is known that heavy metal pollution can inhibit microbial metabolism (Marabottini et al., 2013). In general, microbial respiration rates were very low due to a particularly extreme environment (high carbonates and moderately alkaline pH) not favouring microbial metabolism. The majority of soil microbes thrive in neutral pH (6-7) due to the high availability of most nutrients in this pH range and with a higher soil organic matter level.Lower respiration rates are recorded in the samples from the control, maquis and *Quercus* where As concentration was significantly higher. The highest microbial respiration was instead recorded in the *Pinus halepensis* samples which were the samples with the least As content. An inverse correlation of microbial basal respiration to bioavailable As has

been found (r=0,538; p<0,05) a also reported in another study on As polluted soils (Marabottini et al., 2013).

4.2.3. Enzyme activities

Table 4.2shows the enzyme activities measured in Control, *Pinus*, Maquis, *Quercus* and N.P. samples.

	Control		Pinus		Maquis		Quercus		N.P.	
	nmoles MUF g^{-1} h^{-1}									
Cellulase	$31,4^{b}$	±10,6	$102,0^{a}$	±18,7	$50,4^{b}$	±9,2	$35,5^{b}$	±1,3	$47,4^{b}$	±5,0
Chitinase	$48,0^{c}$	±1,6	$136,6^{a}$	±5,0	$89,4^{b}$	±7,5	$68,1^{bc}$	±3,0	$71,7^{bc}$	±10,1
β-glucosidase	$402,7^{a}$	±23,8	$486,5^{a}$	±67,3	$503,1^{a}$	±43,4	$304,8^{a}$	±15,9	$318,1^{a}$	±36,2
α–glucosidase	$18,3^{a}$	±2,6	$23,7^{a}$	±2,3	$28,2^{a}$	±4,4	$18,5^{a}$	±1,8	$23,0^{a}$	±3,6
Ac. Phosph.	$103,0^{b}$	±15,9	$149,2^{a}$	±7,8	$152,0^{ab}$	±10,9	$159,6^{ab}$	±13,1	$189,3^{a}$	±29,0
Arylsulph.	$59,5^{b}$	±3,5	$113,0^{a}$	±16,2	$123,6^{a}$	±6,8	$129,8^{a}$	±5,4	$94,8^{ab}$	±11,3
Xylosidase	$63,2^{b}$	±19,4	$120,8^{a}$	±4,4	$83,6^{ab}$	±3,2	$87,1^{ab}$	±9,2	$112,1^{a}$	±4,7
Butyr. est.	1510^{b}	±216	1980^{a}	±141	2085^{bc}	±45	2210^{bc}	±82	2057^{a}	±227

Table 4.2: Enzyme activities measured in Control, *Pinus*, Maquis, *Quercus* and Not Planted soils. Standard errors are reported. Different letters in the same row mean significant differences (P<0,05). Ac.phosph: acid phosphatase, Arylsulph.: arylsulphatase

A significant enhancement of enzyme activity was observed in the soils of the Botanical gardens with respect to the control. The average percentage variations of the samples with respect to the control were: *Pinus halepensis* = +90%, maquis = +57%, *Quercus spp* = +36% and the Not planted area = +45%. The control has the lowest enzyme activity because of the high As content and lack of plants growing in the area as opposed to the other samples with plants that have a favourable medium around the rhizosphere for enzyme activity.

The composition of the plant cover is one of the factors which mostly affect soil organic matter (SOM) properties, microbial biomass and its activity within the soil (Traversa et al., 2008). Coniferous litter is low in bases, and high in resins resulting in deep acid un-decomposable organic material accumulation on the soil surface; conversely, litter from hardwood forest returns annually to the soil a large amount of bases (Bonneau, 1988). In addition, hard wood litter usually decomposes more quickly and completely, and has fewer intermediary decomposed products than coniferous litter (Binkley, 1995). Such differences

suggested a possible influence of plant cover on recalcitrance and stability of SOM in the epipedons, which in turn could cause detectable modification of soil profile, physical, chemical and biochemical characteristics. Recalcitrance is linked not only to the presence of compounds that are scarcely appealing for microorganisms, but also to the different reactions leading to humification processes (Jastrow et al., 2007); therefore the microbial activity, the quantity of humic substances as well as their properties affect soil C turnover.

Sorption competition is known for anions, such as phosphate (Geelhoed et al., 1998), but also for dissolved organic carbon (Bauer and Blodau, 2006 and Redman et al., 2002). Natural organic matter influences arsenic mobility in several ways (Wang and Mulligan, 2006). Dissolved humics induce As redox transformation (Tongesayi and Smart, 2006), form chemical bonds with aqueous As (Thanabalasingam and Pickering, 1986) and influence the stability of As bearing colloids (Ritter et al., 2006). Finally organic matter promotes microbial activity, which changes the redox conditions and may induce methylation of arsenic (Huang and Matzner, 2006).

4.2.4. MicroResp™

Table 4.3 shows the results of microbial functional diversity, expressed in terms of carbon substrate utilization efficiency, and measured by means of MicroResp™ technique. Figure 4.8 also shows information on the variation (%) of the microbial substrate utilization for each site with respect to control soils.

	Control		**Pinus**		**Maquis**		**Quercus**		**N.P.**	
	$\mu g\ CO_2\ g^{-1}\ h^{-1}$									
Citric ac.	0,887^{c}	0,090	5,057^{a}	0,216	1,472^{bc}	0,032	4,356^{bc}	0,328	2,193^{a}	0,676
Oxalic ac.	2,587^{b}	0,053	6,161^{b}	0,212	3,703^{ab}	0,294	3,311^{b}	0,137	2,869^{a}	0,556
Ascorbic ac.	0,946^{bc}	0,112	1,900^{a}	0,333	1,609^{bc}	0,093	3,424^{c}	0,111	0,965^{b}	0,317
Butyric ac.	0,486^{b}	0,023	0,487^{a}	0,152	0,285^{b}	0,047	1,269^{b}	0,030	0,235^{b}	0,058
Arginine	2,045^{b}	0,077	5,645^{a}	0,692	2,109^{b}	0,222	4,676^{b}	0,155	1,676^{a}	0,103
Glycine	1,490^{b}	0,166	1,885^{a}	0,294	1,631^{ab}	0,094	3,048^{ab}	0,113	2,003^{ab}	0,008
Leucine	2,257^{bc}	0,344	1,400^{a}	0,215	2,976^{b}	0,139	3,956^{c}	0,068	1,498^{c}	0,065
Aspartic ac.	2,546^{b}	0,292	2,371^{a}	0,188	2,214^{bc}	0,092	4,772^{c}	0,045	1,359^{b}	0,082
Glutamic ac.	1,396^{b}	0,073	2,435^{a}	0,275	1,171^{b}	0,085	3,127^{b}	0,067	1,480^{a}	0,154
Galactose	2,474^{bc}	0,309	1,930^{a}	0,393	3,623^{b}	0,475	5,471^{bc}	0,241	3,274^{c}	0,179
Arabinose	1,815^{b}	0,117	1,735^{a}	0,349	1,676^{b}	0,035	3,302^{b}	0,076	1,645^{b}	0,118
Glucose	0,954^{b}	0,063	0,846^{a}	0,091	0,297^{c}	0,021	1,652^{bc}	0,066	0,638^{b}	0,059
Fructose	0,464^{c}	0,039	1,752^{a}	0,351	0,757^{c}	0,042	2,601^{c}	0,039	0,451^{b}	0,112
Vanillic ac.	1,134^{c}	0,091	1,596^{a}	0,232	2,103^{b}	0,208	3,930^{bc}	0,116	0,235^{bc}	0,184
Siryingic Phenolic ac.	1,047^{b}	0,124	0,732^{a}	0,152	0,962^{b}	0,124	2,725^{b}	0,036	0,451^{b}	0,049

Table 4.3: CLPP-MicroResp measured in Control, *Pinus*, Maquis, *Quercus* and Not Planted soils. Standard errors are reported. Different letters in the same row mean significant differences (P<0,05).

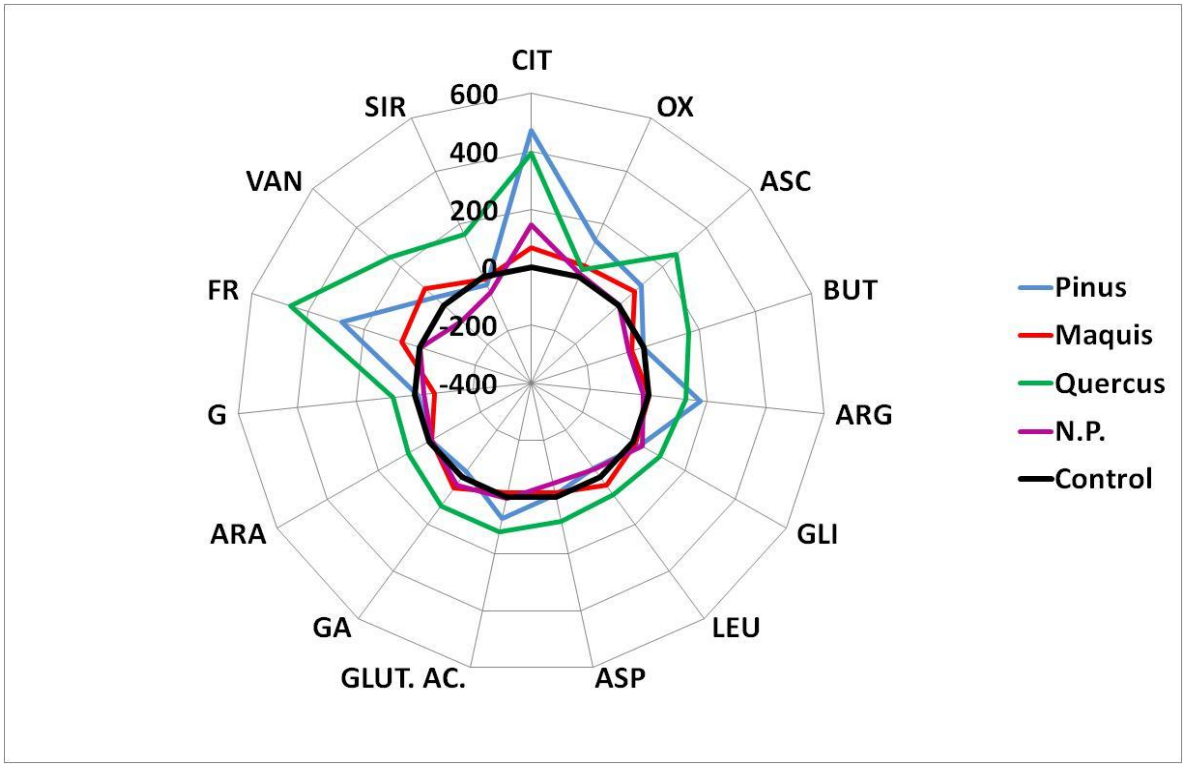

Fig 4.8. Carbon substrate utilization efficiency, measured by means of CLPP-MicroResp™ in Control, *Pinus*, Maquis, *Quercus* and Not Planted sites. The radar graph shows the percentage variation for each substrate utilization with respect to control site. See table 4.3 for acronyms of substrates.

Microbial C utilization performances increased by 80% for *Pinus halepensis*, 18% for Maquis, 170% for *Quercus spp*. The control showed the least carbon substrate use efficiency while *Quercus spp* had the highest functional diversity as seen in their carbon substrate utilization efficiency. There was no significant difference in the functional diversity of the Maquis and Not planted area. The reduced functional diversity of the Control site (highest As content) is evidence of reduced microbial substrate utilization efficiency under metal stress. A study from Liao and Xie, (2007) on the effects of metal toxicity on the efficiency of substrate utilization patterns of microbial communities indicated a lot of variability in the utilization of different substrates suggesting the possibility that the metal contamination may result in a community that was more variable and less stable.

4.2.5. Microbial functional diversity: Shannon Index

Figure 4.9 shows the Shannon diversity Index measured using enzyme activities and MicroResp data; in both cases it provides a measure of microbial functional diversity.

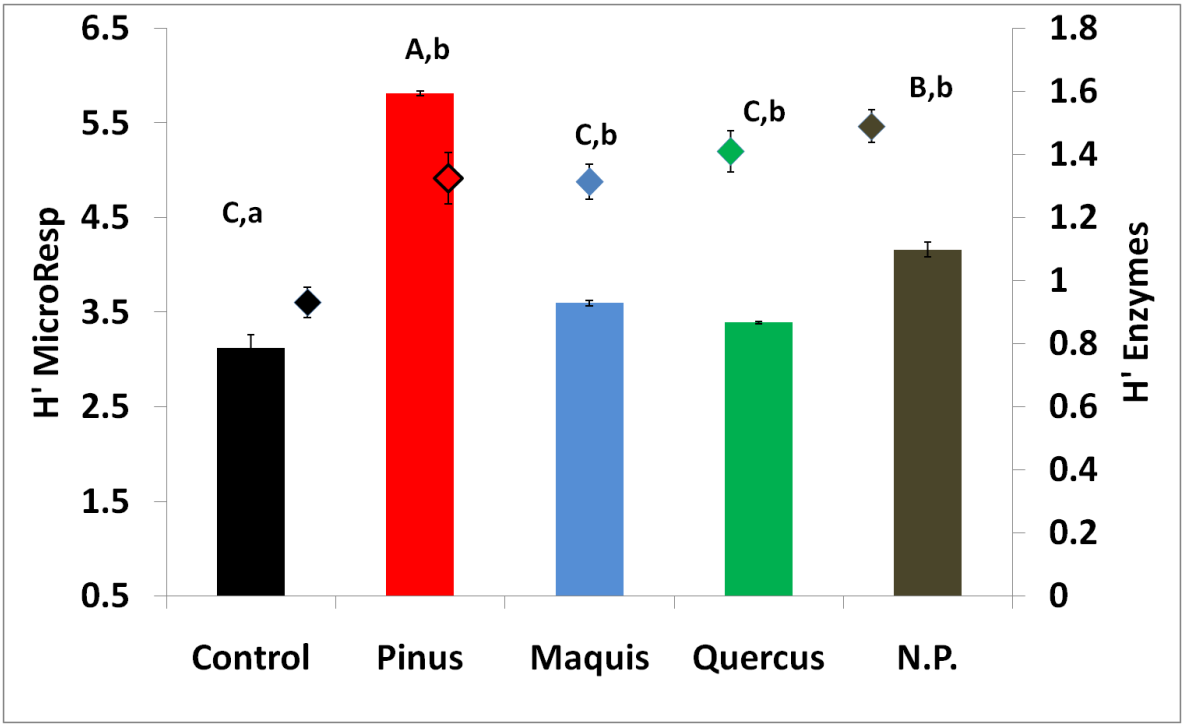

Fig 4.9: Shannon Diversity Index (H') calculated using enzymes activities (diamonds) and MicroResp™ (bars). Error bars are reported. Different letters mean significant difference (P< 0,05). Uppercase letters for H' MicroResp and lowercase letters for H' enzymes.

Both approaches pointed to an increase of microbial functions due to the presence of plants. In particular, under *Pinus*, microbial community processes are highly different. As discussed for enzyme activities, the complexity of chemical compounds released with litter combined to a low As content may account for the high microbial functional diversity found under *Pinus.*The pine needles deposited on the ground from the *Pinus halepensis* together with the vegetation present in this area must have contributed to enhance the presence and variability of different organic substrates present in the soil available for utilization by the microbial community. The response by microorganisms to heavy metals stress has been measured by analyzing microbial respiration and enzyme activities

(Nannipieri et al., 1994). A study from Tyler (1981) reported a negative correlation between microbial functional diversity (enzyme activities) in soils and their heavy metal contents as it is reflected in the high amount of functional diversity under *Pinus* that has the lowest amount of arsenic pollution.

5. CONCLUSIONS

The preliminary results, obtained with this study, suggested the following considerations:

- This study offered an interesting opportunity to evaluate the complex interactions of arsenic-calcareous soils-plants-microbes in a naturally polluted area
- A considerable amount of Arsenic is associated to the $CaCO_3$ fraction
    - It may become unstable under changing environmental conditions and/or the diverse plant inputs to soil
- The presence of plants differently affects As content in soil
    - The different plant covers may have influenced soil properties and processes such as organic matter accumulation and parent material weathering thus affecting As distribution
    - Conifer plant cover reduces total arsenic content and its potential bioavailability in soil:

        Acidification process induced by litter and rhizodepositions was buffered by carbonates thus releasing As

        As could be accumulated in *Pinus* roots and needles (to be verified)
- Microbial functional diversity
    - The presence of plants and plant products favours a higher diversity of microbial processes.

        H' Enzymes points to differences due to plant cover

        H' MicroResp points also on other soil properties (parent material)

Future activities and perspectives

- Determination of microbial structural diversity by means of ELFA technique.
- Determination of As content in plant material
- As speciation (As III and As V)

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