UNIVERSITY OF CATANIA

BIOLOGICAL, GEOLOGICAL AND ENVIRONMENTAL SCIENCES DEPARTMENT

PHD IN GEOLOGICAL, BIOLOGICAL AND ENVIRONMENTAL SCIENCES

XXIX CYCLE

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PHD THESIS

INNOVATIVE TECHNOLOGIES OF PHYTOREMEDIATION FOR CONTAMINATED SOILS

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2013-2016

DEDICATION

I would like to thank my husband Michele, my son Gabriele Rino, and my family for their love and their patience. It was very ‘hard’, but I finished this experience.

I thank my supervisor, Dr Salvatore Antonino Raccuia, for this very green idea, really close to my ideal of life and my respect for the environment.

I thank my colleagues for their help and their support.

Finally, I would like to express my sincerest gratitude to my supervisor at the Univeristy of Edinburgh, Dr Bryne Ngwenya, for his dedication to my work. He was for me a friend in a foreign land.

He also gave me a great opportunity to work in a very prestigious location, Diamond Light Source in Didcot-UK, with one of the few sinchrotrons in the world.

PREFACE

This work was done in collaboration with:

- ✓ CNR - AGROFOOD Department - Institute for Mediterranean Agriculture and Forest Systems of Catania, which funded all the thesis work.

- ✓ SCHOOL of GEOSCIENCES, University of Edinburgh. A part of this work was done with the research group of Dr Bryne Tendelo Ngwenya, reader in Microbial Geochemistry of School of Geosciences, University of Edinburgh.

- ✓ DIAMOND LIGHT SOURCE, Didcot-UK. **STANDARD Proposal Science Case Template – AP20, (**Proposal number: SP15231).

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1. ABSTRACT

Heavy metals pollution has become a global problem in all industrialized countries. Since the industrial revolution, a continuous release of heavy metals has led to a severe contamination of the soil. There are many techniques available for the remediation of contaminated soils, that to date represent a constantly evolving field, absorbing a lot of resources for research and development. Phytoremediation is a technique that uses plants to clean up metals and other contaminants from the soil or to make them harmless or less dangerous. *Cynara cardunculus* L. (cardoon), a perennial species from *Asteraceae* family, native to Mediterranean countries, is a crop studied as a metal accumulator in several researches. In this work, two experiments were performed to evaluate the effects of Cadmium (Cd) and Arsenic (As) on growth of different cardoon subspecies and to determine if this crop can be used for the remediation of polluted soils, combining this application with energy production. Different As and Cd concentrations were tested in *Cynara cardunculus* L. var. *altilis* in Experiment 1 with the aim to study the biological response of cardoon to heavy metals stress. Pot trials were carried out under controlled environment conditions and were exposed to As (0, 6.5, 13 mM), Cd (0, 6.5, 13 mM) and As+Cd (0+0, 6.5+6.5, 13+13 mM) up to 60 days. In cardoon, the biomass production and Cd and As concentrations were determined in 4 different stages of the biological cycle in different parts of plant. The results showed that the cardoon was a plant that could tolerate the presence of Cd and As, even in high doses. Under Cd treatment, the Cd concentration decreased in the roots while increased in the leaves over time. Under As treatment, the As concentration in cardoon tissues increased with increasing As concentration; in particular after 15 days of treatment, the plants treated with As, showed a several reduction in the production of biomass and a significant accumulation of As in both roots and shoots, which subsequently killed the plant. In the combined Cd and As treatments, the plants improved resistance to As and Cd and the presence of Cd increased the ability of cardoon to tolerate As up to 45 days after artificial contamination. In the second study (Experiment 2), three accessions belonging to var. *altilis* (Gen 1) and var. *sylvestris* (Gen 2 and Gen 3) were compared and different concentrations of As (0, 500, 2000 μM), Cd (0, 500, 2000 μM) and As+Cd (0+0, 500+500, 2000+2000 μM) were used. The aim of this work was to assess the concentration and bioaccumulation of As and Cd in the soil and in different parts of the plant, to understand the effects of Cd and As comparing different varieties and genotypes of cardoon plants and to study the specific speciation of As and Cd into plants. The results showed that plants were considerable

tolerant to Cd and As, suggesting that this species was able to tolerate low doses of these toxic elements. The growth parameters showed that all the plants survived until the end of experiment (45 days). For all genotypes, in As treatments, arsenic was accumulated mainly in the roots and the root arsenic concentrations increased significantly with increasing As contamination in the soil.

Otherwise Cd concentrations in old leaves were higher than those in roots with a value of 18.72 mg kg^{-1} dry weight (DW) under Cd 2000 µM in Gen 3. Under As+Cd contamination, the presence of Cd increased the ability of the plants to absorb As and translocate it to old leaves. Furthermore the concentrations of both metals were always greater than those in treatments of As and Cd alone. The As and Cd concentrations in roots and leaves increased significantly with increasing the levels of both metals in the soil.

Moreover, as shown in the values of bioaccumulation factor, cardoon plants had the ability to accumulate large quantities of metal contaminants in its tissue. The results regarding the speciation of As and Cd suggested that exposure of plants to toxic metals appeared to induce the synthesis of sulfur-rich ligands such as phytochelatins, a cysteine-rich oligopeptide, that strongly bound metals. The presence of As upregulated the production of these specific proteins/ligands that bound and traslocated Cd into the plant tissue suggesting that the two metals interacted to magnify phytochelatin production, leading to sequestration of both metals and consequently increasing the tolerance to both.

In conclusion cardoon was a plant that could tolerate the presence of heavy metals including Cd and As. The combination of As+Cd treatment, however, increased the resistance of plants allowing them to survive. The results showed, at least partly, that the plant, had the higher remedying efficiency compared with the other slow-growing hyperaccumulators, for its characteristic of fast growing. Also cardoon plants, contained strong chelators that bound the metals in a non-toxic form promoting the plant growth. Furthurmore, *Cynara Cardunculus* var. *sylvestris* was the best subspecies that could tolerate high levels of As and Cd in its tissues and bioaccumulate greater concentrations of both metals than var. *altilis.* It would be useful to continue the trials with the selected Genotype 3 in future works, with the aim to test for more years, its remediation efficiency in polluted soils and exploit its biomass for energy purposes.

2. INTRODUCTION

The biosphere, also called the ecosphere, is the natural environment of living things: the complex biological epidermis of the Earth (Kabata-Pendias, 2010) and the global ecological system integrating all living beings and their relationships, including their interaction with the elements of the lithosphere, hydrosphere, and atmosphere (Fig. 1).

Figure 1. A beach scene on Earth, simultaneously showing the lithosphere (ground), hydrosphere (ocean) and atmosphere (air). Source: website Wikipedia.

The terrestrial environment, the freshwater environment and the marine environment are three main biosphere ecosystems that include several smaller systems of variable dimensions and conditions.

More than 90% of all living matter is composed mainly of organic compounds and water, a basic constituent of all life. Organomineral compounds and mineral compounds form a relatively small portion of living matter (Kabata-Pendias, 2010).

The chemicals C, O, H, and N are the four organic basic elements of the living organisms; other elements such as K, P, Ca, Mg, S, Na, and Cl are the major elements and represent about 5%. These elements, called essential elements, are required, at different concentrations, for the growth, development and health of organisms. There are also trace elements (sometimes called micronutrients) that are necessary to living organisms. Lastly there are "non-essential" metals (Al, As, Au, Cd, Cr, Hg, Pb, Pd, Pt, Sb, Te, Tl and U) (Jadia and Fulekar, 2009**)**, which have no known biological function (Djingova and Kuleff, 2000) (Fig. 2).

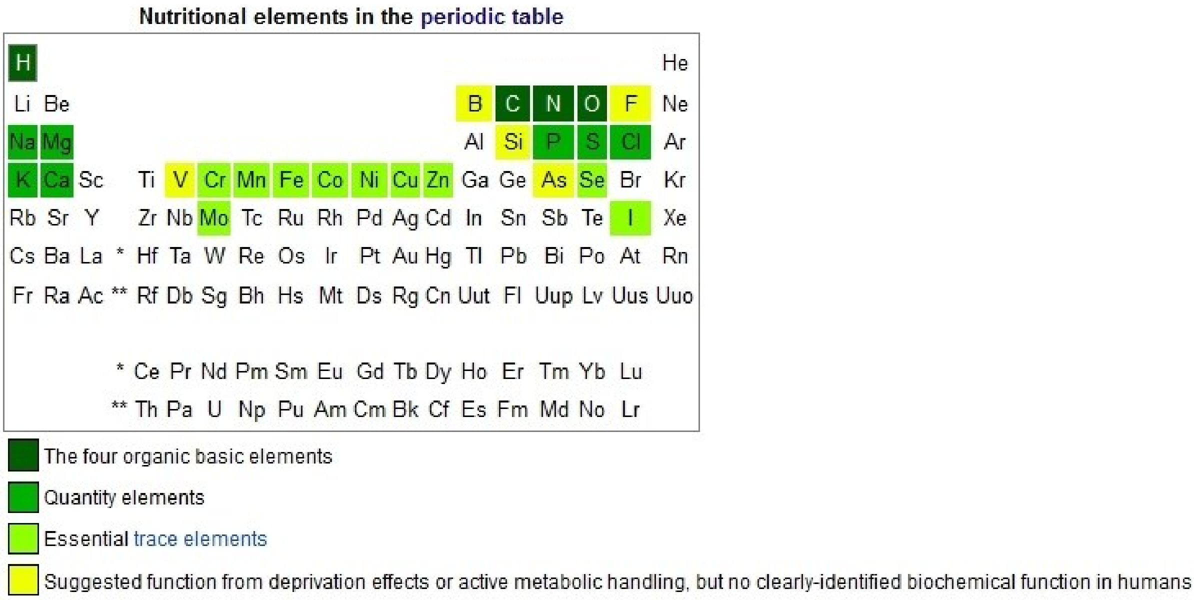

Figure 2. Classification of natural elements.

Source: Impactful Elements - CPALMS.org.

All living organisms, and plants in particular, show a natural ability to select chemical elements (Kabata-Pendias, 2010). However high concentrations of these elements cause considerable problems for plants, animals, and humans; for this reason, it's need to identify and quantify those species in soils that pose the greatest potential threat to organisms and understand how the plants take up essential and non-essential elements from soil (Tabatabai *et al*., 2005**)**.

Sun energy regules the biological processes governing the transfer of elements among the environmental compartments. Each essential element in various forms flows from the nonliving (abiotic) to the living (biotic) components of the biosphere and back to the nonliving again. These cycles vary from one element to another, but each cycle consists of basic phases: gaseous, solution, and sedimentary. Trace element cycles are closely associated with major element cycles but are much less understood (Kabata-Pendias, 2010). (Fig. 3).

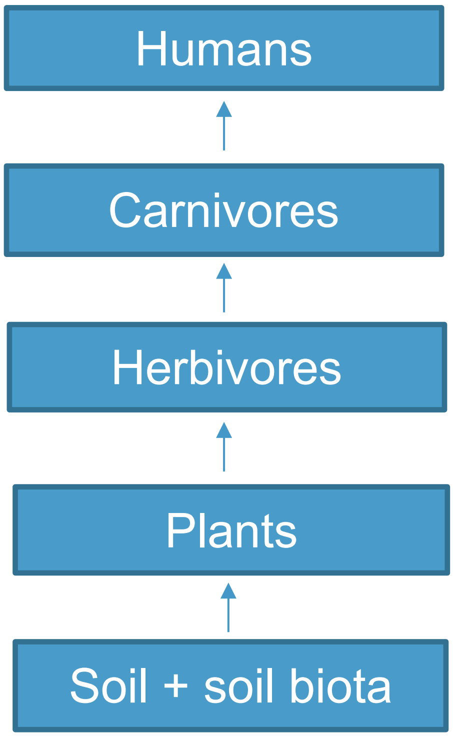

Figure 3. The transfer of chemical elements in a schematic terrestrial trophic chain. Source: Kabata-Pendias, 2010.

3. ENVIRONMENTAL POLLUTION

Humanity has already modified a significant part of the ecosystems in a considerable way, and such modification will continue. Man's impact on the biosphere is very complex (Kabata Pendias, 2010) and it has caused irreversible changes that have accumulated extremely quickly in recent years.

Environmental pollution, by chemicals, in particular, is one of the most effective factors in the destruction of biosphere components and increases at a very rapid rate yearly, causing serious and irreparable damage to the earth (Kabata Pendias, 2010).

Environmental pollution refers to changes that alter the atmosphere and oceans and to local changes (Hooke and Duque, 2012) including those occurring in climate, in composition of air and water, in biodiversity, and in land use (Vitousek, 1992; Rockström *et al.*, 2009).

In this thesis, the attention is focused to soil pollution.

3.1. SOIL POLLUTION

Soil is a very specific component of the biosphere because it is a natural buffer controlling the transport of chemical elements and substances to the atmosphere, hydrosphere, and biota (Kabata Pendias 2010). Despite that, the productivity of soil is the most important role for human survival. Trace elements occur naturally and typical concentrations for some metals found in soils, are at levels that are regarded as trace (<1000 mg kg^{-1}) and rarely toxic (Pendias and Kabata-Pendias 2000; Wuana, 2011). Nonetheless, the overexploitation of the soil to increase its productivity, has had an impact on the concentrations of trace elements, causing an imbalance of all nutrients and destruction of the natural properties of the soil (Kabata Pendias 2010).

Soil pollution, is defined as the build up in soils of persistent toxic compounds, chemicals, salts, radioactive materials or disease causing agents that have adverse effects on plant growth and animal health. The most common chemicals involved in causing soil pollution are petroleum hydrocarbons, herbicides, pesticides, chlorinated hydrocarbons and heavy metals.

Heavy metal pollution has become a global problem in all industrialized countries. From the industrial revolution, a continuous release of heavy metals has led to a severe contamination of the soil (Pedron *et al.*, 2013). Also, the heavy metal contamination is permanent. The heavy metals as elements, are not degradable in the same way of the organic pollutant that is oxidized to carbon dioxide and H_2O.

Geological and anthropogenic activities are sources of heavy metal contamination (Dembitsky and Rezanka, 2003). Anthropogenic source of metal contaminations into the soil, include discharge of industrial waste, percolation of contaminated water, fuel production, mining, smelting processes, rupture of underground storage tanks, solid municipal waste, landfill, utilization of agricultural chemicals, brick kilns and coal combustion (Shen *et al*., 2002).

Recently, many researches have focused on urban soil pollution caused by traffic. The accumulation of trace metals in soil contaminated by automobile service varies with the weather (Kabata Pendias, 2010). The soil pollution is pronounced, especially in the case of Hg, whose content in soil is higher during rainy season (4.8 mg kg^{-1}) than during dry season (2.7 mg kg^{-1}) (Onweremadu *et al*., 2007; Kabata Pendias, 2010).

Still, soils have historically received inputs of metals through agricultural practices in crop farms. Some phosphate fertilizers contain potentially toxic elements, including As, Cd, Cr, Pd, Hg, Ni, and V (Mortvedt, 1996) and some pesticides have contained Cu and As as part of their formulation (Quinton and Catt, 2007). The excessive application of fertilizers and manure affect the concentrations of trace metals, and especially of Cd in the wheat-maize rotations (Ju *et al*., 2007).

High levels of metals in soil influence the growth and development of plants having a negative impact on processes such as respiration, photosynthesis, electron transport and cell division (Wójcik *et al*., 2009; Pourrut *et al*., 2011; Muszyńska and Hanus-Fajerska, 2015).

Also the presence of metals on the crops and the animals with the ingestion of soil during grazing, consequentely causes pollution in the food chain. The possible contamination of human food is an especially urgent problem.

People can be exposed to high levels of toxic metals by breathing air, drinking water, or eating food that contains them (Zovko and Romic, 2011). However the most common route of human exposure to heavy metals is through ingestion from both food and water sources (Pickering and Owen, 1997). Soil remediation is necessary to eliminate serious risk to human health that has resulted from Cd , Se, and Pb in soil (Lasat, 2000).

4. BIOCHEMICAL CHARACTERISTICS OF THE ELEMENTS

From a chemical point of view, the term heavy metal is strictly ascribed to transition metals with atomic mass over 20 and specific gravity above 5. In biology, "heavy" refers to a series of metals and also metalloids that can be toxic to both plants and animals even at very low concentrations. Here the term "heavy metals" will be for these potentially phytotoxic elements.

Two of the most toxic metals, arsenic (As) and cadmium (Cd), that are considered environmental contaminants and require major remediation strategies, were studied in this work.

As is a non essential toxic element of great environmental pollution, due to its toxicity and abundance (Peralta-Videa *et al*., 2009). It is a metalloid widely distributed in the earth's crust and combined rapidly with many metals and non-metals. Arsenic can exist in four oxidation states (-3, 0, +3 and +5). Under reducing conditions, the state of valence +3, arsenite, is the dominant form, while the valence +5, such as arsenate, is the most stable form under oxidizing conditions.

As levels are less than 10 mg kg^{-1} and the natural causes are principally the pedogenic processes, biological and volcanic activity. Trace quantities are in more than 200 minerals, composed by arsenates (60%), sulfates (20%), and the other 20% by arsenites, oxides, silicates and elemental arsenic (As) (Herath *et al*., 2016). Arsenic is generally bound to iron, carbon, oxygen and sulfur, forming inorganic and organic arsenic compounds in different oxidation states.

Regarding the origin of arsenic due to human activities, it is released into the environment from smelting and mining processes, agricultural practices, fabrication and consumption of wood preservatives, and food additives (Aldrich *et al*., 2003).

Many places all over the world are facing problem of arsenic contamination as it is present in high concentrations in different countries: in Asia alone 13 countries are affected by arsenic and the continent has what is considered the worst situation, globally (Kumar *et al*., 2015).

As is also found at high concentration in ground water and surface soil in Europe, Africa, North America, and Australia (Chen *et al*., 2006; Kumar *et al*., 2015).

In some areas of Argentina, Bangladesh, Chile, China, Hungary, India, Mexico, Romania, Taiwan, Vietnam and in many parts of America, the arsenic concentrations are greater than 50 μg L^{-1} (Kumar *et al*., 2015). In particular, in different areas of Argentina, Japan, New Zealand, Chile, Iceland, France, USA, arsenic is present in the thermal waters. In Ghana, Greece, Thailand and the USA, the problems, related to the presence of arsenic, exist in the areas affected by mining activity. The presence of As in groundwater, occurs in oxidising and reducing conditions and in humid-temperate and arid climates. Bangladesh suffers a particular environmental situation in which many contaminated rural wells are used to irrigate crops of rice (Mandal 2002; Kumar *et al*., 2015) (Fig. 4).

Figure 4. Arsenic situation in the world. Source: Kumar *et al*., 2015.

In Italy, the presence of arsenic in soils and waters is generally caused by natural phenomena, but in different regions, including Lombardy, Tuscany, Lazio, Sardinia, Campania and Trentino, its presence is abnormal with concentrations greater than 50 µg L^{-1}, due to human activities (ISPESL-INAIL, 2010).

In Sicily the As situation is dangerous in specific sites: in 2002 three areas of Sicily (Gela, Augusta-Priolo and Milazzo), were declared "high risk of environmental crisis" (Fig.

5).

The WHO, the World Health Organization, based on a study conducted in 2005, decided to start a project to monitorate the human health linked to environmental situation.

Furthurmore, the area of Gela was included among the 57 Italian polluted sites of national interest for environmental remediation because of its widespread contamination from a petrochemical complex (Pasetto *et al*., 2012**)**. It was documented that soil and shallow water in Gela were severely contaminated by metals (Musmeci *et al*., 2009). Maximum concentrations of arsenic were some orders of magnitude higher than the threshold values (Musmeci *et al*., 2009; Directive 2006/118/EC).

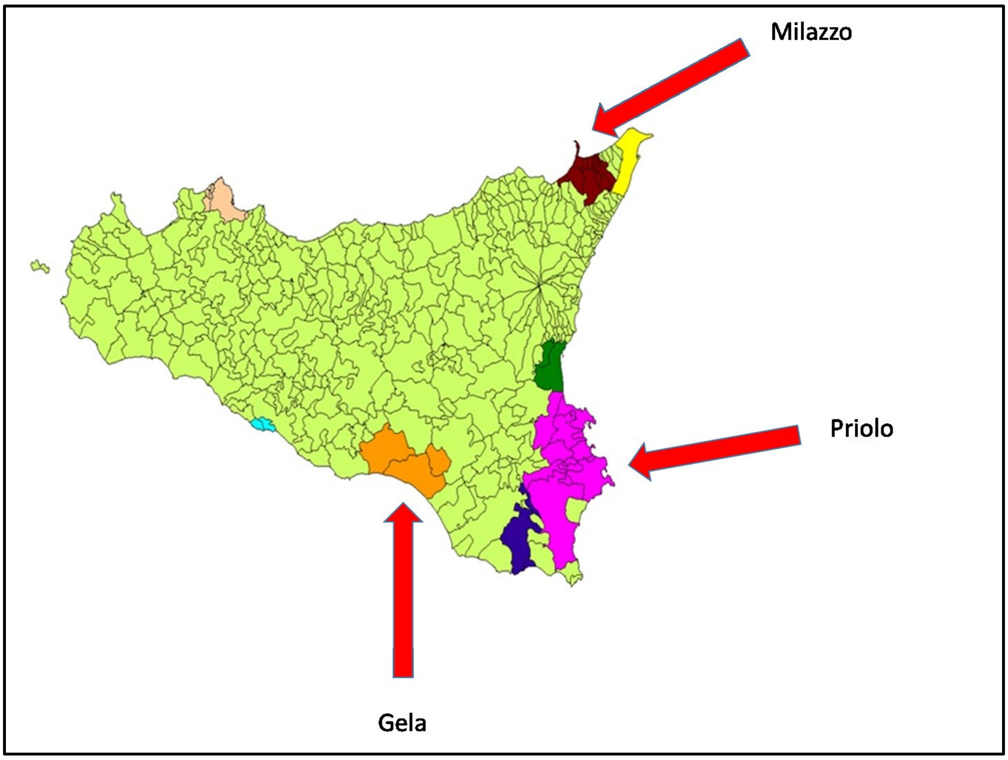

Figure 5. Highly polluted areas of Sicily. Source: Assessorato Regionale Territorio e Ambiente, Regione Sicilia, 2009.

Humans are exposed to many different forms of inorganic and organic arsenic species (arsenicals) in food, water and other environmental media **(**Mandal, 2002**).**

The toxicity of inorganic and organic As compounds has been of global concern due to their probable role in promoting bladder, lung, skin, and prostate cancer in humans, among others (Peralta-Videa *et al*., 2009) (Table 1).

Table 1. Effect of arsenic and cadmium on mammals. Source: Peralta-Videa *et al.,* 2009.

Element	Effects	References
Arsenic	Acute: nausea, vomiting, “rise-water” diarrhea, encephalopathy, multi-organ dysfunction, syndrome, long QT syndrome, painful neuropathy	Soghoian and Sinert (2008)
	Chronic: diabetes, hypopigmentation/hyperkeratosis, cancer: lung, bladder, skin, encephalopathy	Soghoian and Sinert (2008)
	Toxic concentration: 24-h urine: ≥50 μg L^{-1}, or 100 μg g^{-1} creatinine	Soghoian and Sinert (2008)
	Other effects: promotes bladder, lung, skin, and prostate cancer	García Salgado *et al.* (2006)
Cadmium	Acute: pneumonitis (oxide fumes)	Soghoian and Sinert (2008)
	Chronic: proteinuria, lung cancer, osteomalacia	Soghoian and Sinert (2008)
	Toxic concentration: proteinuria and/or ≥15 μg g^{-1} creatinine	Soghoian and Sinert (2008)
	Other effects: kidney and bone damage,	WHO (1992)
	inhibition of progesterone and estradiol,	Zhang *et al.* (2008)
	alterations in uterus, ovaries and oviduct,	Massányi *et al.* (2007)
	progesterone synthesis of ovaries,	Zhang and Jia (2007)
	endocrine disruption,	Henson and Chedrese (2004)
	acts as estrogen in breast cancer,	Brama *et al.* (2007)
	excess risk of cardiovascular mortality	Järup (2003)

Cadmium has no biological function (Sànchez-Pardo *et al*., 2015) and is one of the most dangerous trace elements for human health because it spreads easily in air, water, in animals and plants.

Cd occurs in the earth’s crust at an abundance of 0.1–0.5 ppm and is commonly associated with zinc, lead, and copper ores (Agency for Toxic Substances and Disease Registry, ATSDR, 2002**)**. It is also a natural constituent of ocean water, with average levels between <5 and 110 ng L^{-1}; with higher levels reported near coastal areas and in marine phosphates and phosphorites (Agency for Toxic Substances and Disease Registry, ATSDR, 2002). Natural emissions of cadmium to the environment can result from volcanic eruptions, forest fires, emission of sea salt aerosols, or other natural phenomena (Agency for Toxic Substances and Disease Registry, ATSDR, 2002**).**

Nevertheless, the human activities contribue to increase the spread of cadmium pollution.

The most significant use of Cd is in Ni/Cd batteries, such as rechargeable or secondary power sources exhibiting high output, long life, low maintenance, and high tolerance to physical and electrical stress (Wuana and Okieimen, 2011).

High concentrations of Cd are found in sewage sludge, pesticides, manufacture and application of phosphate fertilisers, fossil fuel combustion, waste incineration and disposal (Agency for Toxic Substances and Disease Registry, ATSDR, 2002); but the main source of Cd intake is through smoking and food (Jarup, 2003). Vegetables, particularly leafy vegetables such as lettuce (0.051 mg kg^{-1}) and spinach (0.124 mg kg^{-1}), have the highest concentrations of cadmium; the concentrations of cadmium in all vegetables ranged from 0.001 to 0.124 mg kg^{-1} (Agency for Toxic Substances and Disease Registry, ATSDR, 2002). Peanuts, soybeans, and sunflower seeds have naturally high levels of cadmium (Agency for Toxic Substances and Disease Registry, ATSDR, 2002); the mean concentration of cadmium in legumes and nuts ranged from 0.001 to 0.054 mg kg^{-1} (Agency for Toxic Substances and Disease Registry, ATSDR, 2002).

Tobacco leaves naturally accumulate cadmium (Agency for Toxic Substances and Disease Registry, ATSDR, 2002). Its levels in cigarettes vary greatly depending on the source of production (Agency for Toxic Substances and Disease Registry, ATSDR, 2002). Tobacco contains approximately 0.5–2.0 μg cadmium per cigarette, and about 10% is inhaled when smoked (Agency for Toxic Substances and Disease Registry, ATSDR, 2002).

Regarding human health, Cd represents serious environmental hazards because it can be absorbed via the alimentary tract, penetrates through placenta during pregnancy, and damages membranes and DNA (Kabata-Pendias, 2004).

Moreover, Cd may cause kidney and bones damage, and also affects the female reproduction system, which implies a serious threat for mammals and humans (Peralta-Videa *et al*., 2009) (Table 1). Furthermore, it is the only metal that might pose human or animal health risks at plant tissue concentrations that are not generally phytotoxic (Peijnenburg *et al*., 2000).

Due to its ubiquity and its toxicity, cadmium is in the list of pollutants to be monitored for the health of Sicily environment (DECRETO 18 settembre 2009).

5. HEAVY METAL TOXICITY

Plants, whose essential physiological processes are seriously impaired, are among the organisms affected (Muszyńska and Hanus-Fajerska, 2015). Elevated concentrations of heavy metals have a negative impact on processes such as respiration, photosynthesis, electron transport and cell division (Wójcik *et al*., 2009; Pourrut *et al*., 2011; Muszyńska and Hanus-Fajerska, 2015**).**

Complex biochemical reactions occur in plants stressed by heavy metal/metalloid (Peralta-Videa *et al*., 2009). As seen in Figure 6, heavy metals bind sulfuric group of proteins, replace the protein cationic centers (in both cases the heavy metals change the protein folding and the protein becomes inactive), or increase the reactive of oxygen species causing oxidative stress (Peralta-Videa *et al*., 2009).

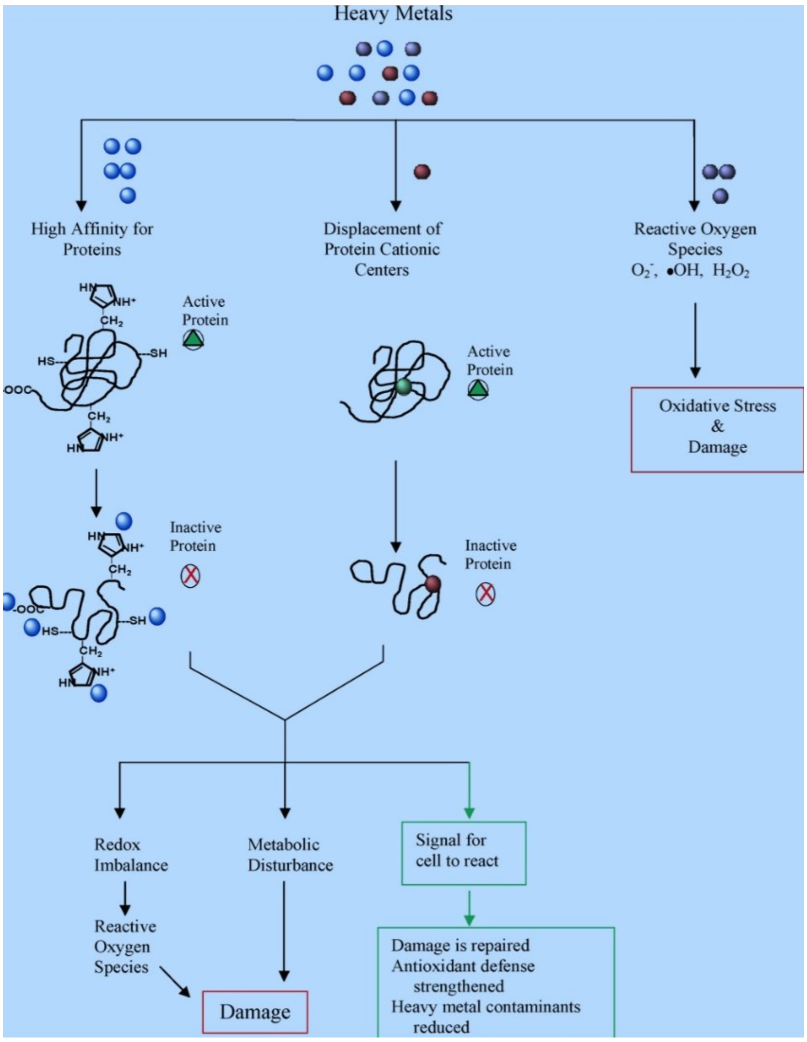

Figure 6. The biochemical reactions of heavy metal in plants that cause stress and damage. Source: Peralta-Videa *et al*., 2009.

The toxicity and bioavailability of a heavy metal depend in part on reactivity and solubility, which are determined by the speciation or chemical form of the element (Brown, 1999).

5.1. METAL MOBILITY AND BIOAVAILABILITY IN SOIL

Metals are chemically very reactive in the environment, which results in their mobility and bioavailability to living organisms (Zovko and Romić, 2011). The metals mobility into the soil depends generally on soil pH (Brallier *et al*., 1996) that solubilizes the metal ions that normally are in a form not bioavailable for the uptake by the plants. Low values of pH increase the mobility of most of heavy metals (Kabata-Pendias, 2010) while high pH values decrease metal mobility in the soil (Tills and Alloway, 1983b; Garcia-Miragaya, 1984; Ram and Verloo, 1985; Sanchez-Camazano *et al*., 1994; Chuan *et al.*, 1996). Moreover, acid rain and sulfate deposition in the soil as a result of melting activity, contribute to increase soil acidity.

Once metals introduced into soil environments, occur complex reactions between solid or liquid phases of the soil and the metal present as free metal ion or complexed to organic or inorganic ligand. Both the free ion and the metal-ligand complex can be exposed to one of several pathways, including: uptake by plants, mineral surfaces, and organic matter; transport through the vadose zone; precipitation as a solid phase; and diffusion into porous material (Tabatabai *et al*., 2005) (Fig. 7).

For the uptake by plants, trace elements have to be bioavailable (ready to be absorbed by roots) (Lasat, 2000). In soil solution the bioavailability depends on metal solubility that differs between metals; for this reason heavy metals can be divided into: (1) readily bioavailable (Cd, Ni, Zn, As, Se, Cu); (2) moderately bioavailable (Co, Mn) and (3) least bioavailable (Pb, Cr) (Ali *et al*. 2013; Muszyńska and Hanus-Fajerska, 2015).

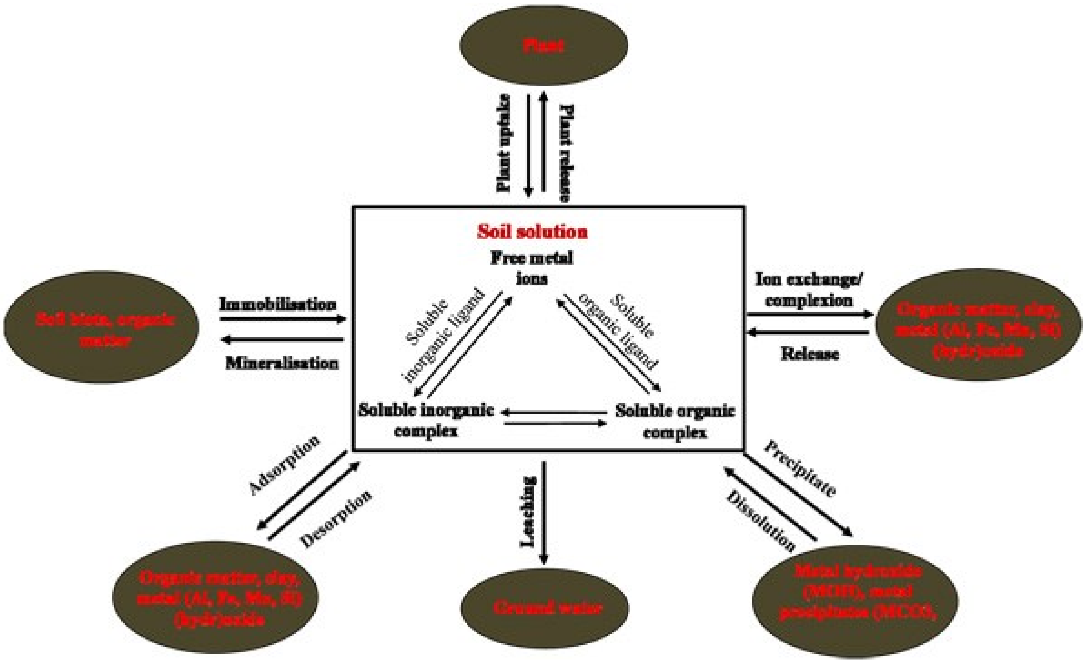

Figure 7. Various chemical and physical pathways a metal ion may encounter once introduced into the soil environment.

At low pH values (pH 4), As is found complexed with iron whereas at high pH values (pH 6–8) it is mostly bound to calcium (Fayiga *et al*., 2007). Moreover, the presence of Fe and manganese oxides also increases As mobility and availability in soil (Zavala and Duxbury, 2008; Peralta-Videa *et al*., 2009).

The bioavailability of Cd in soil depends on its concentration, pH, organic matter content, clay content, soil moisture conditions, and availability of macro- and micronutrients (Welch and Norvell, 1999). Cadmium in soil tends to be more available when the soil pH is low (4.0-4.5) and a drop in pH of merely 0.2 units results in a 3–5 times increase in Cd labile pool (Kabata-Pendias, 2010). During weathering, Cd goes readily into mobile pool and may form several types of complex ions and organic chelates (Kabata-Pendias, 2010).

5.2. SPECIATION OF HEAVY METALS AND UPTAKE BY PLANTS

The term ‘‘speciation” refers to (i) the identity of the element, (ii) its oxidation state, (iii) its physical state (association and complexes to solids and dissolved species), (iv) its empirical formula, and (v) its detailed molecular structure (Brown *et al*., 1999).

The uptake by plants and the toxicity of metal depend of chemical form of trace elements.

The toxicity of arsenic compounds can vary greatly; forms of arsenic that are more rapidly absorbed, are more toxic, while those most rapidly eliminated, tend to be less toxic.

Arsenite [As(III)] and arsenate [As(V)] are the phytoavailable forms of inorganic As in soil solution. Arsenate is taken up by plants via phosphate transporters in the plasma membrane of root cells, and it is rapidly reduced to arsenite once inside the cytoplasm. Then, it can be biotrasformed to less toxic organic compounds, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), or complexed with sulfur ligands and transported into the vacuole. In both cases the plant can be detoxificated from arsenic. The same situation occurs in the leaves, where arsenate (As (V)) is taken up via phosphate transporters, reduced to arsenite, complexed with sulfur ligands and carried as As(III)-tris-glutathione complex into the vacuole (Fig. 8).

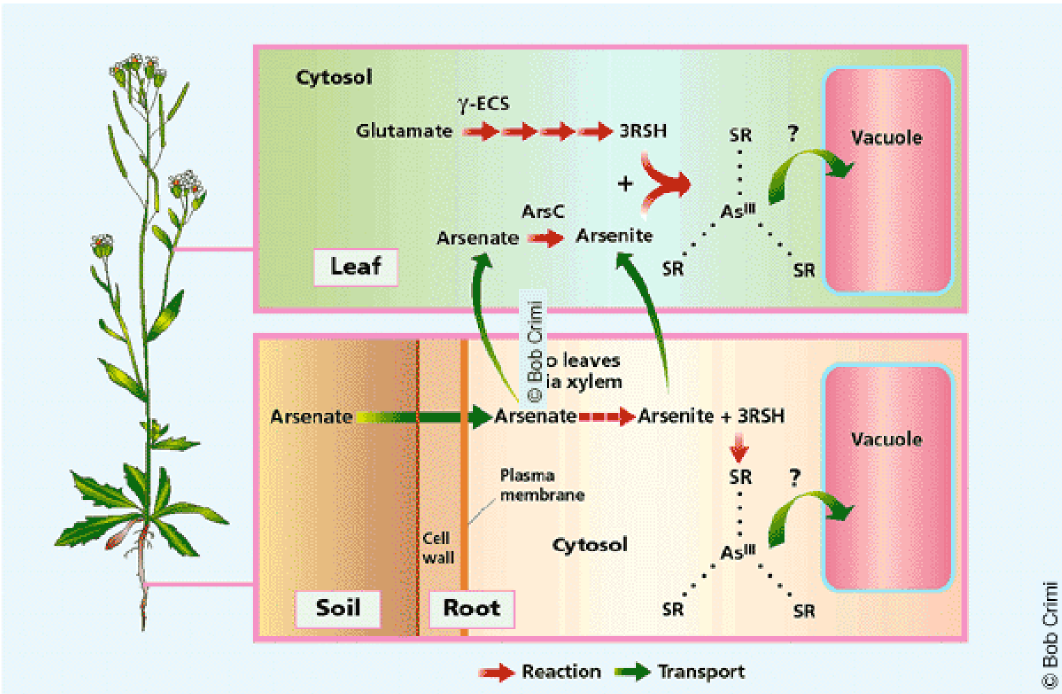

Figure 8. Mechanism of Arsenic biotrasformation by the plant. Source: Doucleff and Terry 2002.

Since arsenate and phosphate behave as analogues with respect to their uptake, arsenate toxicity is linked to phosphorus nutrition and high levels of phosphate can mitigate arsenate toxicity (Esteban *et al*., 2003).

In soil cadmium may precipitate as insoluble cadmium compounds, or form complexes or chelates by interaction with organic matter (Agency for Toxic Substances and Disease Registry, ATSDR, 2002**)**. Different studies suggest that organic matter is more effective than inorganic constituents in keeping cadmium unavailable **(**Agency for Toxic Substances and Disease Registry, ATSDR, 2002).

The uptake and transport of Cd in plants is governed via specific and unspecific transporters of essential bivalent cations such as Ca^{2+}, Zn^{2+} or Fe^{2+} (Llugany *et al*., 2012).

By contrast, the uptake of Cd by plants is controlled more by soil factors than total soil Cd (Siebers *et al*., 2013), suggesting passive uptake that depends mostly on bioavailable Cd or solution concentrations (Smolders *et al*., 1998). Figure 9 shows the absorption of Cd present in soil; its transportation, accumulation and detoxification (Nazar *et al*., 2012).

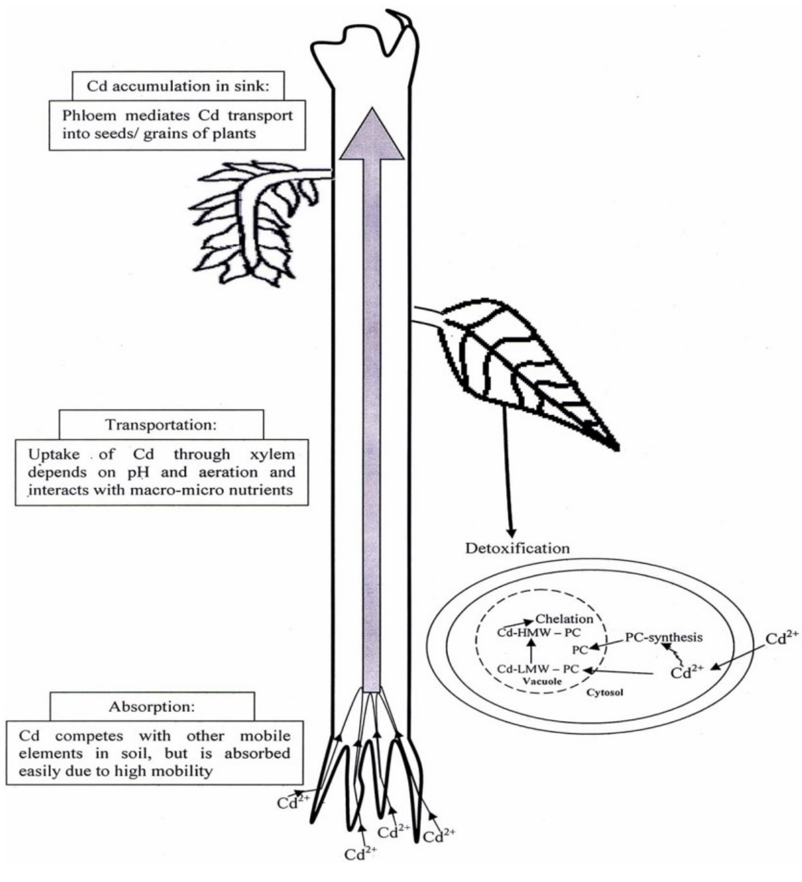

Figure 9. An overview of Cd absorption, transportation, accumulation and detoxification in plants. Source: Nazar *et al*., 2012.

5.2.1. SYNCHROTRON-BASE METHOD: A TECHNIQUE FOR METALS SPECIATION

Spectroscopic approaches to plant and soil sciences have provided important information for several decades. However, many of these approaches suffered from a number of limitations. The introduction to synchrotron radiation and to the fundamentals of some widely used synchrotron-based techniques (Lombi and Susini, 2009**),** have become key components to study the mechanisms in metals uptake and metabolism in plants (Sarret *et al*., 2013). In particular it is useful to study the distibution and the speciation of the metals in plants.

High detection sensitivity, lateral or spatial resolution, chemical speciation capability, limited sample preparation and the possibility to work on hydrated samples, are the best advanteges of synchrotron techniques.

In particular, X-ray absorption spectroscopy (XAS) and micro-X-ray fluorescence (μXRF) that are synchrotron tecniques, used in plant sciences.

μXRF reveals information for imaging the distribution of elements in plant tissues and cells, and quantifying them (Sarret *et al*., 2013).

XAS is specific for the chemical form of metal such as its oxidation state. It is applied at synchrotron radiation facilietes that provide intense and tunable X-ray beams. The synchrotron beam energy is tuned through the absorption edge of an element of interest, and modulations in the absorption are measured.

X-ray Absorption Fine-Structure (XAFS) is the modulation of the x-ray absorption coefficient at energies near and above an x-ray absorption edge. XAFS is divided into 2 regimes:

- XANES, X-ray Absorption Near-Edge Spectroscopy which provides information about geometry and oxidation state
- EXAFS, Extended X-ray Absorption Fine-Structure which provides information about metal site ligation

XAS is usually performed by measuring the photons transmitted through the sample; however, for dilute analytes, such as trace metals in biological samples, XAS is performed in the fluorescence mode, which is much more sensitive than the absorption spectroscopy (Gunter *et al*. 2002; Ortega *et al*., 2009).

5.3. PHYTOREMEDIATING PLANTS

Although high levels of metals disturb the growth of plants, there are different plant species that are able to tolerate them and survive, grow and reproduce on soils contaminated with heavy metals (MuszyńSka and Hanus-Fajerska, 2015).

Since the level of accumulation of elements differs between and within species (Baker, 1981), the plants can be classified into different categories. There are "excluders" species that grow in metal-contaminated soil, retain and detoxify most of the heavy metals in their root tissues and minimize ion translocation to the shoots (Ghosh and Singh, 2005). Examples of excluder plants are *Armeria maritima*, *Plantago lanceolata*, *Silene vulgaris* and *Dianthus carthusianorum* that grew on metalliferous soils of Poland (Wierzbicka *et al*., 2004; Wójcik and Tukiendorf, 2014; MuszyńSka and Hanus-Fajerska, 2015).

Among the category of "indicators" plants, are species able to regulate the uptake and transport of metals to the shoot so that internal concentration reflects external levels (Peralta-Videa *et al*., 2009).

Other species called "accumulators" are able to concentrate metals in the aerial part of biomass. Plants that accumulate high concentrations of metals in their above-ground organs are called hyperaccumulators (Fig. 10).

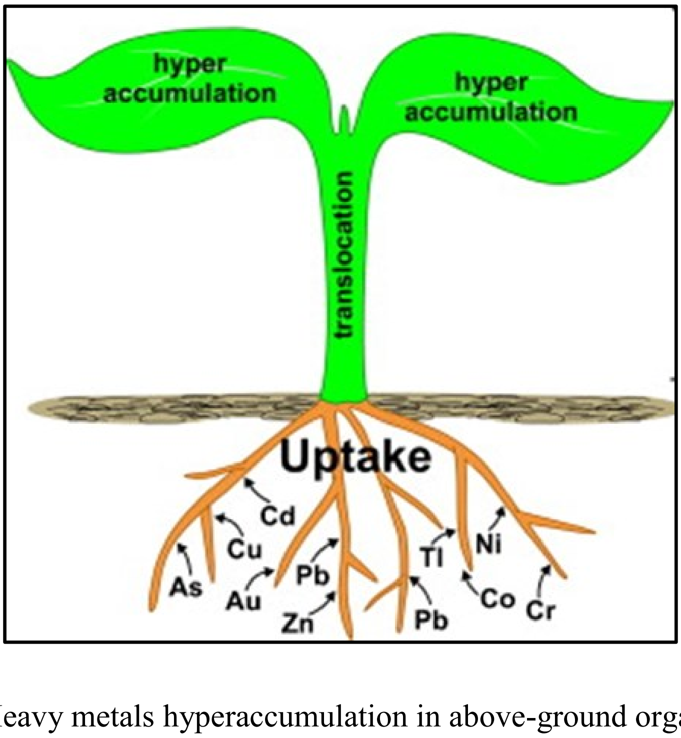

Figure 10. Heavy metals hyperaccumulation in above-ground organs.
Source: Rascio and Navari-Izzo, 2011.

This term was first used by Baker and Brooks (1989) to define plants that accumulate more than 1000 µg g^{-1} of nickel in dry leaves. Hyperaccumulators are plants that accumulate heavy metals from 100 to 1000-times higher than those found in non-hyperaccumulating species, without suffering any discernible phytotoxic effect (Jabeen *et al*., 2009; MuszyńSka and Hanus-Fajerska, 2015).

In hyperaccumulating plants, the toxic effects of heavy metal at high accumulation are minimised, under the influence of different mechanisms such as storage and detoxification/ sequestration of heavy metals (Tran and Popova, 2013), in the shoot, mainly based on chelation and subcellular compartmentalisation (Yadav, 2010; Tran and Popova, 2013). The detoxification/ sequestration consist mainly of heavy metal complexation with ligands such as, for As(III) and Cd^{2+}, thiols, present in glutathione and phytochelatins (PC) (Tran and Popova, 2013). The tripeptide glutathione (Glu- Cys-Gly), GSH, can bind to several metals and metalloids such as Cd, and is also involved in redox defence. However, increasing GSH (and PC) synthesis alone seems to be insufficient to achieve more than marginal enhancements of Cd and As tolerance or accumulation (Tran and Popova, 2013**).** The small ligands, such as organic acids, have a major role as detoxifying factors. These ligands may be instrumental to prevent the persistence of heavy metals as free ions in the cytoplasm and even more in enabling their entrapment in vacuoles where the metal–organic acid chelates are primarily located (Tran and Popova, 2013**).**

Approximately 400 plant species from at least 45 plant families have been reported to hyperaccumulate metals (Lasat, 2000; Ghosh and Singh, 2005). Some of the families are *Brassicaceae, Fabaceae, Euphorbiaceae, Asteraceae*, *Lamiaceae* and *Scrophulariaceae* (Salt *et al*., 1998). Crops like alpine pennycress (*Thlaspi caerulescens*)*, Ipomea alpine, Haumaniastrum robertii, Astragalus racemosus, Sebertia acuminate* have very high bioaccumulation potential for Cd/Zn, Cu, Co, Se and Ni, respectively (Lasat, 2000). Willow (*Salix viminalis* L.), maize (*Zea mays* L.), Indian mustard (*Brassica juncea* L.), and sunflower (*Helianthus annuus* L.) have reportedly shown high uptake and tolerance to heavy metals (Schmidt, 2003).

Other authors showed examples of the most important plants as hyperaccumulators (Table 2).

Table 2. Examples of hyperaccumulating plant species belonging to different families. Source: Muszyńska and Hanus-Fajerska, 2015.

Family	Species	Heavy metals	References

Asteraceae	Berkheya coddii	Ni	Mesjasz-Przybyłowicz *et al*. 2004; Orłowska *et al*. 2011
Brassicaceae	Alyssum bertolonii	Ni	Galardi *et al*. 2007; Mengoni *et al*. 2011
Brassicaceae	Alyssum markgrafii	Ni	Bani *et al*. 2010
Brassicaceae	Alyssum murale	Ni	Bani *et al*. 2010; Lucisine *et al*. 2014
Brassicaceae	Arabidopsis halleri	Zn Cd	Maestri *et al*. 2010; Huguet *et al*. 2012; Verbruggen *et al*. 2013
Brassicaceae	Biscutella laevigata	Tl	Pošćić *et al*. 2012; Babst-Kostecka *et al*. 2014
Caryophyllaceae	Minuartia verna	Pb	Maestri *et al*. 2010
Crassulaceae	Sedum alfredii	Pb	Tian *et al*. 2010; Lu *et al*. 2013
Fabaceae	Astragalus racemosus	Se	Galeas *et al*. 2006; Lindblom *et al*. 2012
Lamiaceae	Haumaniastrum katangense	Cu Co	Brooks 1977
Myrthaceae	Gossia bidwillii	Mn	Fernando *et al*. 2007
Plumbaginaceae	Armeria maritima ssp. halleri	Zn, Pb	Ciarkowska and Hanus-Fajerska 2008; Abratowska *et al*. 2012
Poaceae	Spartina argentinensis	Cr	Redondo-Gomez *et al*. 2011
Pteridaceae	Pteris vittata	As	Wu *et al*. 2009; Wan *et al*. 2013
Violaceae	Viola boashanensis	Cd	Liu *et al*. 2004; Wu *et al*. 2010

The plants with better ability to adjust to the toxicity effects and to survive in heavy metal/metalloid polluted sites, are better candidates for phytoremediation purposes.

5.4. TECHNIQUES FOR THE REMEDIATION OF CONTAMINATED SOILS

There are many techniques available for the remediation of contaminated soils, that to date represent a constantly evolving field that absorbs a lot of resources for research and development.

Conventional methods to remediate metal-contaminated soils (soil flushing, solidification/ stabilization, vitrification, thermal desorption, encapsulation) (Bio-Wise, 2003) can be used in highly contaminated sites but are not applicable to large areas. Also these remediation methods require high energy input and expensive machinery (Schnoor, 1997). At the same time they destroy soil structure and decrease soil productivity (Leumann *et al*., 1995; Jadia and Fulekar, 2009).

Phytoremediation is an innovative and popular plant-based remediation technology with interesting characteristics such as low-cost, low-impact, and environmentally sound

(Cunningham and Ow, 1996). It is a technique that uses plants to clean up metals and other contaminants from the soil.

Phytoremediation includes different processes (Fig. 11):

- Phytostabilization: absorption and accumulation by roots of a plant to remove contaminants from soil sediment, and sludges (United States Protection Agency, 2000). It is useful to limit mobility and bioavailability of contaminants, such as Pb, As, Cd, Cr, Cu and Zn in the soil (Jadia and Fulekar, 2009). One of the advantages of this technology, is that the disposal of hazardous material/biomass is not required (United States Protection Agency, 2000).

- Rhizofiltration: removal of metals by the roots of terrestrial and aquatic plants, filtering surface water, extracted ground water or wastewater with low contaminant concentrations (Ensley, 2000) through absorption, concentration or precipitation in the roots of plants. This processe can be used for Pb, Cd, Cu, Ni, Zn, and Cr, which are primarily retained within the roots (United States Protection Agency, 2000).

- Phytodegradation: uptake and degradation of contaminants through the metabolism of plant that produces different enzymes such as dehalogenase and oxygenase that help to catalyze degradation (Vishnoi and Srivastava 2008**)**.

- Phytovolatilization: uptake from the soil of toxic elements by a plant, trasforming them into volatile forms and releasing them through the leaves into air. Because phytovolatilization implies the transfer of contaminants into the atmosphere, products released in the air, should be less toxic than the initial contaminants; moreover it should be necessary to analyze the impact of this transfer on the ecosystem and on human health (Vishnoi and Srivastava 2008**)**.

- Phytoextraction: metals extraction by the roots of a plant and traslocation to the shoot. It is dependent on the plant's ability to grow in an environment that is not ideal for normal plant growth, to uptake the heavy metals from soil and translocate them from root to leaves.

  Hypraccumulating plants are mainly used for this process, which should occur during several season to obtain an effect.

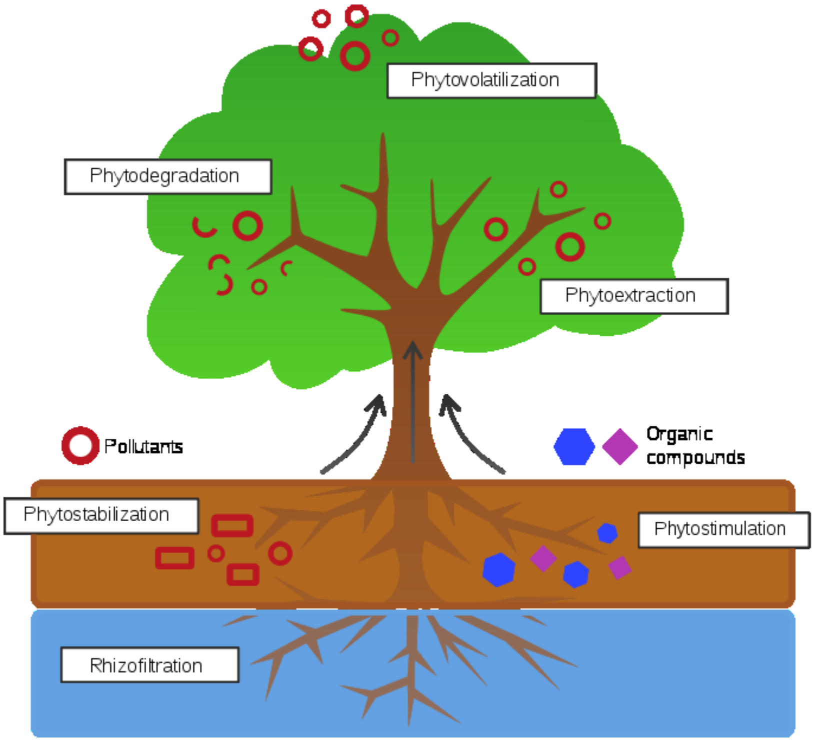

Figure 11. A range of processes mediated by plants or algae that are useful in treating environmental problems. Source website Wikipedia.

The need to minimize soil disturbance and bioavailable contaminants and to increase the yield of plants that hyperaccumulate metals from soils is fundamental to phytoremediation, as well as the development of adequate technologies for the utilization of plant materials (Kabata-Pendias, 2010).

6. *CYNARA CARDUNCULUS* L.

In this research the attention is focused on *Cynara cardunculus* L. (cardoon), a perennial species native to Mediterranean countries. It comprises different subspecies, *C. cardunculus* L. subsp. *scolymus* (L.) Hegi = *C. cardunculus* L. subsp. *scolymus* (L.) Hayek (globe artichoke) and two botanical varieties *C. cardunculus* L. var. *altilis* DC. (domestic cardoon), and *C. cardunculus* L. var. *sylvestris* Lam. (wild cardoon) that is considered to be the wild ancestor of globe artichoke (Rottenberg and Zohary, 1996; Raccuia *et al*., 2004) (Fig. 12).

Figure 12. Cardoon subspecies: *C. cardunculus* L. subsp. scolymus (L.) Hegi. (Artichoke) (A), *C. cardunculus* L. var. *altilis* DC (domestic cardoon) (B), *C. cardunculus* L. var. *sylvestris* Lam. (wild cardoon) (C).

In the Mediterranean environment the choice of cardoon species is linked to the environmental conditions and its cultivation is well documented (Raccuia and Melilli, 2007).

The cultivated cardoon is a less important perennial herbaceous plant and it has been cultivated for many years as a traditional food source in some parts of the southern Europe, particularly in Italy, followed by France and Spain.

The wild cardoon is a robust thistle with a characteristic rosette of large spiny leaves and branched flowering stems. Recent studies, on morphological, biological, and productive characteristics and on intraspecific variability for seed germination under salt and moisture stresses of Sicilian populations, revealed variability among populations (Raccuia *et al*., 2004a). Wild cardoon is fully cross compatible and fully interfertile with the globe artichokes and with the cultivated cardoon, and may be used to improve the globe artichoke genetic pool (Basnizki and Zohary, 1994; Rottenberg and Zohary; 1996; Raccuia *et al*., 2004b).

An outline of the main aspects of the cultivation system follows. The establishment of the plantation is carried out from seed in the first year. Every year the aerial biomass is harvested at the end of the growth cycle. During that time, the plant canopy dries up and the fruits become ripe. Later on - when the climate conditions are favourable - some buds of the plant stock sprout and, gradually, a leaf rosette is formed. This is the beginning of a new growth cycle. The main plant stages and their approximate dates in Mediterranean areas, are: (1) plant sprouting in September –October; (2) winter leaf rosette in November; (3) stem elongation in April – May; (4) full blossom in June; (5) ripe fruits in July; (6) fully dry aerial biomass in August (Fig. 13).

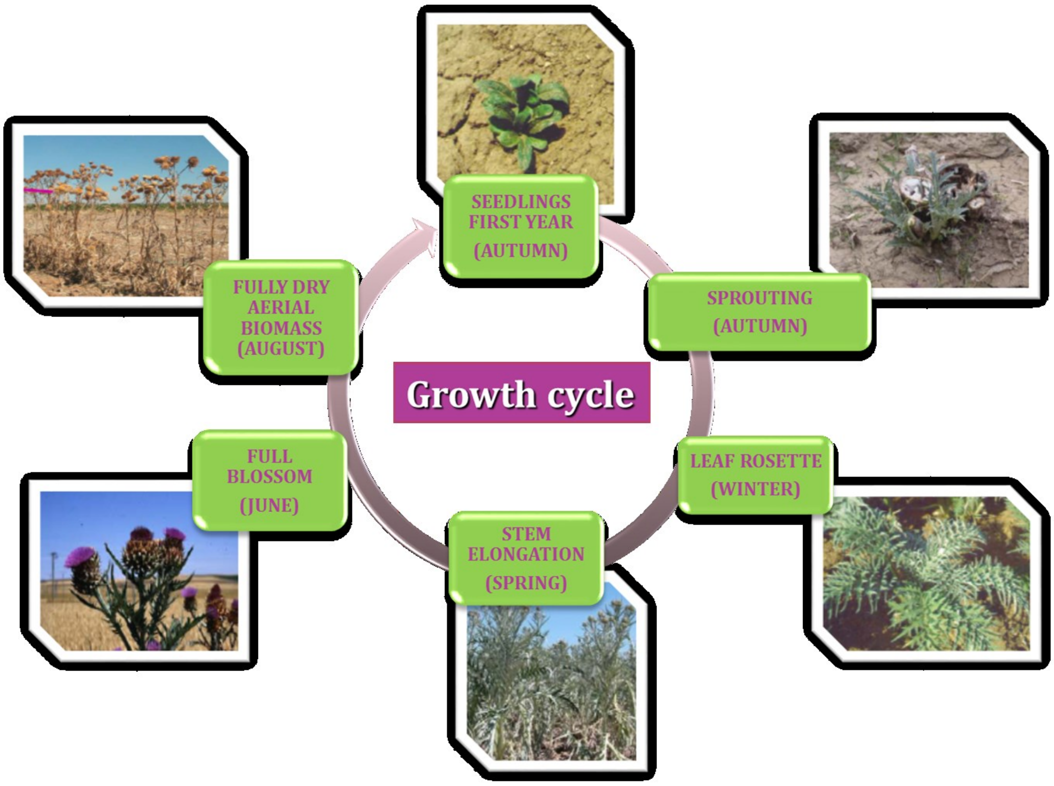

Figure 13. Growth cycle of cardoon plants.

This calendar enables the plant to escape unfavourable environmental conditions because they coincide with the resting stage of the buds, which are attached to underground storage organs. Thereafter the aerial parts die and the plants remain in a state of rest until the next growing season. The growth strategy is based on a large supply of reserves in the form of storage organs. Generally, carbohydrates are the major reserves within the storage organs (Raccuia and Melilli, 2010). Two major functions of these reserves in this growth strategy are to supply carbon and energy for resuming growth following the seasonal dormancy and to make the plant independent of the climate of its habitat (Raccuia and Melilli, 2011; Raccuia *et al*., 2013).

Thanks to the growth cycle, *C. cardunculus* L. helps to conserve the fragile agro-systems of the Mediterranean area, because of the effect of soil erosion control (Raccuia and Melilli, 2007).

A research (Raccuia and Melilli, 2004a) showed the different behavior between domestic and wild cardoon for biomass production and its partitioning; domestic cardoon was characterized for a strong accumulation in leaves, while wild cardoon accumulated mainly in roots. At the end of the crop annual cycle, the root biomass is about 40-50% and the aboveground biomass is about 50-60%; the epigeal biomass consists on average of 30% stalks, 30% leaves and 40% capitula (Raccuia and Melilli, 2004a). The aboveground biomass chemical composition includes about 40% cellulose, 20% hemicellulose, 30% lignin, 10-15% ash and 10% extractives (Antunes *et al*., 2000). Biomass production depends upon the rainfall of the agricultural season (Mehmood *et al*., 2016). On average rain conditions (450 mm y^{-1}) cardoon may yield at the rate of 14 t dry matter ha^{-1} y^{-1} (Angelini *et al*., 2009).

All these characteristics and its good adaptability to the Mediterranean climate suggested its potential use for industrial applications and energy purposes (Toscano *et al*., 2016).

Toscano *et al*., 2016 described how cardoon is cultivated for industrial applications. *C. cardunculus* L. is grown in the same way as in its natural growth pattern that is a perennial field crop in dry farming. The aboveground biomass produced is harvested once a year, in summer time (Fernandez *et al*., 2006). The labors needed for the establishment of the crop (only for the first year) are basal dressing before sowing, soil preparation (subsoiling, ploughing and harrowing), sowing, pre-emergence herbicide treatment and pest control. In the years following the crop establishment, the labours needed for the crop are: fertilization restoration, pest control, harvesting and biomass transport (Toscano *et al*., 2016).

C. cardunculus L. plants offer a wide spectrum of different biomass utilizations. The first proposal is the utilization of the lignocellulosic biomass for alternative energy production (solid biofuel) by combustion, pyrolysis and gasification (Gonzalez *et al*., 2004a; Ochoa and Fandos, 2004). The theoretical caloric value ranged from 16500 to 17028 kJ kg^{-1} of dry matter (Piscioneri *et al*., 2000; Encinar *et al*.,2002a; Encinar *et al*., 2002b), and for paper pulp (Antunes *et al*., 2000; Gominho *et al*., 2001). Biomass residues pellets combustion for domestic heating was demonstrated by Gonzàlez *et al.* (2004b).

Plant fruits (achenes) can also be used in different ways. The evaluation of whole cardoon seed for feeding ruminants was conducted by Cajarville *et al*., (2000). The nutritive value of cardoon seed is mainly conditioned by its high hull proportion (45%), that is higher than in other oil seeds, such as rape seed (15±20%) or sunflower seed (until 30%) (Toscano *et al*., 2016). As this hull shows very high levels of fibre and lignin (similar to those of sunflower hulls), the concentration of these constituents in the whole seed is much higher than in the indicated seeds. The whole cardoon seed presents both high soluble and undegradable fractions. The undegradable fraction should be composed basically of residual hulls because of their very high contents in fibre, lignin and fibre bound nitrogen. On the contrary, the others fractions (soluble and insoluble but fast degraded) should be composed mainly by the kernel, which has a great percentage of cellular contents and very low values of N-fibre bound (Toscano *et al*., 2016). In this way, degradation of whole seeds will be the resultant of the very different patterns of degradation of both their components: the kernel highly degradable and the hulls shortly.

The possibility to use the grain for oil production arises from the fact that this oil is characterised by an optimal ratio of unsatured acid (about 5.7), balanced linoleic/oleic ratio (about 1.8) and absence of erucic acid (Toscano *et al*., 2016). The oil contains a great amount of α-tocopherol, which offers a great warrants of stability against oxidation (Maccarone *et al*., 1999). These characteristics make *Cynara* oil suitable for human consumption. *Cynara* seed oil can be easily extracted by cold pressing (20/25 °C); in this way, the oil composition is not altered and the product can be used for food application (Fernandez *et al*., 2006).

After oil extraction from grain, the residual flour could be used for animal feed, both for the quantity and quality of its proteins (Fernandez and Manzanares, 1990; Foti *et al*., 1999; Maccarone *et al*., 1999).

Roots could be used for extraction of inulin (Raccuia and Melilli, 2004b; Raccuia *et al*., 2004c; Raccuia *et al*., 2005), a fructose polysaccharide very interesting for food and not-food applications (Ritsema and Smeekens, 2003). *C. cardunculus* L. has also been used for medicinal purposes (Kraft, 1997).

Leaves rich in polyphenols were used in European traditional medicine due to the pharmacological activities of their constituents and extracts (Clifford, 1992; Gebhardt, 1997; Perez-Garcia *et al*., 2000; Jimenez –Escrig *et al*., 2003). Recently, there has been an increase in the use of these polyphenolic compounds in cosmetics (Lupo, 2001; Peschel *et al*., 2006).

In Portugal and bordering regions of Spain, crude extracts from the stigma and stylets of flowers are a successful plant rennet used since ancient times, to prepare the traditional raw ovine milk cheeses (Sousa and Malcata, 1997; Freni *et al*., 2001). Aqueous extracts of dried flowers of *C. cardunculus* L. possess three acid proteases, currently termed cardosins (Campos *et al*., 1990; Faro, 1992; Cordeiro *et al*., 1993; Sarmento et al., 1998; Shimoda *et al*., 2003; Pina *et al*., 2003).

Another possible application of the crop, compatible with the use of the dry biomass for energy production, is to the production of forage in winter-time, explored by Cajarville *et al*. (1999).

Finally, a very innovative application of *C. cardunculus* L. is its use as raw material for green chemistry. Green chemistry is an area of chemistry and chemical engineering focused on the design of products and processes that minimize the use and generation of dangerous substances to prevent pollution and reducing consumption of nonrenewable resources. Starting from selected agricultural raw materials with low levels of environmental impact and using innovative, technology it is possible to create an innovative rage of bio-products to use in numerous sectors (bio-plastics, bio-lubricants, home and personal care products, plant protection, additives for the rubber and plastics industries, food fragrances, etc.), with a positive impact on the environment, on performance, income and integration with traditional chemical products, promoting increased specialization and competitiveness.

With green chemistry both the oil extracted from *C. cardunculus* L. seeds and the lignocellulosic biomass can be used to prepare biodiesel (Toscano *et al*., 2016), which is synthesized by transesterification of vegetable oils or animal fats sources and is a realistic alternative of diesel fuel because it is produced from renewable resources and involves lower emissions than petroleum diesel.

Methyl or ethyl esters are the product of transesterification of vegetable oils with alcohol (methanol/ethanol) using an alkaline catalyst (Toscano *et al*., 2016). In addition, the process produces glycerol, which has large applications in the pharmaceutical, food and plastics industries (Bouaid *et al*., 2005).

It has been suggested that biofuels such as biodiesel and bioethanol can be used to mitigate the problem of environmental pollution and the exhaustion of petroleum supplies (Demirbas, 2008). In this sense, the development of alternative technologies is acquiring importance, and many efforts are being done in the gradual replacement of fossil fuels. Within the renewable sources of energy, the production of liquid biofuels from organic feedstock sources represents a feasible alternative that avoids important modifications of vehicle engines, maintaining most of the infrastructures for the supply chain and can replace partially the petroleum-based fuels (Torres *et al*., 2013).

6.1. CARDOON FOR PHYTOREMEDIATION

The success of phytoremediation depends mainly on the choice of the plant, which must obviously possess the ability to accumulate large amounts of heavy metals (hyperaccumulation) (Moosav and Seghatoleslami, 2013).

Grasses have been more preferable in use for phytoaccumulation than shrubs or trees because of high growth rate, more adaptability to stress environment and high biomass (Malik *et al*., 2010).

C. cardunculus L. is a possible good candidate for phytoremediation because is a crop with a different set of interesting characteristics, such as fast growth and high biomass, extended root system and adaptability to polluted sites, high translocation factor and low imput management.

Papazoglou (2011) tested phytoremediation by cardoon for cadmium and nickel. Under Cd treatment, cardoon growth remained unaffected, while increased Ni soil concentrations inhibited plant growth and were lethal to the highly treated plants. In the combined Cd and Ni treatments, an antagonistic effect was observed between the two metals. Cadmium and nickel concentrations in cardoon tissues rose with increasing metal concentrations in the soil. Mean contents of both metals in the shoots were higher than in the roots and the translocation factor was greater than 1. A possible enhancing effect of nickel on cadmium uptake was observed. Cardoon showed characteristics of a Cd accumulator (Papazoglou, 2011).

Llugany *et al*., (2012) evaluated the tolerance of cardoon plants exposed to 5 μM Cd and to 5 or 10 μM As using controlled-environment conditions and hydroponic culture. The aim was to ascertain whether this species could be potentially useful for phytoremediation of marginal soils with excess Cd or As pollution. The plants exhibited considerable tolerance to Cd and As. Biomass was hardly affected by the potentially toxic concentrations of Cd and As. Cadmium was preferentially accumulated in old leaves. Contrastingly, As was efficiently retained in the roots. Results indicate that *C. cardunculus* can be a useful species for phytoextraction of Cd from polluted soils. On soils rich in arsenic, cardoon could be grown as an energy crop that can help to stabilize these soils (Llugany *et al*., 2012).

In a recent study conducted in Spain, the alleviation of arsenic stress in cardoon plants via the supply of a low cadmium concentration was performed. The effect of As (0–80μM) and of As+Cd (0–80μM+5μM) combinations on plant growth, toxicological variables and As and Cd bioaccumulation was studied in cardoon plants under controlled conditions. Plants grown in the presence of As alone showed less reduction in overall root and shoot development than those exposed to As+Cd, although the main root was shorter than in the latter plants. The effective added concentrations of As that reduced shoot or root dry weight by 50% (EC_{50}) and the critical toxic concentration that caused a 10% reduction in plant growth ($CTC_{10\%}$) were higher in plants grown with As alone. In both treatments (As and As+Cd), the $CTC_{10\%}$ was higher in the roots, but the root EC_{50} was lower than the shoot EC_{50}. The presence of Cd increased the accumulation of As in the shoot, but ≥20 µM As reduced the shoot bioaccumulation of Cd. Thus, the presence of 5µM Cd with As appears to reduce the tolerance of cardoon plants to the latter element, but it increases their As phytoextraction capacity (Sánchez-Pardo *et al*., 2015).

From these studies, the potentiality of cardoon to accumulate heavy metals from polluted soils is totally clear. However, the research evaluating the effect of varieties in Cynara species and within variety and the effect of genotype is lacking. Moreover, the highest concentration of Cd and As that these accessions could tolerate and the behaviour of the plants in presence/absence of one or both metals, should be improved. It could be possible that the wild population of cardoon, for its adaption to growth in adverse climatic conditions, may provide more resistance to heavy metals than the domestic cardoon.

7. AIM OF THE THESIS

The PhD thesis was shaped to use an innovative and alternative tecnology to preserve our environment from pollution. In line with public acceptance for the removal of toxic metals from contaminated lands, an energy crop typical of the Mediterranean environment such as *C. cardunculus* L. was chosen, as it could be useful to generate new bioenergy resources from the biomass production, along with the remediation of contaminated soil.

The general aim of this thesis was the land remediation through the investigation of the effects of Cd and As on different cardoon varieties and the use of these plants to uptake environmental contaminants from the soil.

To reach the aim of the research, different specific objectives have been followed:

1. to prospect and characterize the best accession to use in our environment currently damaged by severe genetic erosion, pollution, urbanization, and bad farming practices
2. to evaluate the maximum tolerance of cardoon plants exposed at critical metals concentrations
3. to study the bioaccumulation of heavy metals in different organs of cardoon plants
4. to analyze in which chemical form, the metals are less toxic in the plants

For this reason the physical, chemical, biological and technological aspects of the phenomenon of phytoextraction, were studied, trying to make this innovative technology a practical technique and not only a research topic.

8. EXPERIMENTAL METHODS

During the three year-period, 2013-2016, two different experiments have been set in order to assess adaptation and potential utilization, for lands remediation, of *C. cardunculus* L. species growing in soil contaminated with Cd and As at different concentrations:

- Experiment 1

  Phytoextraction of Cd and As in *Cynara cardunculus* L. var. *altilis* growing in contaminated soil

- Experiment 2

  Potential use of different cardoon genotypes for phytoremediation of metal contaminated soils

9. EXPERIMENT 1

Phytoextraction of Cd and As in *Cynara cardunculus* L. var. *altilis* growing in contaminated soil

The aim of this experiment was to study the biologic response of one *C. cardunculus* L. genotype, selected for biorefinery purposes **(**Raccuia and Melilli, 2007), the bioaccumulation of Cd and As elements in different parts of plant and to determine the maximum trace elements concentration could be lethal for the cardoon plants.

9.1. MATERIAL AND METHODS

9.1.1. SAMPLE PREPARATION

In November 2013, one genotype of *C. cardunculus* L. var. *altilis* DC, Line 01, selected by Institute for Agricultural and Forest Systems in the Mediterranean of the National Research Council (CNR-ISAFOM), was sown in pots (2 seeds per pot). In December 2013, four-week-old domestic cardoon plants with three or four leaves were transplanted into plastic pots (diam.20) containing 1.3 Kg sample soil previously characterized (Table 3).

Table 3. Characteristic of the initial soil

Parameter	Value
Sand (g kg−1)	350
Silt (g kg−1)	410
Clay (g kg−1)	240
pH	6.0
Organic matter (g kg−1)	12.0
Total nitrogen (g kg−1)	0.50
P (mg kg−1)	21
K (mg kg−1)	95

Note: Method reference: D.M. 13/09/99 GuU SO n. 248 21/10/99

After 5 months from sowing, which allowed the development of the plants, three treatments were applied:

1. **As** (0, 6.5, 13 mM), will be referred as control, As< and As>, respectively:

the salt $Na_2HAsO_4\ 7H_2O$ was weighed (0.5 and 1 g) and dissolved in 250 mL H_2O;

2. **Cd** (0, 6.5, 13 mM), will be referred as control, Cd< and Cd>, respectively: the salt $Cd(NO_3)_2\ 4H_2O$ was weighed (0.5 and 1 g) and dissolved in 250 mL H_2O;
3. **As+Cd** (0+0, 6.5+6.5, and 13+13 mM) will be referred as control, As+Cd< and As+Cd>, respectively: the mixture concentrations were prepared by adding half the amount of each metal used for the individual experiments.

The pots within each treatment were arranged adopting a randomized block experimental design, with three independent replicates. The plants were grown in controlled environmental conditions. Irrigation was targeted to meet the needs of plants. During the biological cycle, were measured different parameters of plant growth (height, number of leaves, possible presence of yellow and dried leaves, and number of shoots) and the plants were observed, in order to detect visible toxicity symptoms. Plants were harvested at 4 different stages of the biological cycle, once every 15 days, until 60 days after the artificial contamination of the soil.

Three plants per treatment were harvested and were separated into shoots (leaves plus stems) and roots. Total leaves number, leaves colour (green, yellow, dry) were determined in these plants. The samples of roots were washed with tap water, then with distilled water and finally with 0.01 M HCl for approximately 5 s in order to remove external metal from the root surface (Gardea-Torresday *et al*., 2004).

Plant material was dried in an oven at 70°C for 72 hours and finely ground for the determination of As and Cd. Fresh weight and dry matter weight (DW) were recorded. All analyses were performed in triplicate for each pot and are reported on a DW basis.

9.1.2. DETERMINATION OF AS AND CD

The powdered dry samples (roots, leaves) were submitted to a process of mineralization by means of a closed-vessel microwave digestion system (Ethos 1, Milestone, Bergamo, Italy) equipped with sensors for temperature and pressure control. The equipment is provided with PTFE vessels capable of pressures of up to 110 bar.

For the mineralization approximately 0.5 g of sample with 1mL of internal Re standard at (1 mg L^{-1}), triplicately digested with 8 mL of HNO_3 (65%, v/v) and 2 mL of H_2O_2 (30%, v/v) in acid-prewashed PTFE vessels.

The digestion was carried out in two steps with a constant microwave power of 1000 W. Firstly temperature was increased to 200°C in 10 minutes (step1), and then it was held to 200°C for 20 minutes (step 2). After cooling down to room temperature, the digested samples were weighed, quantitatively transferred into pre-cleaned 50 mL volumetric flasks, diluted to mark using deionized water, and stored at 4°C until next analysis.

The determination of elements in digested samples was carried out by iCAP Q ICP-MS (Thermo Scientific, Waltham, MA) spectrometer equipped with an autosampler ASX520 (Cetac Technologies Inc., Omaha, NE, USA). The ICP-MS operating conditions were the following: RF power, 1550 W; plasma gas flow rate, 14 L min^{-1}; auxiliary gas flow rate, 0.89 L min^{-1}; carrier gas flow rate 0.91 L min^{-1}; helium collision gas flow rate, 4.5 mL $min^{-1;}$ spray chamber temperature, 2.70 °C; sample depth, 4.27 mm; sample introduction flow rate 0.93 mL min^{-1}; nebulizer pump, 0.1 rps; extract lens 1 voltage, 1.5 V.

Monitored isotopes were ^{75}As and ^{111}Cd. These were chosen to maximize sensitivity and to minimize interferences due to the matrix. ^{73}Ge for As and ^{115}In for Cd, were used as on-line internal standards. To integrate the peaks, 3 point for each mass and 3 replicate acquisitions were taken. All samples were analyzed in batches, with blank samples and known standards.

9.2. STATISTICAL ANALYSIS

Data were subjected to the Bartlett's test for homogeneity of variance and then analysed using the analysis of variance (ANOVA). The means were statistically separated on the basis of Student–Newmann–Kewls test when the 'F' test of ANOVA for treatment was significant at least at 0.05 probability level. Significance was accepted at $P \leq 0.05$ level (Snedecor and Cochran, 1989).

9.3. RESULTS AND DISCUSSION

The results showed that the treatments and the concentration of contaminants have significantly influenced the plant growth parameters.

The analysis of variance of cardoon plants exposed to Cd up to 60 days and to As+Cd up to 45 days after artificial contamination, showed that the harvest "Time" and the "Contaminant Concentration" are the most important factors that influenced the development of the plant; "Number of green leaves" (80% of total in Cd treatment), "Number of dry leaves" (94.6% of total in Cd treatment), "Dry weight of the plant" (95.7% of total in Cd treatment) (Table 4)**,** and "Incidence of the roots" (77.8% of total in As+Cd treatment)**,** (Table 5). The results of As effects were not showed because the plants died after 15 days from the treatment.

Table 4. Mean squares expressed in absolute value (AV) and percent of total (%) in relation to time of exposure (T), contaminant concentration (CC) and TxCC interaction for the studied parameters, in plants subjected to Cd treatment up to 60 days after artificial contamination.

Parameter	Mean squares of treatment					
	Time (T)		Contaminant Concentration(CC)		T X CC	
	AV	%	AV	%	AV	%
Number of green leaves	11.7***	80.2	2.3**	16.0	0.6ns	3.8
Number of dry leaves	33.4***	94.6	0.2ns	0.6	1.7ns	4.8
Dry weight of the plant	2087.4***	95.7	86.2 ns	4.0	8.3 ns	0.4
Incidence of the roots	1822.8***	47.6	218.1 ns	5.7	1787.0***	46.7

ns Non significant.
* Significant at 0.05 probability level.
** Significant at 0.01 probability level.
*** Significant at 0.001 probability level

Table 5. Mean squares expressed in absolute value (AV) and percent of total (%) in relation to time of exposure (T), contaminant concentration (CC) and TxCC interaction for the studied parameters, in plants subjected to As+Cd treatment up to 45 days after artificial contamination.

Parameter	Mean squares of treatment					
	Time (T)		Contaminant Concentration(CC)		T X CC	
	AV	%	AV	%	AV	%
Number of green leaves	0.9ns	8.4	6.7***	60.9	3.4**	30.6
Number of dry leaves	25.3***	56.2	14.8***	32.8	4.9**	11.0
Dry weight of the plant	259.6***	39.2	320.1***	48.3	82.5**	12.5
Incidence of the roots	0.0ns	1.2	0.4***	77.8	0.1***	21.0

ns Non significant.
* Significant at 0.05 probability level.
** Significant at 0.01 probability level.
*** Significant at 0.001 probability level

According to Papazoglou (2011), under Cd treatment, plant growth remained unaffected. In the present work no visible toxicity symptoms were observed and measured parameters were not influenced by the treatment. The plants survived until the end of the trial, the growth of cardoon plants was normal and no phytoxicity symptoms were observed. All measured growth parameters were not influenced by the treatment, indicating tolerance characteristics. In particular "Number green leaves" per plant increased during the cultivation period and no significant differences were observed ($p<0.05$) between treated plants and control with about 5 green leaves per plant (60 days after treatment) (Fig.14).

C. cardunculus L. var. *altilis* is known for its plant growth promoting ability in uncontaminated soils. However, even cardoon plants, exposed to high concentrations of Cd, did not show negative effect on biomass production, exhibiting considerable tolerance to this metal. At the end of experimental trial, plant biomass was 48.51 g DW plant^{-1} (control), 41.41 g DW plant^{-1} (Cd <) and 41.94 g DW plant^{-1} (Cd >) (Fig.15).

Under As treatment, the growth and development of the plants were affected by the high doses of element (Kabata-Pendias and Pendias 2000). Here, severe phytotoxicity symptoms and reduction of plant growth were observed in low and highly treatments. All measured parameters differed from control, and the treated plants were dried and died after 15 days. In particular new leaves did not emerge and the existing ones became yellow and dried. At 15 days from the treatment the control had 5 green leaves and the plants treated with As had 0 green leaves (Fig. 14).

Llugany *et al*. (2012) showed that exposure, for 1 week, to As concentrations, did not adversely affect plant biomass production. In the present work, the biomass production slightly differed from the control at 15 days after the contamination, suggesting mechanisms of resistance and defense by the plant. In particular plant biomass was 10.11 g DW plant^{-1} (control), 9.43 g DW plant^{-1} (As <) and 9.55 g DW plant^{-1} (As >) (Fig. 15). Despite that, the high concentrations of As were lethal for the plants and cardoon were totally dried and died after 15 days of exposure.

Under As+Cd treatment, the effects on all measured growth parameters were lower than those of the metals when applied individually. The presence of Cd decreased the negative effects that arsenic had on the development of the plants. Similar effects were observed in railway beggartick (*Bindes Pilosa* L.) for Cd and As (Sun *et al*., 2009), in cucumbers for Cd, Cu and Pb (An *et al*., 2004).

All measured growth parameters slightly differ from the control up to 30 days from the artificial contamination but the differences became significantly different after 45 days of exposure and the plants dried and died. In particular, the "number of green leaves" per plant resulted similar to the control up to 30 days from the treatment, three green leaves for the control and 3–2 leaves for the treated plants. At 45 days the difference was significant: a reduction in "number green leaves" was observed (one green leaf), but the plants were still vital (Fig. 14).

Similar trend was recorded for "plant biomass". The results showed a reduction of biomass production compared to the control, only at 45 days from the treatment: plant biomass was 32.51 g DW plant^{-1} (control), 10.68 g DW plant^{-1} for the lower concentration of As+Cd and 16.56 g DW plant^{-1} for the highest concentration of As+Cd (Fig. 15).

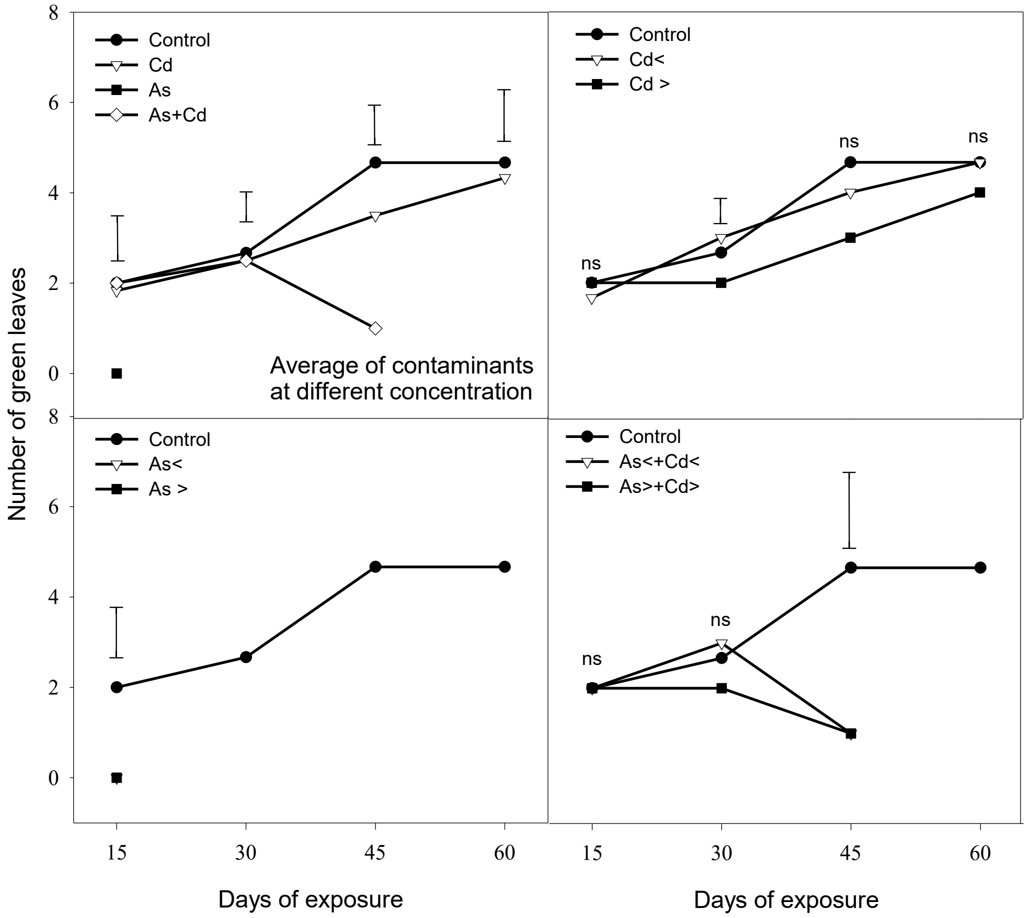

Figure 14. Number of green leaves of *Cynara cardunculus* var. *altilis* exposed to contaminants for 60 days. Values are the means of 3 samples per t reatment. Vertical lines indicate LSD values at P≤0.05.

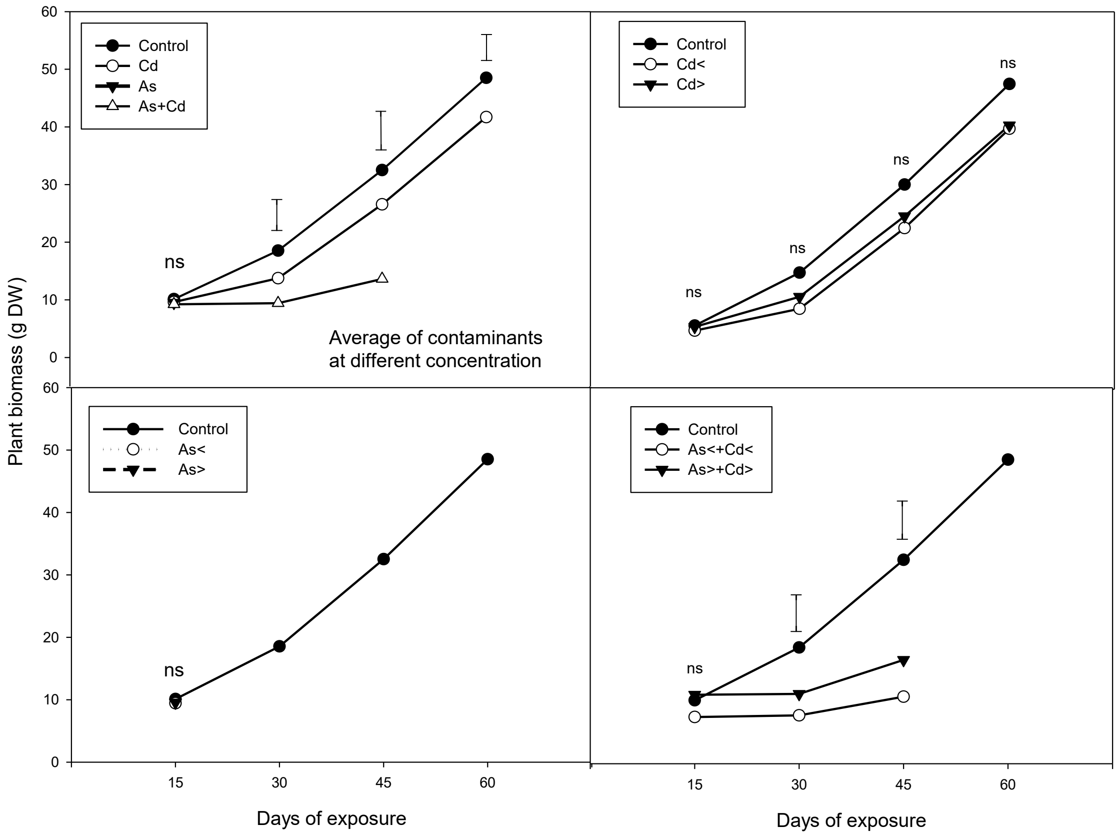

Figure 15. Plant biomass of *C. cardunculus* var. *altilis* exposed to contaminants for 60 days. Values are the means of 3 samples per treatment. Vertical lines indicate LSD values at $P \leq 0.05$.

Regarding the accumulation of Cd and As in the roots and shoots/leaves of cardoon plants, the ICP-MS results showed a different behaviour of plant in response of the type of exposure, Cd and As alone or combined.

During all the trial period, the two concentrations of metals used (6.5 mM, 13 mM) showed a similar trend both in roots and in leaves. Therefore, as shown in Figure 16 and Figure 17, the average value of results was used.

Particularly under Cd treatment, cardoon plants exhibited a high tissue tolerance for Cd: the metal was uptaken by the roots and translocated to the leaves. During time the concentration of this contaminant decreased in the roots (from 147 mg kg^{-1} DW to 59 mg kg^{-1} DW), while increased in the leaves (from 24 mg kg^{-1} DW to 43 mg kg^{-1} DW).

Pollution with only As caused a rapid mechanism of uptake of As by the roots (410 mg kg^{-1} DW) and its accumulation in the aerial parts of the plant (302 mg kg^{-1} DW). The As concentration in cardoon tissues increased with increasing As concentration, resulting in the death of all plants after 15 days (Fig. 16).

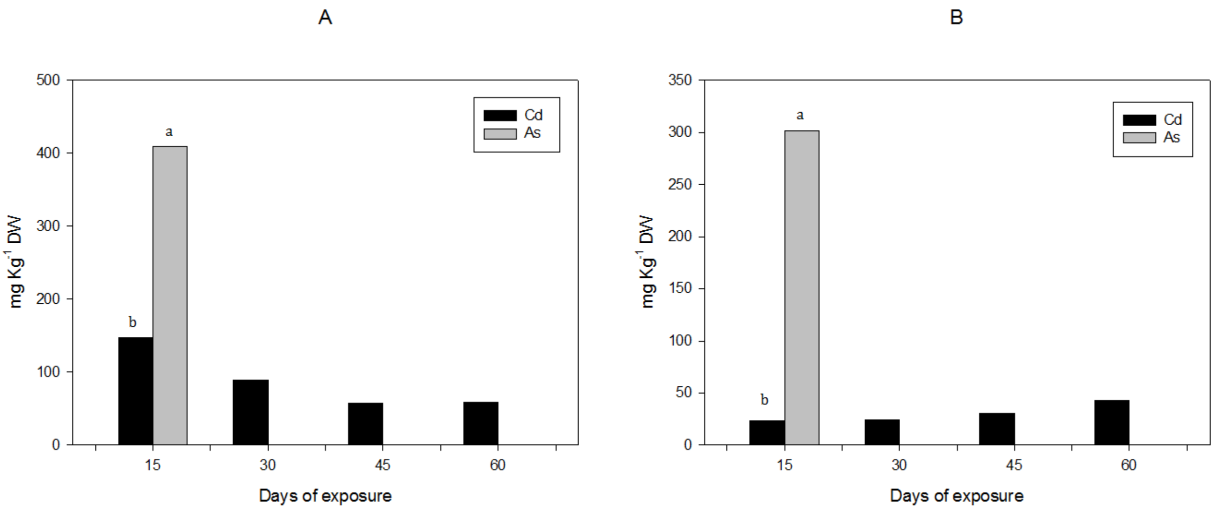

Figure 16. Cd and As accumulation in roots (A) and in leaves (B), on average of metals concentrations, under Cd and As treatments. Different letters indicate significant differences at P≤0.05.

Under the interaction effect of As+Cd the plants absorbed higher quantities of Cd and As, comparable to those absorbed by plants exposed to Cd and As alone; at 15 days after artificial contamination, Cd accumulation value in the roots was 391 mg kg^{-1} DW.

According to Sun *et al*., 2009 and Sanchez-Pardo *et al*., 2005**,** the presence of Cd increased the ability of cardoon to tolerate As and to translocate it from the roots to the shoots up to 45 days after contamination. Cd increased the As concentration on the leaves but the presence of As, increased Cd phytoextraction by plant and its translocation in the leaves (159 mg kg^{-1} DW after 45 days) (Fig. 17).

Under co-contamination conditions, the presence of Cd seemed to mitigate the negative effects to plants exposed to As alone (up to 45 days) and cardoon behaved as accumulators, showing a efficient root to shoot/leaves traslocation of Cd and As.

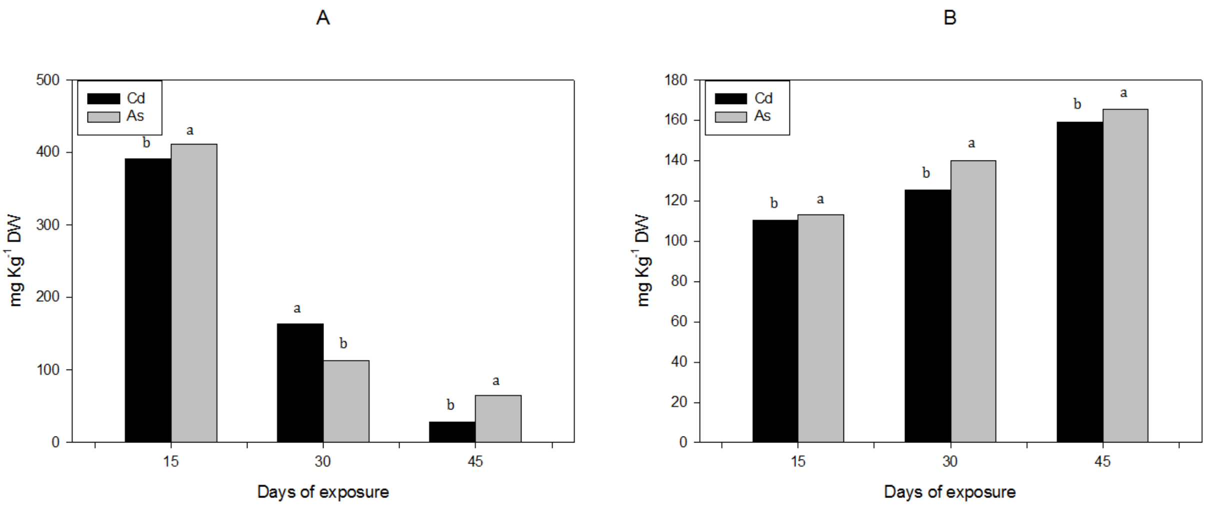

Figure 17. Cd and As accumulation in roots (A) and in leaves (B), on average of metals concentrations, under As+Cd treatments. Different letters indicate significant differences at P≤0.05.

10. EXPERIMENT 2

Potential use of different cardoon genotypes for phytoremediation of metal contaminated soils

In this experiment different accessions of *C. cardunculus* L. were compared and two low concentrations of As and Cd were used to allow the plants survival.

The aim of this experiment was (1) to assess the concentration and bioaccumulation of As and Cd in soil and in different parts of the plant, (2) to understand the effects of Cd and As comparing different varieties and genotypes of cardoon plants, (3) to study in which chemical form were the metals in the plant.

Regarding the elements characterization, the chemical analyses were done in the Microbial Geochemistry Laboratory of School of Geosciences, University of Edinburgh with the supervision of Dr Bryne Tendelo Ngwenya, reader in Microbial Geochemistry.

The metals speciation was determined at Diamond Light Source, Didcot-UK, on September 2016, thanks to the proposal submitted by Institute for Agricultural and Forest Systems in the Mediterranean of National Research Council (CNR-ISAFOM) and School of Geosciences, University of Edinburgh with the principal investigator Dr Bryne Ngwenga. The proposal was viewed by a committee of scientists.

10.1 MATERIAL AND METHODS

10.1.1. PLANT MATERIAL, GROWTH CONDITIONS AND TREATMENTS

In December 2014, seeds from three cardoon subspecies were sown:

1. a domestic cardoon, *C. cardunculus* L. var. *altilis* (**Gen 1**);
2. a wild cardoon population of *C. cardunculus* L. var. *sylvestris* Lam., R14CT (**Gen 2**), collected from native plants found in uncontaminated soils in Randazzo (Catania, Sicily), (754 m a.s.l.);
3. a wild cardoon population of *C. cardunculus* L. var. *sylvestris* Lam., A14CT (**Gen 3**), collected from native plants found in polluted soil, Augusta (Siracusa, Sicily), an industrial area (10 m a.s.l.).

Four-week-old wild and domestic cardoon plants with three or four leaves were transplanted in January 2015, into plastic pots (diam. 45) filled with 13.0 Kg sample soil, previously characterized (Table 3), (1 plant per pot). This soil showed no nutrient deficiency and was not fertilised prior to or during the experimentation.

After 5 months from sowing which allowed the development of the plants, three heavy metals treatments at different concetrations, were performed on the plants. The heavy metals solution was added to the soil of each plant, using the following concentrations:

- **As** (0, 500, 2000 μM), named As 0, As 500, As 2000: the salt Na_2HAsO_4 $7H_2O$ (7.80 and 31.20 g) was dissolved in H_2O and added to the soil;

- **Cd** (0, 500, 2000 μM), named Cd 0, Cd 500, Cd 2000: the salt $Cd(NO_3)_2$ $4H_2O$ (7.71 and 30.85 g) was dissolved in H_2O and added to the soil,

- **As+Cd** (0+0, 500+500, 2000+2000 μM), named As+Cd 0, As+Cd 500, As+Cd 2000: the mixture concentrations were prepared by adding the concentrated solution of As and Cd in H_2O and added to the soil.

The plants were grown from December 2014 (sowing) to July 2015 (last harvested) in controlled environmental conditions. The pots within each treatment were arranged adopting a randomized block experimental design, with three independent replicates. The water supply was standardised among plants, and it was targeted to meet the needs of plants.

During the biological cycle, plant growth parameters (height, number of leaves, possible presence of yellow and dried leaves, and shoots) were measured and each individual plant was observed, in order to detect visible toxicity symptoms.

Cardoon plants were harvested at 3 different stages of the biological cycle, every 15 days until 45 days after the artificial contamination of the soil, from June to July 2015.

10.1.2. ELEMENT ANALYSIS

Two plants per treatment were harvested and were separated into shoots (leaves plus stems) and roots. Upon harvest, total leaves number (n. $plant^{-1}$), stems diameter (mm), roots length (mm), leaves colour (green, yellow, dry) and the total fresh weight (g plant^{-1}) were determined.

A total amount of 13 samples (soil, roots, shoots) for each genotype and for each harvest were collected. After carefully removing soil particles manually, roots were washed with tap water, then with distilled water and finally with 0.01 M HCl for approximately 5 s in order to remove external metals from the root surface (Gardea-Torresday *et al*., 2004).

Root and shoot parts were weighed after drying for at least 72 hours at 70°C. The dry biomass of each plant was determined and the mean single plant mass per pot was recorded. For elemental analyses, roots and shoots from 2 pots, each per genotypes and treatment, were pooled (2 pots = 1 sample), cut with stainless steel scissors and ground in an agate pestle and mortar with liquid nitrogen to obtain homogeneous samples. The powdered dry plant samples were submitted to a process of mineralization by means of a closed-vessel microwave digestion system (MARSXPRESS by CEM Corporation) equipped with sensors for temperature and pressure, 175°C, 1600 W. 0.5 g samples with 1 mL of internal standard, Ytrium (1 mg L^{-1}), were put inside the microwave vessels and triplicately digested in a mixture of 8 mL of HNO_3 (65% V/V) and 2 mL of H_2O_2 (30% V/V). After digestion, the solution was quantitatively transfered into pre-cleaned 50 mL volumetric flasks and diluted until 25 mL using deionized water.

Soil samples were collected from each pot, ari-dried at room temperature and ground to pass a 2.0-mm mesh.

The mineralization of As and Cd in soil samples was determined by triplicated digestion of 0.5 g soil sample in a high pressure microwave system (MARSXPRESS by CEM Corporation: 175°C, 1600 W) with a mixture of 3 mL HNO_3 (65 %) and 9 mL HCl (37 %). After digestion, the solution was quantitatively transfered into pre-cleaned 50 mL volumetric flasks and diluted until 25 mL using deionized water.

Standard reference materials of metals (E-Merck, Germany), were used to ensure the accuracy in the analyses.

Samples were analysed by ICP-MS using an Agilent 7500ce (with octopole reaction system), employing an rf forward power of 1540 W and reflected power of 1 W, with argon gas flows of 0.81 L min^{-1} and 0.21 L min^{-1} for carrier and makeup flows, respectively. Sample solutions were taken up into the Micro mist nebuliser by peristaltic pump at a rate of approximately 1.2 mL min^{-1}. Skimmer and sample cones were made of nickel.

The instrument was operated in spectrum multi-tune acquisition mode and three replicate runs per sample were employed. Each mass was analysed in fully quant mode (three points per unit mass). The following isotopes were monitored: ^{75}As, ^{89}Y,^{111}Cd. ^{103}Rh was added as an internal standard. ^{111}Cd, was analysed in 'nogas'tune and ^{75}As was analysed using Helium tuning to remove any polyatomic interferences. The internal standards ^{103}Rh ^{89}Y, were analysed in both modes.

The ICP-MS operating conditions were the following (Table 6):

Table 6. ICP-MS parameters for Lenses and Quadrupole

Ion Lenses:	**Quadrupole Parameters:**	**Parameters for Helium Mode:**	**Ion lens values that differ form no-gas mode:**	**Quadrupole parameters that differ form no-gas mode:**
Extract 1: 0 V	OctP Bias: -6 V	He gas flow: 6.5 mL min^{-1}	QP focus: 3 V	OctP Bias: -20 V
Extract 2: -110 V	QP Bias: -3 V		Cell Exit: -34 V	QP Bias: -15 V
Omega Bias-ce: -20 V				
Omega Lens-ce: 0 V				
Cell Entrance: -30 V				
QP focus: 3 V				
Cell Exit: -34 V				

A series of standards were prepared by serial dilution of a 1000 mg L^{-1} stock solution with 2% v/v HNO_3 (Merck). An internal standard was added to each standard and sample (spiked at a concentration of 20 ppb). The calibration curve fit (at least five standard concentrations) was of R^2=1.00 in all cases. The mean As concentration in blank digests was 0.07 µg L^{-1} and the detection limit for As was 0.01 µg L^{-1}.

All analyses were performed in duplicate for each pot and are reported on a DW basis.

The As and Cd bioaccumulation factor (BF) was calculated as: BF = the heavy metal concentration in the total (above and below ground) harvested dry plant biomass (mg Kg^{-1}) / the heavy metal concentration in soil (mg Kg^{-1}) at the end of experiment.

10.1.3. SYNCHROTRON ANALYSIS

Plant samples of *C. cardunculus* L. var. *altilis* (Gen 1) and of *C. cardunculus* L. var. *sylvestris,* (Gen 3) were collected at the Synchrotron, Diamond Light Source, Didcot, UK.

For Cd, As and As+Cd treatments, one sample of soil, roots and green-old leaves, was made into pellet for analysis.

Samples spectra were collected on beamline B18, using standard conditions at the Cadmium and Arsenic K-edge.

Spectra were compared to standards of model Cd and As, freshly prepared. The Cd standard solutions (nitrate, phytate, cysteine, citrate, malate and histidine) were prepared at 4 mM (pH 5 for Cd phytate and Cd Cysteine, pH 7 for the other standards) and held in polythene tubes.

The As powder standards (uncomplexed As(V) sodium arsenate heptahydrate, sodium cacodylate As(V), arsenic pentoxide, As_2O_5, arsenic trioxide, As_2O_3) were ground, homogenized in cellulose and made into pellet for analysis, as below:

- Weigh: 0.076g Cellulose+0.01 g As(V) sodium arsenate heptahydrate
- Weigh: 0.075 Cellulose+ 0.009 g sodium cacodylate As(V)
- Weigh 0.075 Cellulose + 0.01 g As_2O_3
- Weigh 0.076 Cellulose+0.01 g As_2O_5

Pt-coated branch with Si 311 monochromator for Cd edge and Cr-coated branch with Si 111 monochromator for As edge were used.

Spectra were acquired in fluorescence mode by means of a 9-element solid state Ge detector. The beamline energy was calibrated using a Cd foil (26711 eV) and As foil (11867 eV). Energy range was collected up to 12 A^{-1} with 0.5 eV resolution. Consecutive spectra from the same point were examined from possible beam damage. Background subtracted EXAFS spectra were prepared using PySpline v1.1 and were modelled using DLexcurv v1.0. μXANES data summed and normalised using Athena.

A preliminary qualitative analysis to compare EXAFS and XANES spectra was performed. For some samples Linear combination Fitting (LCF) was used, with a least-squares algorithm of the sample μXANES and the spectra of the standards. The goodness of the fit was estimated by calculating the residual R factor of the fit: Σ_i (experimental-fit)^2/Σ_i (experimental)^2, where the sums are over 103 data points as flattened μ(E) **(**Adediran *et al*., 2015**)**. A lower R factor represents a better match between the fitted standard spectra and the sample spectrum (Terzano *et al*., 2008).

10.2. STATISTICAL ANALYSIS

Data were subjected to the Bartlett's test for homogeneity of variance and then analysed using the analysis of variance (ANOVA). The means were statistically separated on the basis of Student–Newmann–Kewls test when the 'F' test of ANOVA for treatment was significant at least at 0.05 probability level. Significance was accepted at $P \leq 0.05$ level. (Snedecor and Cochran, 1989).

10.3. RESULTS AND DISCUSSION

C. cardunculus were considerable tolerant to Cadmium and Arsenic suggesting that this specie was able to tolerate low doses of these toxic elements. Combination of the two heavy metals contributed to increase tolerance to the indeed stress.

10.3.1. PLANT GROWTH PARAMETERS

The growth parameters showed that all the plants survived until the end of the trial. When the heavy metals dose is low, growth might be stimulated, according to Cao *et al*., 2004.

At 15 days after contamination, no visible toxicity symptoms were observed and all the leaves were green, similar to the control. In particular the number of new leaves per plant, showed no significantly differences to the control (11 new green leaves) with a reduction of 28% (8-7 new green leaves) in plants treated with Cd alone and a reduction of 19% (9 new green leaves) in plants contaminated with As+Cd.

Under exposure to As alone all the plants at 15 days after artificial contamination, were healthy but the total number of new green leaves dramatically decreased, with a reduction of 50% compared to the control. It had 11 green leaves and the plants treated with As had 6-5 green leaves, in the average of three genotypes.

At 45 days after contamination, the number of new leaves of Gen 1 was significantly different from Gen 2 and Gen 3 for all treatments. No new green leaves were observed but the plants were still vital.

Regarding the biomass production of cardoon genotypes, only the factor “Genotype” influenced significantly the growth of the plants; no significant differences were observed analysing “concentration” and “contaminat” factors. In particular the biomass production of Gen 3 was higher than Gen 2, probably for its good adaptability to grow in adverce soil conditions (Fig. 18).

A severe reduction in roots elongation was observed only on plants treated with high As concentration (Fig. 19).

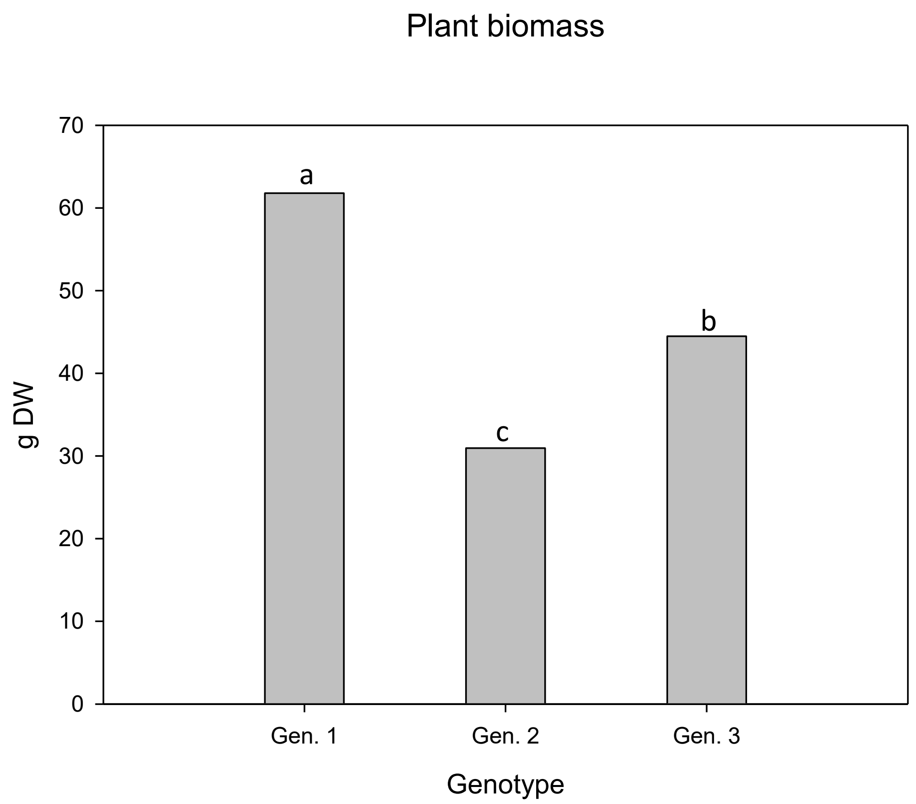

Figure 18. Biomass production of cardoon genotypes on average of contaminant and concentrations at 45 days after contamination. Values are the means ± S.E. (n=3).

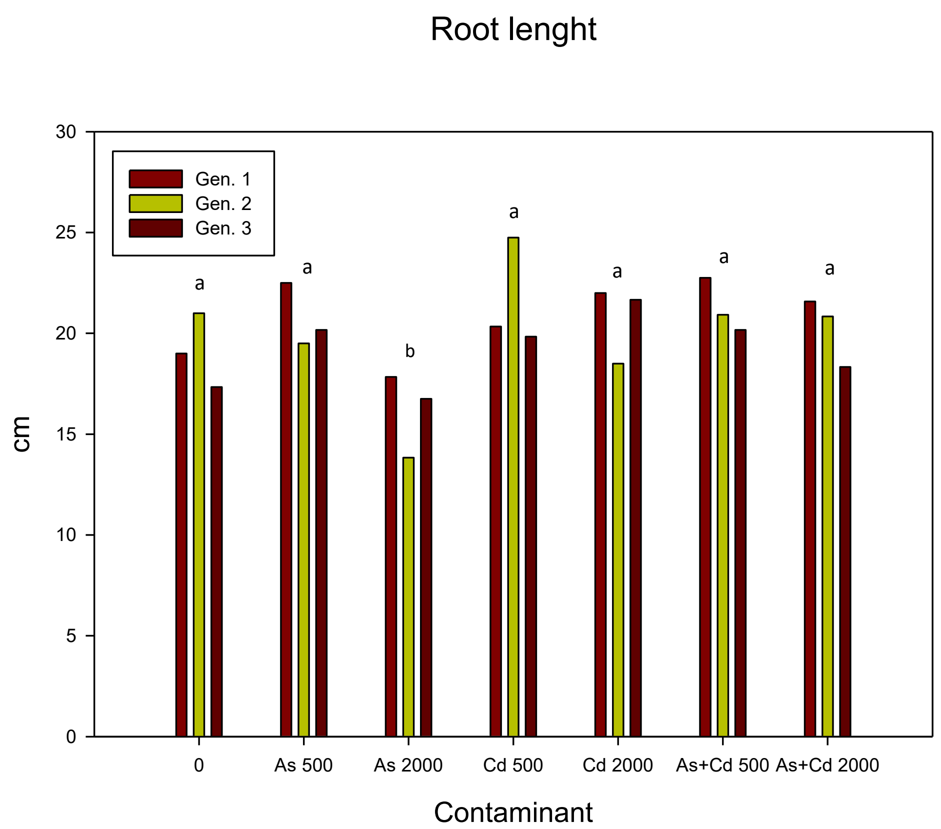

Figure 19. Root lenght of cardoon genotypes in response to different As, Cd and As+Cd exposures, at 45 days after contamination. Values are the means ± S.E. (n=3).

10.3.2. HEAVY METALS ACCUMULATIONS

Arsenic and Cadmium accumulations in the plants were analysed at different concentrations (0, 500, 2000 μM) and at different times of exposure (15, 30, 45 days after contamination). The statistical analysis showed that the times of exposure did not influence the characters studied. For this reason the accumulations of As and Cd at the end of the experiment, were considered. The analysis of variance (Table 7) showed that the factor that most influenced the accumulation of the metals in the plants is "Concentration", for all the characters studied, especially for the concentrations of As and Cd in roots (58%, 60% of total), and for "Cd (mg Kg^{-1} DW) in old leaves" (50% of total). The interactions "Contaminant x Concentration" (CoxConc) and "Genotype x Contaminant x Concentration" are significant in the character "As (mg Kg^{-1}) in old leaves".

Table 7. Mean squares expressed in absolute value (AV) and percent of total (%) in relation to genotype (Ge), contaminant (Co), concentration (Conc), CoXConc and GeXCoXConc interactions for the studied parameters, in plants subjected to As, Cd and As+Cd treatment , at the end of experiment.

	Mean squares of treatment									
Parameter	Genotype (Ge)		Contaminant (Co)		Concentration (Conc)		CoXConc		GeXCoXConc	
	AV	%	AV	%	AV	%	AV	%	AV	%
As (mg Kg^{-1} DW) in roots	$24{,}88^{ns}$	3,26	$142{,}17^{**}$	18,61	$439{,}76^{***}$	57,55	$121{,}35^{**}$	15,88	$35{,}91^{ns}$	4,70
Cd (mg Kg^{-1} DW) in roots	$0{,}15^{ns}$	0,30	$10{,}95^{**}$	22,20	$29{,}81^{***}$	60,44	$4{,}8^{ns}$	9,73	$3{,}61^{ns}$	7,32
As (mg Kg^{-1} DW) in old leaves	57,91*	9,99	$169{,}8^{***}$	29,30	$219{,}51^{***}$	37,88	$94{,}63^{***}$	16,33	37,66*	6,50
Cd (mg Kg^{-1} DW) in old leaves	$101{,}65^{ns}$	9,00	$272{,}32^{**}$	24,11	$568{,}79^{***}$	50,37	$141{,}57^{**}$	12,54	$44{,}93^{ns}$	3,98

ns Non significant.
* Significant at 0.05 probability level.
** Significant at 0.01 probability level.
*** Significant at 0.001 probability level

Regardless of the form of supplied As, cardoon plants accumulated As mainly in the roots (Llugany *et al.*, 2012), suggesting the immobilization of the As root cells. In the present work, for all genotypes, in As treatments, arsenic was accumulated mainly in the roots (Fig. 20-1) and the arsenic concentration was lower in the old leaves (Fig. 20-2) than in the roots. Also the root arsenic concentrations, increased significantly with increasing As contamination in the soil. In particular under As 2000 µM, the highest As concentrations in roots, were 15.32 mg kg^{-1} in Gen 1 and 12.50 mg kg^{-1} in Gen 3 (Fig. 20-1).

Despite that, as shown in Fig. 20-2, Gen 3 accumulated a major quantity of arsenic in the leaves under As 500. It is possible that the lower concentration of contaminant influenced the accumulation of As in the leaves suggesting that Gen 3 exhibited considerable resistance to As, with higher accumulation of As in the leaves.

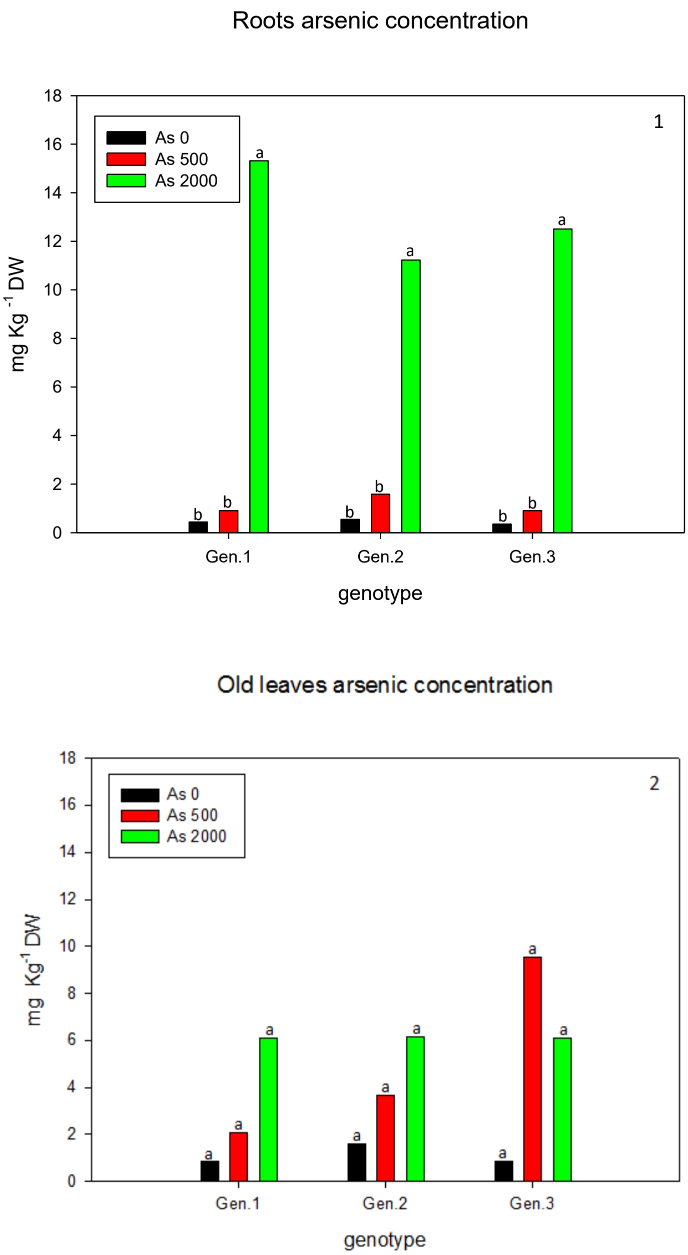

Figure 20. Roots (1) and old leaves (2) accumulation of arsenic under different As concentration in *C. cardunculus*, at the end of experiment. Values are the means ± S.E. (n=3).

Cadmium accumulation in cardoon roots showed the same behaviour of arsenic accumulation and increased significantly with the increase of the Cd concentration in the soil. The highest Cd concentrations in roots were 4.79 mg kg^{-1} in Gen 1 under Cd 2000 µM (Fig. 21-1). However, Cd accumulation was lower than that of arsenic in roots for all genotypes.

Cd concentrations in old leaves were higher than those in roots and the plants accumulated high levels of Cd under highly treatments.

The highest value of 18.72 mg kg^{-1} DW was under Cd 2000 µM in Gen 3 (Fig. 21-2).

This suggests that cardoon had efficient translocation ability to transfer Cd from root to shoots/leaves. Cd was absorbed by roots easily due to its high mobility and was translocated in aerial parts of plants by its interaction with macro-micronutrients (Nazar *et al.*, 2012).

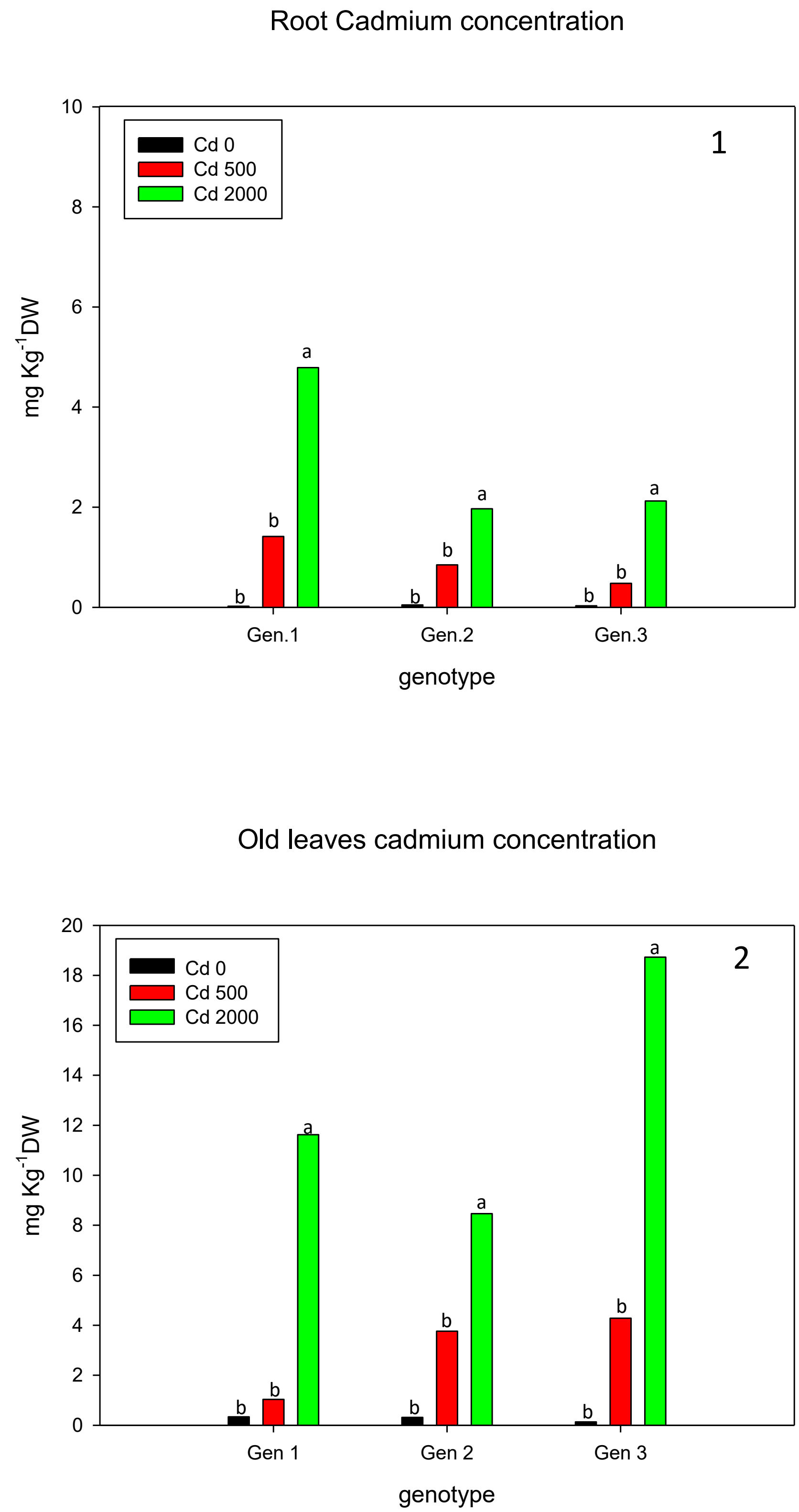

Figure 21. Roots (1) and old leaves (2) accumulation of cadmium under different Cd concentration in *C. cardunculus*, at the end of experiment. Values are the means ± S.E. (n=3).

According to Llugany *et al*., 2012, As was higher in plants grown in the presence of Cd than in those exposed to As alone and the presence of Cd increased the ability of the plants to absorb As and translocate it to old leaves. It could be due to Cd damaging the roots with the loss of specific As binding sites, and as a consequence induces greater traslocation of As to the shoot (Sanchez-Pardo *et al*., 2015). Moreover, in this experiment the results showed that the genotypes were significantly different from each other suggesting that the highest accumulation of metals was in Gen 3. Furthermore, the concentrations of both metals were always greater than those in treatments of As and Cd alone. The As and Cd concentrations in soil and roots increased significantly with the increase of both metals levels in the soil. The highest accumulations in old leaves were of 21.18 mg kg^{-1} for As and 24.62 mg kg^{-1} for Cd in Gen 3 under As+Cd 2000 µM, showing a signicant difference between Gen 3 and Gen 1 (Fig. 22, 1-2).

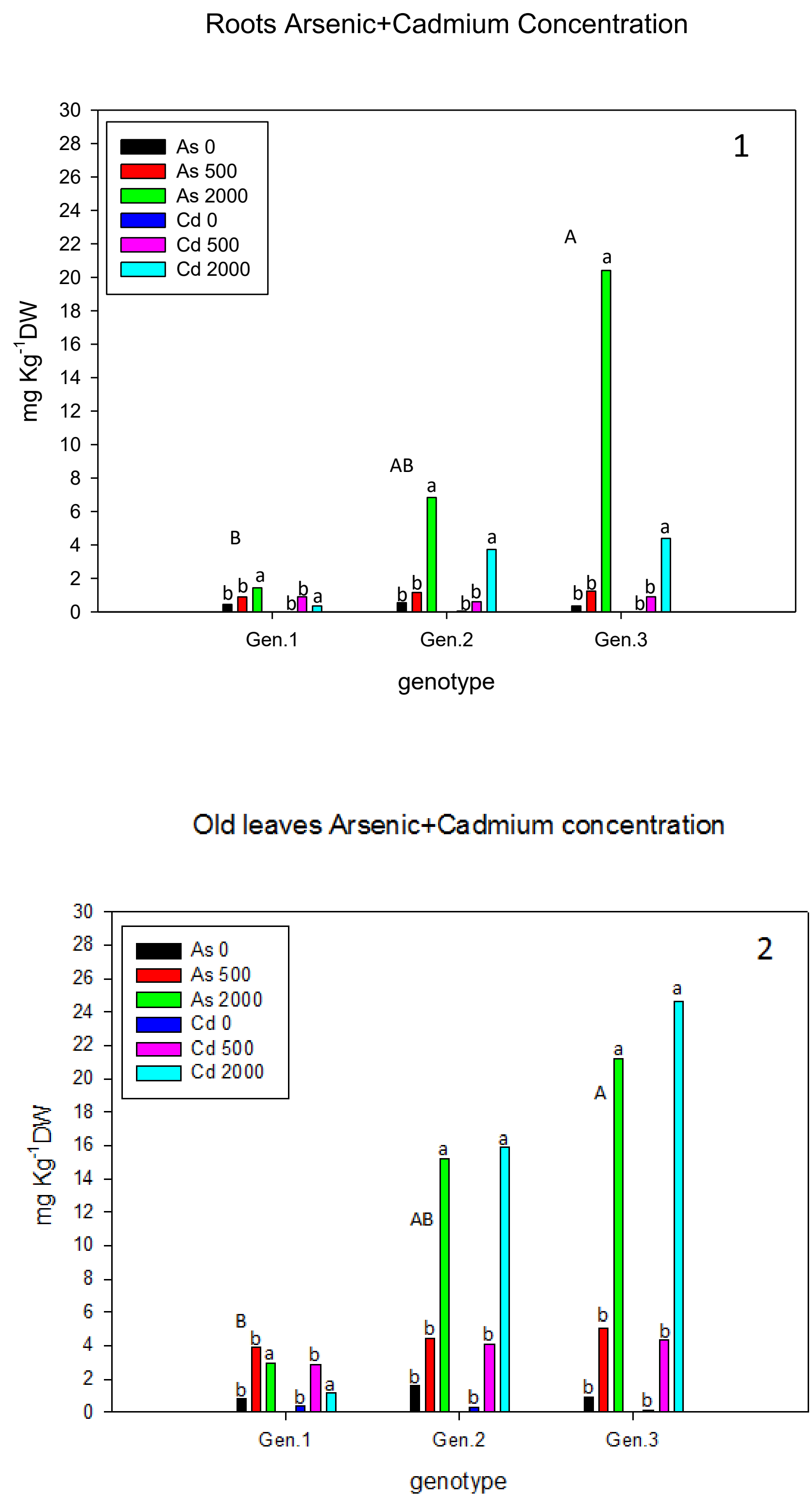

Figure 22. Roots (1) and old leaves (2) accumulation of arsenic under different As+Cd concentration in *C. cardunculus*, at the end of experiment. Values are the means ± S.E. (n=3).

10.3.3. BIOACCUMULATION FACTOR OF ARSENIC AND CADMIUM

Table 8 showed the bioaccumulation factor (BF) of As and Cd from soil. BF is an important parameter for assessing the ability of a plant to uptake metals from contaminated soil. For effective toxic metal phytoextraction, BF should be greater than 1.0 (Wei and Chen, 2006).

BF of all genotypes was between 0.04 and 7.41 and increased with the increase of metals concentration. The highest BFs of cadmium were 2.46 under Cd 2000 in Gen 3 and 7.41 under As+Cd 2000 in Gen 2. Moreover BF for As in plants exposed to As+Cd, was mostly higher than that of plants exposed to As alone.

The BF values would seem lower than those of hyperaccumulator plants but the cardoon plant, due to its high biomass production, has the ability to accumulate large quantities of metal contaminants in its tissue.

Table 8. Bioaccumulation factor (BF) of cardoon genotypes (means ± S.E.) in response to different arsenic, cadmium and arsenic+cadmium supplies in soil, at the end of experiment.

		Bioaccumulation factor (BF)		
Contamination	**Concentration (µM)**	**Genotype 1**	**Genotype 2**	**Genotype 3**
Arsenic	0	0,00	0,00	0,00
	500	0,07	0,12	0,29
	2000	1,28	1,08	1,03
Cadmium	0	0,00	0,00	0,00
	500	0,04	0,06	0,07
	2000	2,20	0,55	2,46
Arsenic in As+Cd	0	0,00	0,00	0,00
	500	0,14	0,12	0,08
	2000	0,27	1,54	1,75
Cadmium in As+Cd	0	0,00	0,00	0,00
	500	0,12	0,72	0,08
	2000	0,10	7,41	2,38

Exclusion and accumulation of metal are two main tolerance mechanisms of plants in response to heavy metal pollution as declared by Revees and Baker (2000). The preferential Cd accumulation in the leaves and the traslocation of arsenic from roots to aerial parts of plant when it was in co-contamination of Cd, suggested the potential ability of cardoon plants for phytoextraction.

10.3.4. METALS DISTRIBUTION IN PLANTS

Cadmium and Arsenic are very toxic for the plants and different authors reported their toxicity (Patra *et al*., 2004**;** Wang and Zhou, 2005; Shri *et al*., 2009).

The toxic effects of metals are mediated by the plant, storing toxic elements in forms recognized as the main survival mechanism in plants under metal toxicity (Kopittke *et al*., 2011). Exposure of plants to toxic metals appeared to induce the synthesis of sulfur-rich ligands such as phytochelatins, a cysteine-rich oligopeptide, that strongly bound metals. This mechanism of detoxification is predominant in non-tolerant plants but is important in hyperaccumulators when metal concentrations reach toxic levels (Huguet *et al*., 2012).

In stems and leaves, Cd was attached to oxygen and sulfur groups. This might imply that some small organic acids are responsible for Cd transport from roots to stems and leaves **(**De la Rosa *et al*., 2004).

In leaf cells of hyperaccumulators, metals are generally sequestered in vacuoles, as observed for Zn and Cd in *N. caerulescens* (Vázquez *et al*., 1994; Küpper *et al*., 1999; Frey *et al*., 2000) and for Zn in *A. halleri* (Küpper *et al*., 2000; Huguet *et al*., 2012). In vacuoles they may be bound to organic acids: *A. halleri* constitutively contains high amounts of malate, citrate and oxalate (Zhao *et al*., 2000; Sarret *et al*., 2009; Huguet *et al*., 2012).

Isaure *et al*., 2006 showed that Cd L3-edge XANES spectroscopy could discriminate between different Cd local structures of O/N ligands and S ligands, but was not sensitive enough to distinguish complexes with similar Cd environments, e.g., Cd-malate versus Cd-citrate, or Cd-cysteine versus Cd-glutathione .

In the present work, EXAFS analysis of Cd spectra showed that old leaves spectrum of Cd, is dissimilar to those of soil and roots spectra of plants under As+Cd treatment (Fig. 23), suggesting that the presence of As upregulated the production of specific proteins/ligands that bound and translocated Cd into the plant tissue; moreover the two metals interact seemed to magnify phytochelatin production, leading to sequestration of both metals and consequently increased tolerance to both.

The Cd reference spectra were not significantly different and it was not possible to distinguish, in this preliminary analysis, the dominant form of Cd in the powdered fractions of the leaves samples. Other studies are required for Cd speciation in *C. cardunculus*.

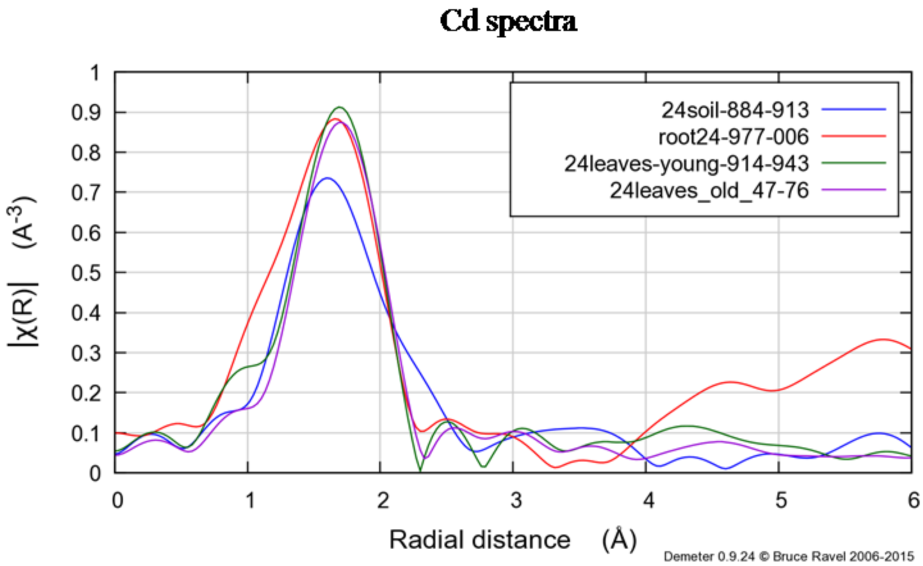

Figure 23. EXAFS spectra of Cd of soil, root, and young-old leaves samples under As+Cd treatment.

μXANES analysis showed that As in roots was mainly stored as sodium arsenate heptahydrate (66%), arsenic pentoxide (26%) and arsenic trioxide (0.8%). The LCF fits for As are those for roots sample determined with the spectra of the selected standards (R=0.082). (Fig. 24).

These As forms suggest the immobilization of the As, binding the metal to root cell walls and the limitated interaction of the toxic metal with vital plant tissues. In addition, the As form such as arsenic trioxide, suggests that Arsenate (As (V)) is taken up via phosphate transporters, is reduced to arsenite, is complexed with sulfur ligands and carried as As(III)-tris-glutathione complex into the vacuole.

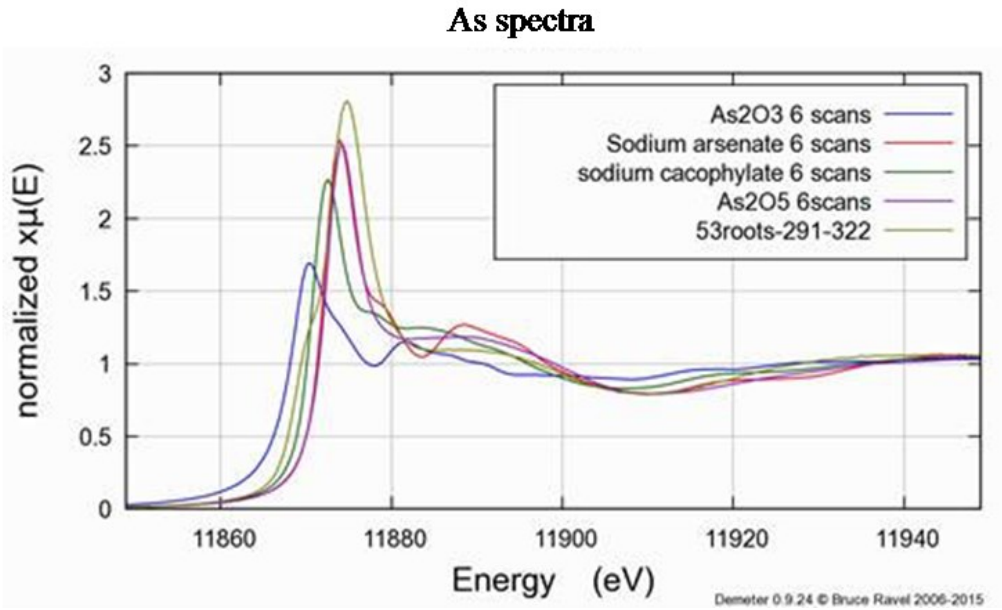

Figure 24. As K-edge XANES fitting R-factor and % As-compound composition for As roots sample under As treatment.

From these preliminary results no significant difference in Cd and As speciation was observed between two varietes of cardoon plants but other studies are required to improve the understanding of the different responses of cardoon genotypes and the ability to select appropriate genotypes for metal phytoextraction in environments contaminated with different metals.

12. CONCLUSION

An ideal plant for phytoextraction application should have high metal tolerance and high accumulation capacity in its tissues, especially in harvestable parts (Nabulo *et al*., 2007; Zandsalimi *et al*., 2011).

Cardoon is a plant that can tolerate the presence of Cd and As through several defence mechanisms such as, cross protection by activation of antioxidants, acclimation by activation of stress-specific defenses and amelioration by substrate interactions (Poschenrieder *et al*., 2013).

Based on the findings of both experiments, the presence of Cd alone did not influence, even at high levels of contamination, the growth and the development of cardoon plants: the plants accumulated Cd in their tissues specially in leaves instead.

Conversely, the tolerance and the accumulation of As, dependeded on the concentrations of contaminants. In the first experiment, at high levels of As the plant growth was inhibited; at 15 days after artificial contamination, metal concentration in plant tissues were elevated, and the plants were dried and died. In experiment 2, the lower concentrations of As allowed the plants to survive until the end of the trials. Moreover, the plants accumulated high levels of As in roots and As accumulation increased with increasing the concentration of As in soil.

The interaction effect of As+Cd has increased the resistance of plants to these metals, allowing the plants to survive, even in presence of high concentrations of both metals. Furthermore the accumulation of metals was mostly higher in plants exposed to co-contamination of As and Cd than that of plants under As or Cd alone. Also cardoon, under As+Cd contamination, translocated more As from root to shoots/leaves.

Therefore, depending on metals concentration and the presence or absence of Cd, these plants could respectively be used as excluders of As in As-contaminated sites, and as accumulators in sites co-contaminated by As and Cd.

Lastly, comparing the varieties and the genotypes of cardoon, the results demonstrated that *C. cardunculus* L. var. *sylvestris*, A14CT (Gen 3), collected from polluted soil, accumulated high levels of both contaminants suggesting its use in future works to remediate our Sicilian soils from these toxic elements.

Although many studies have focused on the metals tolerance and on the accumulated concentration in hyperaccumulators (Malakootian *et al*., 2009, Reza and Singh, 2010), another important aspect for the phytoextraction application in contaminated fields is biomass production of plants. Remediation factor (Rf), represents percentage of element removed per year from a determined volume of soil in respect to plant element concentration and plant yield (Fischerová *et al*., 2006).

It is well documented that cardoon is a fast-growing plant with high biomass production; cardoon, with lower heavy metal concentration in shoot but higher biomass production, exhibites higher phytoextraction efficiency in comparison to some other species of hyperaccumulator plants such as Thlaspi caerulescens, with higher metals concentration and low biomass production (Escarré *et al.*, 2000; Vázquez *et al.*, 1992). Also cardoon contains strong chelators that bind the metals in a non-toxic form promoting the plant growth.

Then the biological cycle and the high yield of cardoon allow using this crop for the remediation of polluted soils, combining these applications with energy production.

It would be useful to continue the trials with the selected Genotype 3 in future works, with the aim to test for more years its remediation efficiency in polluted soils, and exploit its biomass for energy purposes.

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