UNIVERSITY OF MILANO-BICOCCA

DEPARTMENT OF EARTH AND ENVIRONMENTAL SCIENCES

ASSESSMENT OF COMBINED TOXIC AND GENOTOXIC EFFECTS OF SOIL METAL POLLUTANTS: A LABORATORY AND A FIELD EXPERIMENT USING THE TEST PLANT *TRIFOLIUM REPENS* L

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ABSTRACT

The use of bioindicators as early warning systems represents a powerful approach for assessing and interpreting the impact of natural or anthropogenic perturbations in soil ecosystems on environmental quality and human health. Living organisms are sensitive to the cumulative effects of environmental stressors and contaminants and, as such, bioindicators provide information on environmental contamination that is complementary to direct physical and chemical measurements (Heger et al., 2012).

Trifolium repens is a pollutant-sensitive plant, often used as a bioindicator for a number of environmental contaminants. Specifically, its exposure to environmental contaminants followed by a DNA analysis with molecular markers allows the detection of sublethal levels of genotoxic compounds in the environment (Piraino et al., 2006). However, given the limited information available on the joint genotoxic-effect of multiple contaminants, the interpretation of biomonitoring results is often difficult. There is, then, a clear need to improve our understanding of the combined effects of stressors on bioindicators.

Starting from these considerations, the objective of the first part of my PhD research was to study the combined toxic and genotoxic effects of soil Cd and As, two of the most dangerous compounds for both environmental and human health, whose joint action is still unknown.

To do this, I exposed white clover (*Trifolium repens* L) plants to soil spiked with increasing concentrations of cadmium sulfate (20, 40 and 60 mg Kg^{-1}) or sodium arsenite (5, 10 and 20 mg Kg^{-1}) separately and in their combinations for 15 days, after which I assessed plant growth by measuring plant dry weight (roots and shoots) and mortality. In addition, I extracted DNA from the experimental plants in order to evaluate DNA damage using Random Amplified Polymorphic DNA (RAPD) techniques. DNA sequence damage induced by arsenic or cadmium and by their concomitant presence was evaluated by calculating the percentage of polymorphism (P %), which represents the ratio between the number of polymorphic bands and the total detected bands ×100.

During the experiment, I also assessed the bio-availability of As and Cd in the soil and their concentration in plant roots and shoots using the method of Lindsay and Norwell (1969) and the USEPA 3051a protocol, respectively.

The results from this experiment showed that individual and joint toxicity and genotoxicity were related to the concentration of Cd and As measured in plant organs and that As concentration was the most relevant variable. Joint effects on plant growth were additive or synergistic, whereas joint genotoxic effects were either additive or antagonistic. The interaction between Cd and As occurred at both soil and plant level: in soil the presence of As limited the bioavailability of Cd, whereas the presence of Cd increased the bioavailability of As. Nevertheless, only As bioavailability determined the amount of As absorbed by plants. The amount of Cd absorbed by plants was not linearly correlated with the fraction of bioavailable Cd in the soil, suggesting the involvement of additional factors, such as plant uptake mechanisms. These results revealed that the presence of both Cd and As in the soil, although producing an additive or synergistic reduction of *Trifolium repens* L. growth, caused less DNA damage. The reduction in growth was most likely due to a combination of the toxic effects of Cd and As, and to plant response to the high DNA damage, which led to a temporary arrest of cell divison providing a longer time for DNA repair and for production of scavenging free radicals. This would be consistent with the antagonistic genotoxic effect observed in most of the combined treatments, although the antagonistic interaction of Cd and As could also be linked to the similar genotoxic mechanisms of the two heavy metals.

In the second part of my PhD, I used the information and the techniques described above to assess the genotoxicity of soils in the Lombardy Region (Italy). I carried out a biomonitoring experiment in collaboration with the Catholic University of Piacenza and the European Research Centre of Ispra.

I analyzed a total of 67 samples of surface soil (0-30 cm in depth) which were collected in 7 different agricultural areas of concern within the Lombardy with assistance from AEFORIA, a Catholic University spin-off. The 7 different areas and the number of soil samples collected and analyzed are shown in the table below:

Area	Sample name	N° samples
Pieve Fissiraga (LO)	V/visc/2012 (V1-V8)	8
Autostrada Origgio (VA)	O/auto/2012 (O1-O8)	8
Broni (PV)	IT/cem/2012 (IT1-IT9)	9
Brescia Agricola	S/sin/2012 (S1-S8)	8
Boario Terme (BS)	F/fond/2012 (F1-F8)	8
Treviglio (BE)	CR/plume/2013 (CR1-CR14)	14
Parona (PV)	P/term/2013 (P1-P12)	12

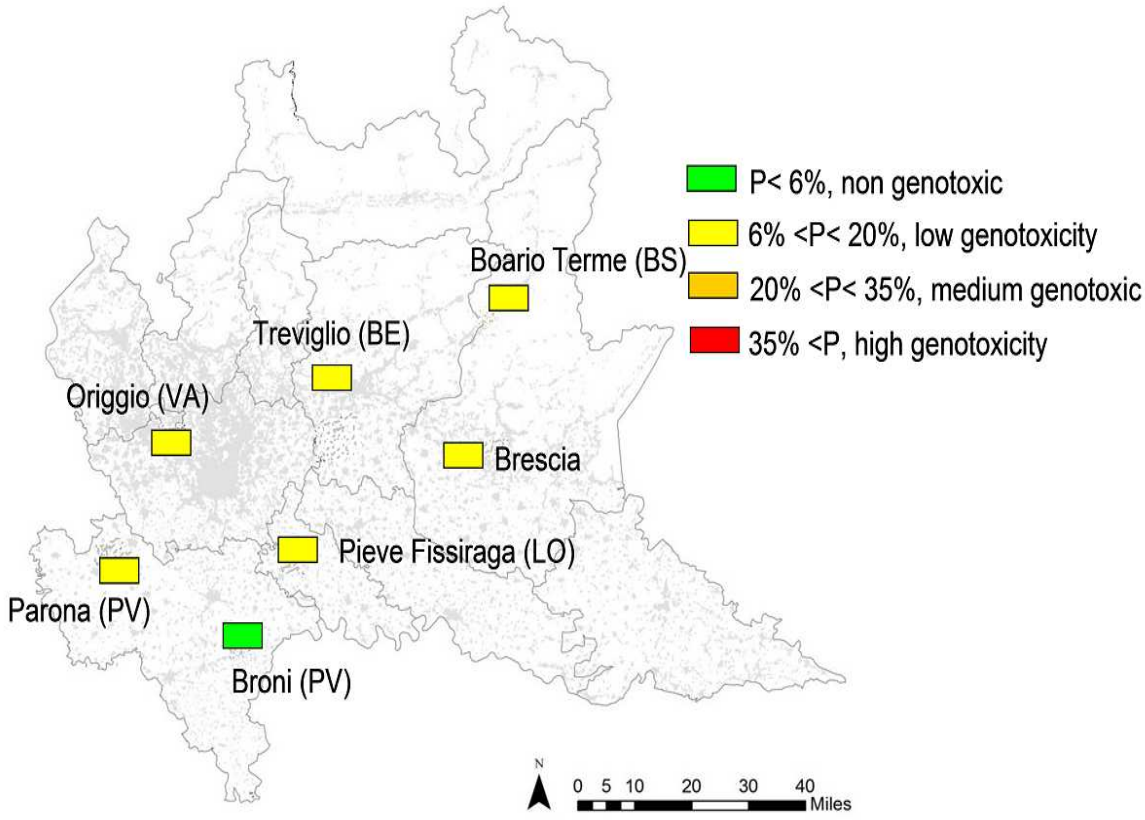
After soil collection I exposed clover plants to all these soils for two weeks and then at the end of the exposure assessed plant growth and DNA damage following the procedures described above. In order to better interpret the bioindication results I also took into account the soil properties (pH, EC, organic matter content and soil texture) and the concentrations of inorganic and organic compounds which were determined for the same soils by other research groups from the Catholic University of Piacenza and from the European Research Centre of Ispra.

The results showed that most soils did not affect the survival of the test plants, excepted for the soils CR3 and CR6 (from the area of Treviglio) and O1 (from the Origgio area close to the highway). Furthermore, no statistically significant difference was observed in the growth of seedlings (measured in terms of dry weight), except for some soils from the Treviglio area (CR2, CR3, CR6 and CR14) and for IT5 soil from the Broni area (PV). Although other soils from the latter area led to a reduction in root growth, they were considered to be not potentially toxic as the shoot growth of the test plants was not affected and the soil characteristics were not appropriate for white clover development.

Regarding the soil genotoxic potential, in general all soils except those from Broni area were found to contain bioavailable genotoxic compounds and were classified as "moderately genotoxic” on the basis of the polymorphism (P) scale shown below:

P < 6%	Non genotoxic
6% < P < 20%	Low genotoxicity
20% <P <35%	Medium genotoxic
35% < P	High genotoxicity

The genotoxicity results are summarised below:



DECLARATION

I, Nguyen Van Tho, declare that this PhD thesis entitled "Assessment of combined toxic and genotoxic effects of soil metal pollutants: a laboratory and a field experiment using the test plant *Trifolium repens* L" was carried out by me for the degree of Doctor of Philosophy in Environmental Science under the guidance and supervision of Prof. Sandra Citterio and Dr. Alessandra Ghiani, Department of Earth and Environmental Sciences (DISAT), University of Milano Bicocca, Italy.

This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

February 19th, 2015

LIST OF ABBREVIATIONS

AAS	Atomic absorption spectroscopy
APX	Ascorbate peroxidase
BaP	Benzo[a]pyrene
CAT	Catalase
CEC	Cation exchange capacity
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTPA	Diethylenetriaminepentaacetic acid
DW	Dry weight
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GPX	Guaiacol peroxidase
GR	Glutathione reductase
GSH	Glutathione
IARC	International Agency for Research on Cancer
IQ	Intelligence quotient
IPCS	International Programme on Chemical Safety
Naph	Naphthalene
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo para dioxins
PCDFs	Polychlorinated dibenzofurans
PCR	Polymerase chain reaction
PDW	Plant dry weight
POPs	Persistent organic pollutants
RAPD	Random Amplified Polymorphic DNA
RDA	Redundancy analysis
ROS	Reactive oxygen species

SCGE	Single-cell gel electrophoresis
SOC	Soil organic carbon
SOD	Superoxide dismutase
SOM	Soil organic matter
TCDD	Tetrachlorodibenzo para dioxin
TEL	Tetraethyl lead
UNEP	United Nations Environment Programme
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
WB	World Bank
WHO	World Health Organization

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1. Introduction

1.1 General properties of soil

As defined by Soil Survey Staff (1999): “Soil is a natural body comprised of solids (minerals and organic matter), liquid, and gases that occurs on the land surface, occupies space, and is characterized by one or both of the following horizons, or layers, that are distinguishable from the initial material as a result of additions, losses, transfers, and transformations of energy and matter or the ability to support rooted plants in a natural environment”. Soil is a natural medium made up of five major components: mineral particles (clay, silt, sand and gravel), organic matter (decaying plant and animal material), water, air, and living organisms (soil biota- ranging from bacteria, fungi and earthworms) (DEPI, 2014). Soil types are different, depending on the parent materials from which they came and from the surrounding environment. Soils are dynamic, forming continuously over a long period of time. The way in which soil forms depends on: parent material, climate, topography, living organisms and time (Harrison and Strahm, 2008). Soil has many environmental functions, for example the capacity to remove contaminants from the environment by filtration and adsorption. Soil has some different meanings (Fitzpatrick, 2013): (1) for soil scientists, soil is made up of different-sized mineral particles (sand, silt, and clay) including organic matter and has complex biological, chemical, physical, mineralogical, and hydrological properties that are always changing over time. So, it is ubiquitous and is dynamic, teeming with organisms, and is an integral part of both terrestrial and aquatic environments, (2) but for farmers, gardeners, and agronomists, soil is just a medium for growing crops, pastures, and plants, (3) and for engineers, soil is a material to build on and excavate. Thus soils can be both naturally occurring, comprising natural minerals and organic materials, and human-made, such as those that often contain very small amounts of manufactured materials, including brick fragments, explosive residues, or paint flecks. Soil has many ecological and socio-economic functions. Soil is a core component of land resources and the foundation of agricultural development and ecological sustainability. It is the basis for food, feed, fuel and fiber production and for many critical ecological services and is a complex, dynamic living system and its suitability varies from place to place (FAO, 2014a).

Soil quality is considered to be important for the assessment of the extent of land degradation or amelioration, and for identifying management practices for sustainable land use (Dexter, 2004). However it is difficult to define the “soil quality” because it is not easy to define exactly the physical chemical and biological properties of soil. There is no clear boundary among these different disciplines. To be simpler in this thesis, physical, chemical and biological features of soil will be considered separately.

1.1.1 Physical property

Physical properties of soil can change gradually over time. It is not always easy to quantify any significant changes over a short time period. Two important physical properties of soils are texture and structure.

Soil texture is a term used to refer to the size distribution of the primary mineral particles in the soil. It is commonly used to designate the proportionate distribution of the different sizes of mineral particles in a soil. It does not include any organic matter. These mineral particles vary in size from those easily seen with the unaided eye to those below the range of a high-powered microscope (UF, 2014). They are: Sand = <2 to 0.05 mm, Silt = 0.05 to 0.002 mm, Clay = <0.002 mm. Sand, silt, and clay constitute the “fine-earth fraction”, and represent inorganic soil particles less than 2mm in diameter. Inorganic soil particles 2mm and larger are called “rock fragments” (USDA, 2014). On the basis of relative sand, silt and clay percentages the soil textural classes are defined (Figure 1).

From textural classes of soils, it is easy to recognize soil general characteristics. For example, sandy soils generally contain low organic matter contents, are well aerated but do not retain moisture and nutrients well, so are generally of low fertility.

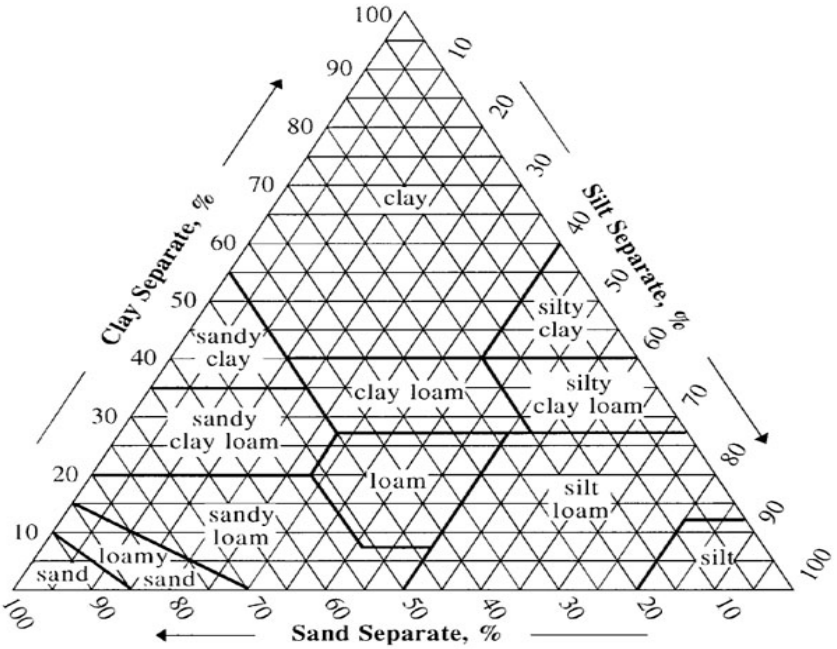

Figure 1: Graph showing the percentages of sand, silt, and clay in the soil texture classes by Soil Survey Staff (1999)

Soil texture plays a key role in soil degradation and water transport processes, controlling soil quality and its productivity (Hillel, 1980) and in determining the extent to which specific soils become compacted and the possible effects on root growth (Alameda and Villar, 2012). It may also be an important factor affecting the mineralization response to dry/wet cycles due to its role in the stabilization of soil organic matter, and effects on pore size distribution that also affect the moisture release characteristics of soils (Harrison-Kirk et al., 2013). Soil texture affects also plant growth by influencing root distribution and the ability to take up water; specifically, the amount of water the soil can hold, the rate of water movement through the soil and nutrients disturbance of soil structure through compaction or tillage can result in the rapid recycling of nutrients, crusting, reduced water and air availability to roots and how workable and fertile the soil is (Bronick and Lal, 2005). If a soil contains a lot of macropores, like coarse sand, it loses water through gravitational drainage easily. As a result, many pores are open for aeration, and little water remains for plant. This can cause drought stress to occur during dry periods. On the contrary, a fine-textured soil, such as a clay loam, has mainly micropores which hold water tightly and don’t release it under gravity. This kind of soil is prone to poor aeration and anaerobic (without oxygen) conditions, which can negatively affect plant growth.

Well-aggregated, loamy soils are best suited for supplying plants with water because they have enough macropores to provide drainage and aeration during wet periods, but also have adequate amounts of micropores to provide water to plants and organisms (McCauley et al., 2005). In addition, soil texture affects the extraction efficiency of bacteria from soils and also their biosynthetic activity, the finer the soil the higher the extraction efficiency of bacteria and the higher the biosynthetic activity and turnover of bacteria (Uhlirova and Santruckova, 2003).

The second important physical property of soil is structure. This term refers to the way soil particles group together to form aggregates which vary in size and shape from small crumbs through to large blocks. Soil structure is a key factor in the functioning of soil, its ability to support plant and animal life, and moderate environmental quality with particular emphasis on soil carbon sequestration and water quality. Along with texture, it affects water availability, nutrient uptake and leaching thereby affecting ground and surface water supplies (Bronick and Lal, 2005). A healthy soil structure is a key factor for crop production because it controls depth-penetration of roots, the extent of soil water storage, and the movement of water, air as well as soil fauna (Pardo et al., 2000). Thus the determination of soil structure is essential for understanding soil functionality and for a proper management of agricultural and environmental problems involving soil compartment (Dexter, 1997). In fact soil structure is the property most frequently evaluated when determining soil quality under different land uses and tillage practices. It is usually evaluated in an indirect way from properties such as soil organic carbon content, bulk density, porosity, soil water retention curve, soil resistance to root growth and infiltration rate (Moncada, 2014). These properties can be used as indicators of soil physical quality.

Soil structure can be improved by enhancing the diversity and quantity of soil flora and fauna (Bronick and Lal, 2005)

1.1.2 Chemical property

Soil cation-exchange capacity (CEC), pH and salinity are the most frequently evaluated parameters to determine soil chemical properties.

Some plant nutrients and metals exist as positively charged ions, or “cations”, in the soil environment. Among the more common cations found in soils are hydrogen (H^+),

aluminum (Al^{+3}), calcium (Ca^{+2}), magnesium (Mg^{+2}), and potassium (K^{+}). Most heavy metals also exist as cations in the soil environment. Clay and organic matter particles are predominantly negatively charged (anions), and have the ability to hold cations from being "leached" or washed away. The adsorbed cations are subject to replacement by other cations in a rapid, reversible process called "cation exchange" (USDA, 2014).

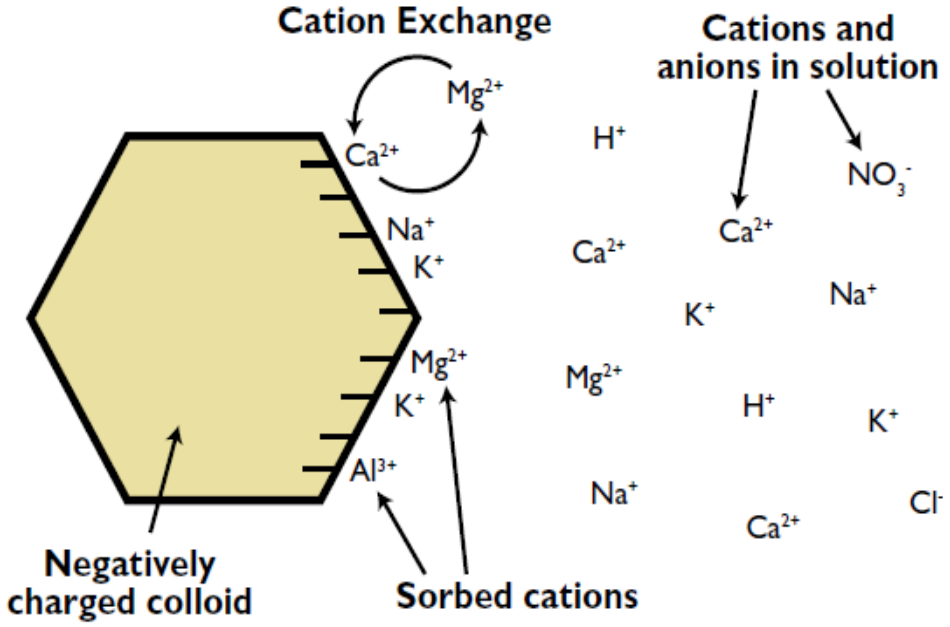

Figure 2: Soil cation-exchange capacity (McCauley et al., 2005).

Soil cation-exchange capacity (CEC) is the maximum quantity of total cations that a soil is capable of holding, at a given pH value, available for exchange with the soil solution. CEC is used as a measure of fertility, nutrient retention capacity, and the capacity to protect groundwater from cation contamination. It is expressed as centi-mol of hydrogen per kg ($cmol_c/kg$ or $meq_c/100g$). Most of the soil's CEC occurs on clay and humus (FAO, 2014b). The greater the clay and organic matter content, the greater the CEC should be, although different types of clay minerals and organic matter can vary in CEC.

Along with ion exchange property, an important index of soil chemical properties is soil salinity. Soil salinity is one of chemical properties of soil which affects plant growth. The salt concentration in the water extracted from a saturated soil (called saturation extract) defines the salinity of this soil. If this water contains less than 3 grams of salt per liter, the soil is said to be non saline. If the salt concentration of the

saturation extract contains 3-6, 6-12 and more than 12 g/l, the soil is said to be slightly, medium and highly saline respectively (FAO, 2014c). Salts can be transported to the soil surface by capillary transport from a salt laden water table and then accumulated due to evaporation. Salinization occurs when irrigation practices are carried out without due attention to drainage and leaching of the salts out of the soil. Salts can also accumulate due to seawater intrusion, or may occur naturally. As soil salinity increases, salt effects can result in degradation of soils and vegetation. The most common salts are combinations of the cations: sodium, calcium, magnesium and potassium with the anions chlorine, sulfate and carbonates. Salinity has a pronounced negative effect on soil organic matter decomposition, irrespective of soil texture (Setia et al., 2011). Most crops do not grow well on soils that contain salts. One reason is that salt causes a reduction in the rate and amount of water that the plant roots can take up from the soil. Also, some salts are toxic to plants when present in high concentration. The highly tolerant crops can withstand a salt concentration of the saturation extract up to 10 g/l. The moderately tolerant crops can withstand salt concentration up to 5 g/l. The limit of the sensitive group is about 2.5 g/l. (FAO, 2014c).

Soil pH is another important factor to define the chemical properties of a soil. It is an indication of the acidity or alkalinity of soil and is measured in pH units. Soils with high acidity (pH <5.5) tend to have toxic amounts of aluminium and manganese. Soils with high alkalinity (pH >8.5) tend to disperse. Soil organisms are hindered by high acidity, and most agricultural crops do best with mineral soils ranging from 6.0 to 6.8 pH. (FAO, 2014b)

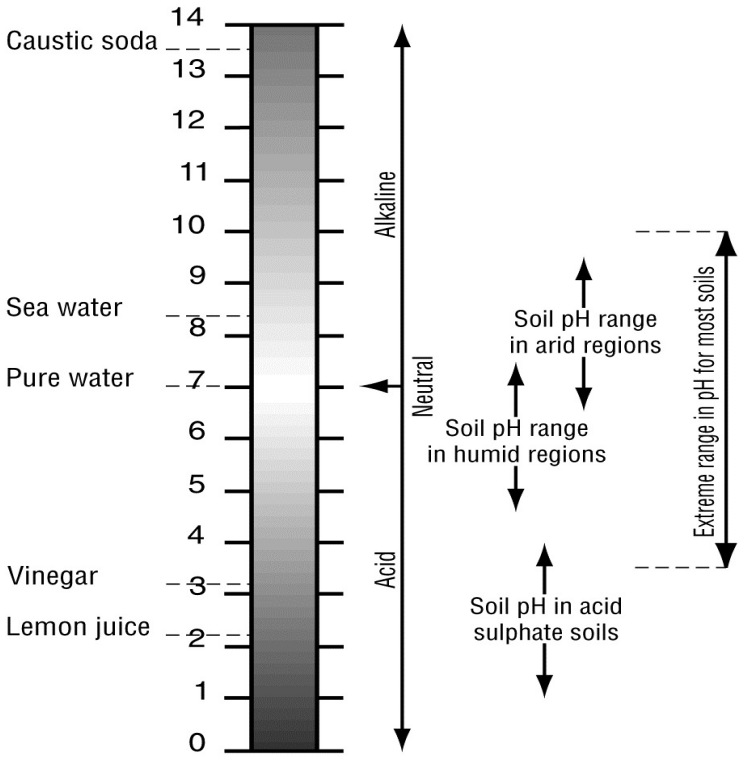

Figure 3: The range of pH values found in soils (QG, 2014)

Among soil properties (for example organic matter content, cation exchange capacity (CEC), the contents of clay minerals and so on), soil pH was found to play the most important role in determining metal speciation, solubility from mineral surfaces, movement, and eventual bioavailability of metals, due to its strong effects on solubility and speciation of metals both in the soil as a whole and particularly in the soil solution (Zeng et al., 2011). Some nutrients become unavailable if the soil pH remains at extremely acid or extremely alkaline conditions (Osman, 2013a). A pH range of 6.0 to 6.8 is ideal for most crops because it coincides with optimum solubility of the most important plant nutrients. Some elements for example Mo and Mg are more available at higher pH than other elements. However, heavy metal cations are most mobile in acid soils. This means that metal contaminants are more available for uptake by plants, or to move into the water supply (EC, 2014) and thereby posing a threat to human health (Zeng et al., 2011).

1.1.3 Biological property

Together with physical and chemical properties, soil biological properties are very important in assessing soil quality. Biota plays a crucial role in biological property of

soil, especially soil organic matter which is produced from biota. Soil organic matter (SOM) which is derived from residual plant and animal material at various stages of decomposition, ranging from fresh undecomposed materials through partially decomposed and short-lived products of decomposition to well-decomposed humus by microbes under the influence of temperature, moisture and ambient soil conditions, plays an importance role in maintaining soil functions because of its influence on soil structure and stability, water retention, soil biodiversity and as a nutrient source for plant (EC, 2012; Osman, 2013a). The primary constituent of soil organic matter (SOM) is soil organic carbon (SOC). It is a main component of global carbon cycle, and also plays a major role in regulating and maintaining ecosystem functions, including atmospheric exchanges of CO_2 (Nocita et al., 2013). It was proved that soil organic carbon originating from plants, animals and microorganisms, and their exudates enhance aggregation through the bonding of primary soil particles (Bronick and Lal, 2005). Dissolved organic matter is the most mobile soil organic matter or humus fraction. It plays an essential role in soil formation and mineral weathering, binding a variety of compounds ranging from small charged compounds such as metals to larger hydrophobic substances including pesticides and polycyclic aromatic hydrocarbons (Nierop et al., 2002). In addition, it also supplies organic chemicals to the soil solution that can serve as chelates and increase metal availability to plants (Zeng et al., 2011).

Activity of soil microfauna (mainly protozoa and nematodes) is important in the formation of organo-mineral complexes and aggregation (Bronick and Lal, 2005). Soil organisms have different important functions in energy transfer and nutrient cycling (Nannipieri et al., 2003). They may act as a nutrient source and are involved in humification processes, degradation of pollutants, and maintenance of soil structure (Marcin et al., 2013). They produce organic matter, consume organic matter, and decompose them usually the most active in the surface soil zone of 0–15 cm or in the plowed player because this zone has accumulation of organic residues and available nutrients. As the soil microfauna, soil microbial communities play an important role to plant. For example, they break down organic matter making nutrients available for uptake by plants. In addition, they also contribute to soil C and N transformations and nutrient cycles and thus affect soil biological, chemical, and physical properties

because the activity of soil microorganisms determines not only soil C sequestrations and emissions, and the decomposition and accumulation of soil organic matter but also N^{2-} fixation, nitrification and denitrification, and the accumulation of plant available $NH4^{+}$ and NO_3^{-} (Chen et al., 2014). The nutrients stored in the bodies of soil organisms prevent nutrient loss by leaching (FAO, 2014b).

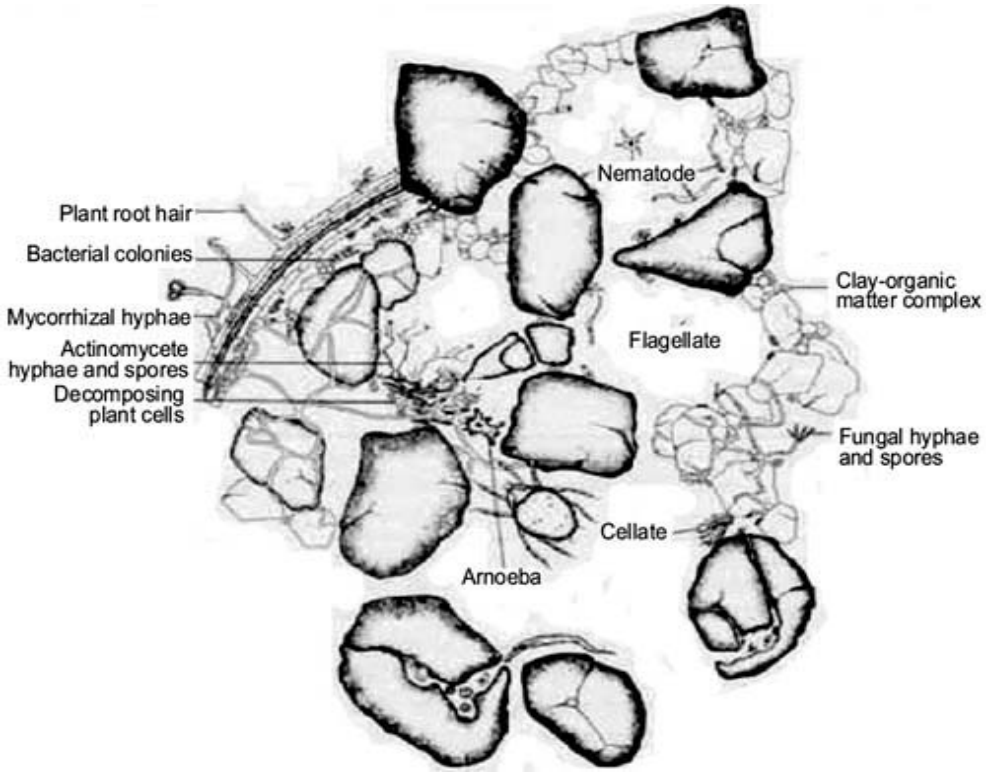

Figure 4: Soil organisms make up the diversity of life in the soil, play an important role in decomposition and accumulation of soil organic matter (Rose and Elliott cited by FAO, 2014d)

1.2 Soil pollutants and sources

1.2.1 Heavy metals and metalloids

Nowadays heavy metals are the environmental priority pollutants and are becoming one of the most serious environmental problems with increasing industrialization and disturbance of natural biogeochemical cycles (Fu and Wang, 2011). Heavy metals enter the environment from natural and anthropogenic sources. The most significant natural sources are weathering of minerals, erosion and volcanic activity while anthropogenic sources include mining, smelting, electroplating, use of pesticides and (phosphate) fertilizers as well as biosolids in agriculture, sludge dumping, industrial discharge, atmospheric deposition, etc (Ali et al., 2013).

It is difficult to quantify the real extent of local contamination as many European countries lack comprehensive inventories and there is a lack of EU legislation obliging Member States to identify contaminated sites (EC, 2012). However, according to what estimated by European Commission in 2007 (EC, 2014), following over 200 years of industrialization, soil contamination has become a widespread problem in Europe: approximately three million European sites are potentially affected by activities that can pollute soil with the most frequent contaminants, heavy metals and mineral oil (Figure 5) and approximately 250,000 of these sites may need a urgent remediation. Heavy metal contaminated soils have been estimated to represent about the 35% of polluted soils.

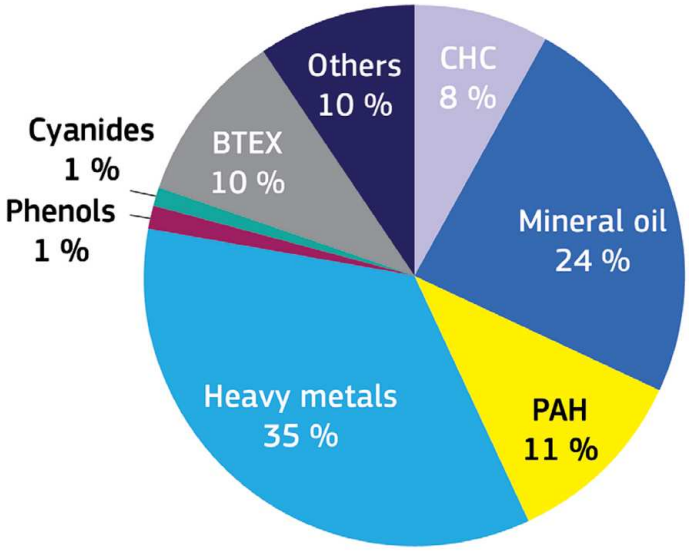

Figure 5: Most frequent soil contaminants in Europe (EC, 2014)

The accumulation of contaminants and in particular of heavy metals in soils and waters poses a risk to the environmental and human health. Heavy metals accumulate in the body tissues of living organisms (bioaccumulation) and their concentrations increase as they pass from lower trophic levels to higher trophic levels (a phenomenon known as biomagnification) (Ali et al., 2013). Several metals are essential for animal and plant life whereas other do not have any known biological function. For example Zinc, Fe and Cu are essential elements and they become toxic only at high concentrations. Cd^{2+}, Pb^{2+}, Hg^{2+}, Ag^{+} and As^{3+} are instead non-essential compounds and are toxic to plants and animals and react with the body's bio-molecules often

forming extremely stable biotoxic compounds which are difficult to dissociate (Hashim et al., 2011).

1.2.1.1 *Cadmium (Cd)*

Cadmium is of concern because of its toxic effects, accumulation and persistence in the environment, causing a risk to plants, animals and human. Cadmium is widespread in soils, water and atmosphere. It is released into the environment by heating systems, metallurgic industries, waste incinerators, urban traffic, and cement factories (Sanita di Toppi and Gabbrielli, 1999). It is also known that fertilizers may contain trace element contaminants such as cadmium that can be inadvertently introduced into soils. In particular, phosphorus (P) fertilizer, applied over the long-term, can serve as an important source of trace elements such as arsenic, cadmium and lead that can potentially accumulate in plants and soils (Altansuvd et al., 2014). Because of a strong demand for Cd worldwide, particularly in the nickel–Cd battery industry, approximately 30,000 tones of Cd are released into the environment each year, with an estimated amount of 4,000–13,000 tones coming from industrial activities (Gallego et al., 2012). However, its usage in developed countries has begun to decline because of its toxicity.

The amount of cadmium in soil varies with location due to differences in soil formation, management practices and exposure to pollution sources, but the level of Cd in the soil appears to be increasing over time (Grant et al., 1998).

Cadmium concentrations in crops increased with increasing soil Cd concentrations, all other factors being constant (Six and Smolders, 2014). In Europe, analysis of archived soil samples from experimental stations in UK, France and Denmark revealed that soil Cd increased by factors 1.3–2.6 during the 19th and 20th century. Mass balances (input–output) reflecting the period 1980–1995 predicted larger Cd inputs via phosphate (P) fertilizers and atmospheric deposition than outputs via crop uptake and leaching (Six and Smolders, 2014). The gradual increase of soil cadmium concentrations in European soils during the 20^{th} century has prompted environmental legislation to limit soil cadmium (Cd) accumulation (Six and Smolders, 2014). In numerous regions of the world food and feed crops are cultivated in soils contaminated by plant-available heavy metals. This leads to economic losses and have negative

effects on human health (Meers et al., 2010). For example, rice is staple food in Asia and is also staple food of half the world population. However, in the recent years, millions of tons of Cd contaminated rice have been discarded in rice bowls of Asia such as Thailand and China because of the application of phosphate fertilizer contaminated with Cd and irrigation of rice fields using wastewater released from mines (Sebastian and Prasad, 2014).

1.2.1.2 *Arsenic (As)*

Most environmental arsenic problems are the result of mobilization under natural conditions such as weathering reactions, biological activity and volcanic emissions (Zhang et al., 2002). However, man has had an important additional impact through mining activity, combustion of fossil fuels in coal- and oil-fired power plants, municipal solid waste, the use of arsenical pesticides, herbicides, algicides, wood preservatives, crop desiccants and the use of As as a growth stimulants for plants, additive to livestock feed, particularly for poultry (Eisler, 1988; Smedley and Kinniburgh, 2002; Zhang et al., 2002). The production of antifungal wood preservatives is a significant industrial source of arsenic that can contaminate soil (EC, 2014). The natural contents of arsenic in the soils are different depending on parental material. Arsenic concentrations in uncontaminated soil are generally in the range 0.5–40 mg kg^{-1}, with lowest concentrations in sandy soils and those derived from granites, whereas larger concentrations are found in alluvial and organic soils (Mandal and Suzuki, 2002). As contamination of surface soils ranging from 50 to >15000 mg As kg^{-1} by anthropogenic activities has been documented, but the occurrence of As concentrations >15000 mg As kg^{-1} is not common in the soil environment (Smith et al., 2014).

Although the use of arsenical products such as pesticides and herbicides has significantly decreased in the last few decades, their use for wood preservation is still common and the impact on the environment of the use of arsenical compounds, at least locally, will remain for some years (Smedley and Kinniburgh, 2002). It was also reported that ground water contaminated with high arsenic contents are used for drinking water in many areas in the world especially in India, West Bengal,

Bangladesh, China, Taiwan and in many countries in South East Asia (Berg et al., 2006; Khan and Yang, 2014).

In Italy, high As concentrations have been detected in the soil and groundwater of several regions (Campania, Lombardia, Puglia, Calabria Lazio, Toscana, Emilia-Romagna, Veneto, and Sardegna; Sommella et al., 2013).

Natural arsenic contamination in groundwater is widespread and there are a number of regions in the world where arsenic contamination of drinking-water is significant. More than 100 million people worldwide ingest excessive amounts of arsenic through groundwater enriched from natural geogenic sources (Buschmann and Berg, 2009). In Asian countries, an estimate by World Bank in 2005 showed that nearly 65 million people are at risk of ingesting unsafe levels of arsenic through drinking water (WB, 2014).

1.2.1.3 *Lead (Pb)*

Natural sources of lead are mainly from volcanic activity, geochemical weathering and sea spray emissions. However, lead pollution in the environment is mainly from human activities such as use of leaded petrol (gasoline), production of lead-acid batteries and paints, jewellery making, mining, smelting, refining and informal recycling of lead, leaded glass manufacture in informal and cottage (home-based) industries, electronic waste and use in water pipes and solder (WHO, 2014c). The anti-knock properties of tetraethyl lead (TEL) were discovered in 1921 and it had been introduced into the market as leaded gasoline in 1923. The commercial use of leaded gasoline has led to environmental effects which are evident over the entire globe (Walraven et al., 2014). In Europe, anthropogenic source of atmospheric lead has dominated over the geogenic source since industrialization (Thevenon et al., 2011). Globally, in the late 1980s, anthropogenic emissions of Pb were estimated more than 20 times greater than natural emissions, an enrichment factor far greater than any other trace metal (Watmough and Hutchinson, 2004). Although leaded gasoline is not in use in most of the European countries anymore, lead is still widely found in vegetation and topsoil (Tomašević et al, 2013).

1.2.1.4 *Mercury (Hg)*

Mercury is a metallic element that exists naturally in the earth's crust. It is released into the environment through natural processes such as volcanic activity, forest fires, weathering of rock, and biologic processes and so on. Mercury releases in the environment result also from human activity, mainly from coal-fired power stations, residential heating systems, waste incinerators and as a result of mining for mercury, gold and other metals (WHO, 2014c). The global anthropogenic Hg emission to the atmosphere was estimated to be 2190 tons in 2000 to which the Asian countries contributed about 54% (especially China with more than 600 ton of Hg contributing about 28% to global emissions; it was the top ten country with the highest Hg emissions from anthropogenic activities), followed by Africa (18%) and Europe, including the European part of Russia (11%) (Pacyna et al., 2006). Global natural mercury emission was approximately 1800–5800 tons/year (Li et al., 2009). According to Pirrone et al (2010) on an annual basis, natural sources accounted for 5207 tones of mercury released to the global atmosphere (including the contribution from re-emission processes, which are emissions of previously deposited mercury originating from anthropogenic and natural sources, and primary emissions from natural reservoirs) and anthropogenic mercury emission sources were estimated to be 2320 tones annually, which include a large number of industrial point sources.

It was believed that the timing of long-term increases in mercury levels found in ocean life could be tied to historical events. For instance, significant increases in marine mercury levels beginning in the 19th century were likely to have been caused by industrialization in Europe and North America, whereas recent jumps in the amount of mercury found in the seabirds' eggs from the South China Sea were consistent with Asian industrialization (EC, 2014). In addition, it was also revealed that Arctic marine animals have 10-12 times higher concentrations of mercury in their bodies than before 1800 (EC, 2014).

1.2.1.5 *Chromium (Cr)*

Chromium occurs in each of the oxidation states from -2 to +6, but only the 0 (elemental), +2, +3, and +6 states are common (IPCS, 2014). Chromium (Cr) is considered a metal of increasing concern regarding environmental health although

only the form Cr(VI) is toxic (EC, 2014). Cr(VI) has unique properties of corrosion resistance, hardness and color and therefore finds large number of applications as well as a rapid increase in the utilization in industries (Itankar and Patil, 2014). USEPA's Toxic Release Inventory listed 1,762 industrial facilities that released a total of 52,600 metric tons of Cr into the environment (Choppala et al., 2013). Cr is found in all phases of the environment, including air, water, and soil. Naturally occurring in soil, Cr ranges from 10 to 50 mg kg^{-1} depending on the parental material (Shanker and Venkateswarlu, 2011). Human activities are also a source of Cr; they include industrial, commercial and residential fuel combustion (coal, and oil), emissions from metal industries, and wastewaters from industries such as electroplating operations, leather tanning industries, and textile manufacturing (Wise Sr et al., 2009). Industries all over the world have used Cr for more than a century, and at present Cr is a primary pollutant at over half of all hazardous waste sites (Shanker and Venkateswarlu, 2011).

1.2.2 Organic pollutants

Apart from heavy metal(loid)s, soil organic pollutants are also concerned. Most organic pollutants are persistent organic pollutants (POPs). POPs are chemicals that are extremely stable and persist in the environment, bio-accumulate in organisms and food chains, are toxic to humans and animals and have chronic effects such as the disruption of reproductive, immune and endocrine systems, as well as being carcinogenic (UNEP, 2014). An increasingly industrialized global economy over the last century has led to dramatically elevated releases of anthropogenic organic chemicals into the environment (Gerhardt et al., 2009). Soil plays an important role in the fate and distribution of organisc pollutants and can act as a sink or a source because once released into air or water, they will end up in soils, with the exception of those that are deposited at the bottom of oceans (EC, 2014). Prevalent organic pollutants found in soils include polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins and dioxin-like chemicals, herbicides, pesticides, organic fuels (gasoline, diesel), etc.

1.2.2.1 Polycyclic aromatic hydrocarbons (PAHs)

PAHs are a group of organic compounds containing only carbon and hydrogen and constituted by two or more aromatic rings fused together. They enter the environment via the atmosphere during industrial processes, especially incomplete combustion of products like coal, oil, gas, and garbage (USEPA, 2014b). Many PAHs have been identified as being carcinogens, with possible genotoxic properties (EC, 2002). They do not burn very easily and can stay in the environment for long periods of time. Most of them do not break down easily in the water (USEPA, 2014b). PAHs have been detected in vegetables contaminated by the deposition of airborne particles or grown in contaminated soil (EC, 2002).

1.2.2.2 Dioxins

Dioxins are a group of chemically-related compounds that are highly toxic and persistent environmental pollutants (POPs). The name "dioxins" is often used for the family of structurally and chemically related polychlorinated dibenzo para dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (EC, 2014). The chemical name for dioxin is: 2,3,7,8- tetrachlorodibenzo para dioxin (TCDD) which has a wide range of effects and was classified by the WHO's International Agency for Research on Cancer (IARC) in 1997 and 2012 as a human carcinogen. However, TCDD does not affect genetic material (WHO, 2014b). Several dioxin-like polychlorinated biphenyls (PCBs) with similar toxic properties are also included under the term "dioxins". There are 419 types of dioxin-related compounds that have been identified but only about 30 of these are considered to have significant toxicity, with TCDD being the most toxic (WHO, 2014b).

When dioxins enter soils, they remain (i) in the very top layer (0.1 cm) with a half-life of 9-15 years and (ii) at deeper soil levels persisting for 25-100 years (EC, 2014). Vietnam is thought to be the site of the world's largest and most significant dioxin contamination event (dioxins include polychlorinated dibenzo-p-dioxin [PCDD] and polychlorinated dibenzo-furan [PCDF]; Tawara et al., 2011) which was caused by US army.

1.3 Effects of pollutants on plant and human health

1.3.1 Effects on plant

1.3.1.1 *Principal inorganic pollutants*

Many metals such as Ca, Co, Cr, Fe, K, Mg, Mn, Na, Ni, and Zn are known as essential elements or micronutrients in small amounts and play important roles in the biological processes of organisms. Some other metals such as Ag, Al, Cd, Au, Pb, and Hg are non–essential and potentially toxic to organisms. At high concentrations, both essential and non–essential metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions, and damage the structure of DNA (Cukurluoglu and Muezzinoglu, 2013), inhibit transport processes and basic metabolism of plant (Ovečka and Takáč, 2014). Once metals are added to soil, they remain there for thousands of year. Sensitive plants exposed to elevated or toxic concentrations of heavy metal ions, usually exhibit considerably reduced growth, productivity and yields (Ovečka and Takáč, 2014). The toxicity of heavy metals at the cellular and molecular level may result from the binding to sulphydril groups of proteins, leading to an inhibition of activity or disruption of structure, to the displacing of essential elements (Hall, 2002) and it is related to their interaction with the thiol and carboxylate groups and also to their ionophoretic properties and abilities to generate free radicals (Rodríguez-Zavala et al., 2007). Among the effects due to heavy metal compounds, in this chapter the effects of As and Cd will be in particular considered as they are the two metal(loid)s studied in the first part of this PhD research.

Arsenic does not break down but it can change form. Although a variety of natural processes affect its fate and transport in soil and water, including chemical reactions (e.g., oxidation-reduction reactions), ligand exchange reactions, and biotransformations (metabolism by living organisms), inorganic arsenic has been shown to persist in soil over 45 years (USEPA, 2014a).

Toxicity of arsenic highly depends on its chemical speciation. It is well known that inorganic arsenic compounds are more toxic than organic compounds, and trivalent species are more toxic than pentavalent species, inorganic forms are more mobile than organic arsenic species (Eisler, 1988; Adriano et al., 2004). Methylated forms (methylarsonate, MMA and dimethylarsinate, DMA), trimethyl-arsine oxide (TMAO) and tetra- methyl-arsonium (TETRA) are considered only moderately toxic whereas

arsenobetaine (AsB), arsenocholine (AsC) and other arsenosugars (AsS) show no toxicity (Ventura-Lima et al., 2011).

The availability of soil arsenic is determined by soil properties, notably mineral composition, organic matter content, pH, redox potential and phosphate content (Huang et al., 2006). For instance soils with a high content of clay and organic matter favor soil adsorption and restrict As bioavailability (Fernandez et al., 2005). Clayey soils therefore generally have a higher As content compared to more sandy soils, and at the same total soil concentration, clayey soils are less toxic compared to sandy soils because As is more strongly bound (Heikens, 2006). Geogenic As-contaminated (gossans), and mine soils generally have much lower As relative bioavailability compared to soils contaminated through pesticide or insecticide applications (Smith et al., 2014).

As reported above, the solubility and speciation of As are influenced by several factors of which the most important ones are the redox potential and pH. Plants take up AsV and AsIII by different transport systems. As(V) is easily incorporated into plant cells through the high-affinity Pi transport system and cellular As(V) is usually rapidly reduced to As(III) in cells, either enzymatically or nonenzymatically (Verbruggen et al., 2009). Due to its structural similarity to phosphate, As(V) exerts its toxicity by uncoupling oxidative phosphorylation, thereby disrupting ATP synthesis, while As(III) binds to thiol groups, which can result in enzyme inhibition (Poirel et al., 2013). AsIII is absorbed through aquaporin channels since it is found in the environment predominantly as As(OH)3, a neutral compound. This molecule has a great affinity to sulfhydryl groups -SH, important component of enzymes and proteins, which can lead to cell dysfunction and death (Gusman et al., 2013).

The paradox is that, although As is toxic at high concentrations (Shaibur and Kawai, 2009), it is nutritionally essential or beneficial at low doses which stimulate growth and development in various species of plants (Eisler, 1988). If plants are exposed to an excess quantity of As either in soil or in hydroponic cultures, they can exhibit a multitude of symptoms such as (a) inhibition of seed germination and seedling growth, (b) decreases in shoot growth, plant height, chlorophyll content, and tillering, (c) reductions in leaf area and photosynthesis, and (d) lower yields of fruit and grain (Rahman et al., 2014). Visible symptoms of As-toxicity vary depending on the plant

species. For example, in Japanese mustard spinach, toxic symptoms and crop yields may not always be correlated with As concentrations in the medium (Shaibur and Kawai, 2009).

Figure 6: Physiological response of Japanese mustard spinach with different levels of As. Shoot height and root lengths were much affected by the elevated concentrations of As after 14 days of As exposure (Shaibur and Kawai, 2009).

Concerning the effects of As on plant growth, for instance, Duxbury et al (2007) reported that arsenate induced a decrease in maize biomass (Figure 7).

Figure 7: Effect of increasing soil As from 12 to 58 mg kg ¯1 (from left to right in picture) on maize biomass production (Duxbury et al, 2007)

Also the development of rice plants exposed to As added via As(V) in irrigation water was affected by increasing As concentrations leading to a reduction in biomass as well yield (Heikens, 2006).

Figure 8: Effect of soil As concentration (from 12 to 58 mg kg ˉ1, from left to right) on rice growth in buckets (Duxbury et al, 2007)

The amount and localization of As in the plant tissues differs depending on the external conditions and the plant species. For example, As levels in normally-edible parts of the vegetable increased in the approximate order as: peppermint < Indian squash < bottle gourd < cluster beans < spinach < bitter gourd < peas < sponge gourd < okra < brinjal (Baig & Kazi, 2012). Arsenic content in maize leaves was > than arsenic content in maize straw that was > than arsenic content in grain (Duxbury et al, 2007) and As concentrations in rice were ranked as follows: root > straw > husk > grain (Heikens, 2006). Jamali et al (2008) reported that leafy vegetables had high capability to accumulate high levels of trace metals and minerals from soil than other vegetables. Thus, localization of As varies greatly not only among plant species but also among different tissues of cultivars within the same species.

The distribution of arsenic among plants is also affected by the level of As in soil. Villatoro-Pulido et al (2009) reported that radish plants grown on “lower As soil” accumulated more As in the leaves than in the roots, whereas those grown on “higher As soil” had more As in the roots than in the leaves. In addition some plants may

increase As availability by releasing root exudates, including organic acids, which exert their action on for example oxides/hydroxides and ion exchange sites on the soil particles where As is adsorbed because organic acids have a major effect on the mobilization of elements in the rhizosphere (Bergqvist et al., 2014). For example, the hyperaccumulating ferns *Pteris vittata* and *Pteris biaurita* increased the plant-available As in the soil by releasing exudates from their roots (Gonzaga et al., 2009).

Anyway, the translocation of inorganic As from the roots to the above ground parts is usually limited. However, the fern *Pteris vittata* (brake fern) is extremely efficient in extracting arsenic from soils and translocating it into its above-ground biomass with large amounts of arsenic without toxic symptoms. According to Ma et al (2001), fern *Pteris vittata* (brake fern) can take up large amounts of arsenic into its fronds in a short time, arsenic concentration in fern fronds growing in soil spiked with 1500 mg kg^{-1} arsenic increased from 29.4 to 15861 mg kg^{-1} in two weeks. Furthermore, in the same period, ferns growing in soil containing just 6 mg kg^{-1} arsenic accumulated 755 mg kg^{-1} of arsenic in their fronds, a 126-fold enrichment. Brake fern is considered as an arsenic hyperaccumulator which not only has the potential for phytoremediation of arsenic contaminated soil, but also provides an excellent opportunity to investigate plant detoxification mechanisms for arsenic (Zhang et al., 2002). Arsenic hyperaccumulation seems to be confined to the Pteridaceae family of ferns (Verbruggen et al., 2009).

Apart from As, soil characteristics such as soil pH, salinity, humus content significantly influence crop uptake, uptake rate and bioavailability also of cadmium. Cadmium mobility and bioavailability are higher in more acidic soils, and lower in chalky/lime soils (EC, 2014). However, cadmium may be adsorbed on clay minerals, carbonates or hydrous oxides of iron and manganese or may be precipitated as cadmium carbonate, hydroxide, and phosphate. Under acidic conditions, cadmium solubility increases, and very little adsorption of cadmium by soil colloids, hydrous oxides, and organic matter takes place (EC, 2014). Because of its high mobility and water solubility, Cd can readily enter the roots through the cortical tissue and can reach the xylem via an apoplastic and/or a symplastic pathway to form complexes with organic acids or phytochelatins (Salt et al., 1995).

Higher plants can uptake Cd, depending on its availability and concentration, from soil and water; rather little is also taken up directly from the atmosphere (Clemens, 2006). The uptake and transport of Cd in plants varies with species and with cultivars within species (Grant et al., 1998). An (2004) reported that in 4 test species (sweet corn, *Zea may*; wheat, *Triticum aestivum*; cucumber, *Cucumis sativus*; and sorghum, *Sorghum Bicolor*), cucumber retained the greatest amount of cadmium in the roots while the roots of sorghum transported more of their absorbed Cd to the shoots than the other plant species and accumulated the most Cd; Cd was accumulated mainly in the roots and that only small amounts of Cd were transported to the shoot.

Cd uptake in soil by plant roots also depends on the root morphology. Plants with numerous thin roots accumulate more metals than one with few thick roots (Das et al., 1997), and the greater surface area of thin and long roots compared to thick and short roots contribute to more absorption of Cd in plant roots (An, 2004).

Varieties and the presence of elements in soil also affect plant Cd absorbtion. Seth et al (2007) reported that Giant Duckweed (*Spirodela polyrrhiza* L.) accumulated about 1.5-fold more Cd accumulation than As at the same concentration and exposure periods and low accumulation of the metal was recorded in combination when compared with separate exposures.

Plant uptake of Cd at levels present in the soil solution is dependent on a system that is largely metabolically mediated and competitive with the uptake system for Zn and possibly other metals (Grant et al., 1998).

Thus Cd toxicity to plants is affected by the presence of other elements in soil.

Pollutants in soil and water can induce additive, antagonistic or synergistic effects on plant and animal growth (Liu et al., 2007a; Huang et al., 2009a; Huang et al., 2009b). Analyses of chemical mixtures indicate that the toxicity may be equal to the sum of the fractional toxicities of individual components, or higher/lower than the sum due to synergistic/antagonistic interactions. For example, according to Liu and Zhang (2007), a combined exposure to Cd and As produced greater toxicity to wheat than single exposure to each metal separately. However, Sun et al (2008) reported that a combined toxicity of arsenic and cadmium is less severe than that of single As or Cd in terms of rice growth (because Arsenic can mitigate Cd-induced inhibitory effect on plant growth). If the rate of absorption of metals by a plant exceeded the rate of arrival by

convection, a depletion zone was created at the root and the concentration gradient promoted diffusion from the soil to the root (Das et al., 1997). However, excess Cd causes a number of visible toxic symptoms in plants such as growth inhibition, rolled and chlorotic leaves and other toxic symptoms such as inhibition of photosynthesis, induction and inhibition of enzymes, altered stomatal action, water relations, efflux of cations and generation of free radicals (Clemens, 2006).

Some plants can accumulate relatively high levels of cadmium, without adverse effects on growth. They belong to the class of hyperaccumulators. For example, some Cd-hyperaccumulating populations of *T. caerulescens, T. praecox, and Arabidopsis halleri*, all belonging to the Brassicaceae family, and *Sedum alfredii* (Crassulaceae) require Cd for optimum growth (Verbruggen et al., 2009). Plants have evolved several mechanisms to cope with Cd^{2+} toxicity and attempt to adapt themselves to environments contaminated with excess Cd. Some of the prevalent mechanisms of Cd-tolerance are: accumulation, sequestration, their stabilization by sulphide ions, damage rescue by heatshock proteins and phytochelatin constituting organics (Prasad, 1995). A major strategy to detoxify nonessential trace metal(-loid)s is the synthesis of specific low-molecular-weight chelators to avoid binding to physiologically important proteins and to facilitate their transport into the vacuoles (Verbruggen et al., 2009). Plants could develop a detoxification mechanism which eliminates metals from them by translocation of metals from roots to the brown leaves and leaf fall (Dahmani-Muller et al., 2000). Restricted distribution of the metal in sensitive tissues, metal binding to the cell wall are also some of these mechanisms (Gallego et al., 2012). In plants, metallothionines are important components for maintaining homeostasis of essential metals and detoxification of toxic elements like cadmium (Cd) and As (Robinson et al., 1993). To prevent Cd accumulation in shoot tissues, plants can restrict the entry of Cd to the xylem by restricting Cd movement to the xylem through both the symplasmic and the apoplasmic pathways. (Lux et al, 2010). The uptake of Cd from the soil seems to occur mainly via Ca^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} transporters (Verbruggen et al., 2009). If zinc is present, it can reduce cadmium's availability to plants, by inhibiting calcium uptake and preventing it from moving from the roots to the shoots of the plants (EC, 2014).

It was reported that heavy metal pollution induces reactive oxygen species generation in cells that damage plants major cell macromolecules proteins, lipids, and DNA (Gichner et al., 2004). Generally, plants possess several antioxidative defense systems such as NP-SH, cysteine, glutathione (GSH), tocopherols, ascorbic acid, and enzymes like catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPX), glutathione reductase (GR), and ascorbate peroxidase (APX) to scavenge toxic reactive oxygen species to protect them from the oxidant stress (Seth et al., 2007).

Metal hyperaccumulation by plants has an adaptive function, mostly defense against insect herbivory (Boyd, 1998). For example, the retention of high concentrations of arsenic in sori is probably a defensive strategy against pests to sustain the life cycle (Bondada et al., 2004). Hyperaccumulation of heavy metals by higher plants from the soil to the shoots is a complex phenomenon which involves several steps such as: (a) bioactivation of metals in the rhizosphere through root–microbe interaction; (b) enhanced uptake by metal transporters in the plasma membranes; (c) detoxification of metals by distributing to the apoplasts like binding to cell walls and chelation of metals in the cytoplasm with various ligands, such as phytochelatins, metallothioneins, metal-binding proteins; (d) sequestration of metals into the vacuole by tonoplast-located transporters (Yang et al., 2005) shown in Figure 9.

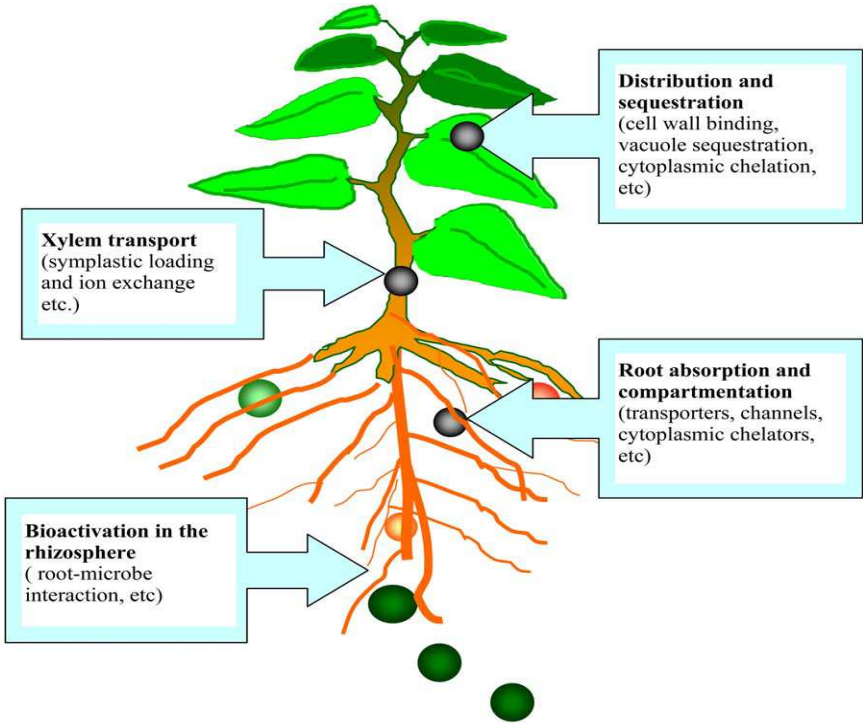

Figure 9: Major processes proposed to be involved in heavy metal hyperaccumulation by plants (Yang et al., 2005)

1.3.1.2 *Principal organic pollutants*

In addition to heavy metal(loid)s, organic compounds have been known to be toxic and genotoxic to plant and their accumulation depends on species and, environmental conditions. Highly persistent compounds, such as PBDE- and PFC-compounds may accumulate in agricultural soil after repeated use of organic fertilizers containing these compounds, and accumulation potential of PBDEs has been demonstrated for example for zucchini, radish, alfalfa, summer squash, pumpkin, maize, and ryegrass (Suominen et al., 2014). Most plants do not bioaccumulate PCBs from contaminated soil due to the presence of a waxy layer, or cuticle, which binds the PCBs and prevents them from being absorbed into the plant (USEPA, 2014c). Many plant species for example, lettuce, potato, tomato, rice, garland chrysanthemum, Chinese cabbage, maize, and soybean, can absorb organic compounds through their roots, but usually very small amounts of these substances are translocated from roots to shoots. However, some species of cucumber family, Cucurbitaceae, are known to accumulate higher levels of POP such as polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF) and PCB in their tissues especially in leaves and fruits, compared with other plant species (Wyrwicka et al., 2014). As a result, they may show secondary oxidative stress in plant cells and may reduce plant growth and development (Wyrwicka et al., 2014).

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) and naphthalene (Naph) are among the most dangerous environmental contaminants due to their toxic, carcinogenic and mutagenic effects. It was reported that they were genotoxic for *Trifolium repens* L, inducing significant changes in root and shoot DNA sequence (Aina et al., 2006). Dihydrophenanthrene has been also proven to inhibit germination of seed, and induced DNA changes in different target sequences of *Arabidopsis thaliana* (L.) Heynh (Labra et al., 2003).

1.3.2 Effects on human health

1.3.2.1 Principal inorganic pollutants

Unlike organic contaminants, heavy metals and metalloids are generally non biodegradable, immutable and persistent in nature. Nevertheless, they can become mobile in soils, sediments and in water to the extent that a fraction of their total mass

can become bioavailable to organisms including plants, animals and humans (Adriano et al., 2004).

Many heavy metals and metalloids are toxic and can cause undesirable effects and severe problems even at very low concentrations (Ali et al., 2013).

Some heavy metals may transform into the persistent metallic compounds with high toxicity, which can be bioaccumulated in the organisms, magnified in the food chain, thus threatening human health. For example, after Hg is deposited in soils and sediments, bacteria and microbes are mainly responsible for changing mercury to methylmercury. Once methylmercury is formed, it cycles in the environment for thousands of years, exposing humans and other species to potentially toxic levels for generations (EC, 2014). Significant examples of these are the outbreaks of severe mercury poisoning in Minamata, Japan, and Iraq in the last centaury had posed the shocked disaster to eco-environment system and human beings (Li et al., 2009). In Minamata Bay, mercury-contaminated effluent was discharged from an acetaldehyde-producing factory over a 30-year period ending in 1965 (from 1958 to 1959, this effluent was discharged into the Minamata River). The total amount of mercury discharged from the chemical plant was reported to be 70–150 t or more, which contained significant levels of methylmercury generated as by-product in the acetaldehyde process (Tomiyasu et al., 2014). Methylmercury is readily accumulated by aquatic biota. Over 90% of the mercury found in fish is methylmercury (EC, 2014). For many years, no one realised that the fish were contaminated with mercury, and that it was causing a strange disease to people who ate the fish in the local community and in other districts. At least 50000 people were affected to some extent and more than 2000 cases of Minamata disease were certified (WHO, 2014c). In the early 1970's mercury's use in agriculture has led to distressing human health incidents. A major methylmercury poisoning catastrophe occurred by consumption of seed grain treated with a fungicide containing mercury, which an estimated 10,000 people died and 100,000 were severely and permanently brain damaged (Li et al., 2009).

Toxic metal ions that enter plant roots and aboveground different organs can pose a potential threat to human health. Metal accumulation in edible parts of crop plants represents the principal route of toxic metal entry into the human food-chain (Clemens, 2006). Cadmium belongs to the metals whose ions are most readily taken

up by plant roots and therefore is the element of greatest concern in food chain (Giller and McGrath, 1988). Much of the Cd taken up by plants is retained in the root, but a portion is translocated to the aerial portions of the plant and into the seed. Some crops such as durum wheat, flax, sunflowers and potatoes can accumulate amounts of Cd which exceed current and proposed maximum acceptable Cd concentrations (Grant et al., 1998). Cadmium and cadmium compounds have been classified as carcinogenic to humans (Group 1) by The International Agency for Research on Cancer (IARC, 2014a), meaning that there is sufficient evidence for their carcinogenicity in humans. Around 90% of cadmium exposure in non-smokers is through food (EC, 2014). Cd is retained for many years in the human body with biological half-life 10–35 years, accumulates primarily in the kidneys (WHO, 2014a), so consumption of foods high in Cd can induce chronic toxicity (Candéiasa et al., 2010). One example for this is Itai–itai disease in Japanese in the 1950s and 1960s, which developed in numerous inhabitants of the Jinzu River basin in Toyama Prefecture, was the most severe form of chronic cadmium (Cd) poisoning caused by prolonged oral Cd ingestion resulting in osteomalacia and bone fractures. Its cause has been clarified to be environmental Cd pollution originating from effluent from zinc mine located in the upper reaches of the river. Inhabitants used water polluted with Cd to supply to their food crops, mainly rice, soybean, and then ate them accumulated with Cd (Inaba et al., 2005). Many studies using cultured animal cells show that exposure to cadmium compounds damages genetic material. DNA strand breaks, mutations, chromosomal damage and cell transformation have been observed *in vitro*. Cadmium compounds inhibit the repair of DNA damaged by other agents, thereby enhancing their genotoxicity (IARC, 2014a).

In addition to cadmium, arsenic is one of the most studied soil pollutants because of its ubiquity, toxicity, and persistence. Arsenic can also enter food chain causing wide spread distribution throughout the plant and animal kingdoms. The concentration of arsenic in cereals, vegetables and fruits is directly related to the level of arsenic in contaminated soil (Zhang et al., 2002). Fish, fruits, and vegetables primarily contain organic arsenic which has low toxicity, less than 10% of the arsenic in these foods exists in the inorganic form, although the arsenic content of many foods (i.e. milk and dairy products, beef and pork, poultry, and cereals) is mainly inorganic, typically 65–

75% (Mandal and Suzuki, 2002). High As levels in farming zones add substantial amounts of As to the diet intake through agricultural product consumption, thus posing risk to human health (Rosas-Castor et al., 2014). Crops for human consumption and animal fodder cultivated on soils and/or irrigated with As-enriched water have shown corresponding high As contents and expose humans to severe health risks associated with high As concentrations in many areas in the world (Bundschuh et al., 2012). Rice is the staple food of many countries in Asia. However, arsenic contamination of rice by irrigation with contaminated groundwater and secondarily increased soil arsenic compounds the arsenic burden of populations dependent on subsistence rice-diets (Sengupta et al., 2006). High As accumulation capacity of rice poses immense health hazards to almost 50% of world population who are dependent on rice as their staple food (Nath et al., 2014). Arsenic and inorganic arsenic compounds are classified as carcinogens to humans (Group 1) by the International Agency for Research on Cancer. This means that there is sufficient evidence in humans for the carcinogenicity of mixed exposure to inorganic arsenic compounds, including arsenic trioxide, arsenite, and arsenate (IARC, 2014b). Arsenic can pass through the placenta, so pregnant women exposed to arsenic through drinking water are at greater risk of miscarriage, stillbirth and pre-term birth (EC, 2014). There is evidence that exposure to arsenic in the womb or in early life increases the risk of lung cancer and other lung disorders (EC, 2014). In general, the ingestion of As by humans can cause a variety of disorders, including skin lesions (e.g., hyperpigmentation, melanosis, keratosis), respiratory system problems (e.g., chronic cough, shortness of breath, bronchitis), nervous system effects (e.g., neuropathy, neurobehavioral, weakened memory, lower IQ, decreased attention), cancers of different organs (e.g., skin, lung, bladder), and reproductive effects (e.g., pregnancy complications, fetus abnormalities, premature deliveries, reduced birth weight) (Kapaj et al., 2006).

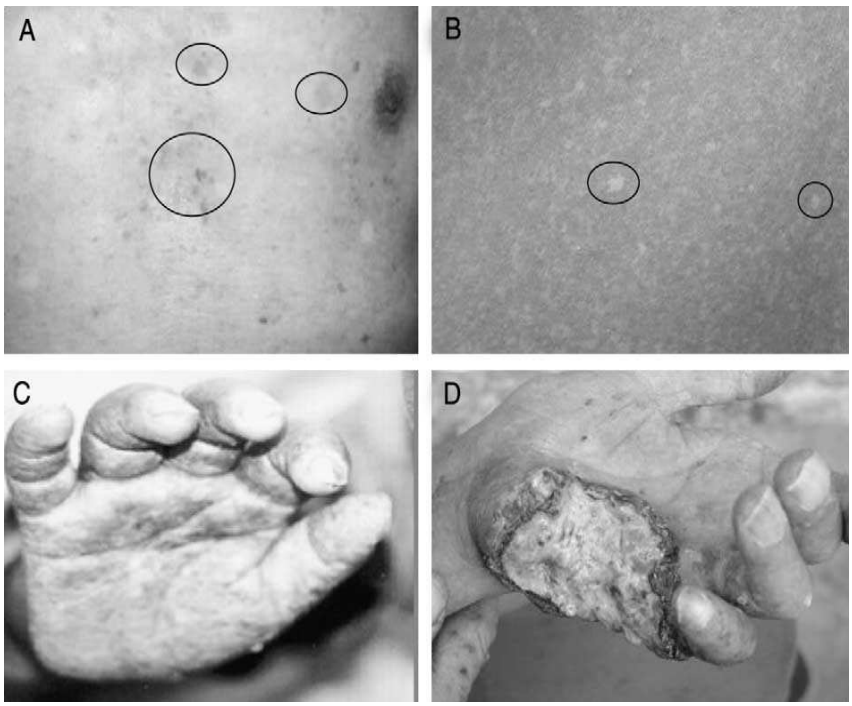

Figure 10: Typical skin lesions and skin cancer found in patients who have been chronically exposed to arsenic: (A) hyperpigmentation, (B) hypopigmentation, (C) keratosis, and (D) skin cancer (Ng et al., 2003).

Levels of lead in the blood began to decline earlier in the Western European and Scandinavian countries than in Eastern Europe, largely because unleaded petrol was gradually introduced earlier in these countries (EC, 2014). However, significant sources of exposure still remain, particularly in developing countries (WHO, 2014e). Fruits and vegetables grown in lead contaminated soil may become contaminated as a result of plant uptake of this metal from soils or direct deposition of leaded dust onto plant surfaces. The occurrence of lead in the edible portion of the plant is of specific interest from a health point of view, since ingestion of the plant may contribute to elevated body burdens of lead (Finster et al., 2004). The International Agency for Research on Cancer (IARC) has classified inorganic lead compounds as probably carcinogenic to humans. It has been estimated that lead exposure was responsible, in 2004, for 143 000 deaths and 0.6% of the global burden of disease (expressed in disability-adjusted life years), taking into account mild mental retardation and cardiovascular outcomes resulting from exposure to lead (WHO, 2014d). Childhood lead exposure is estimated to contribute to about 600,000 new cases of children with intellectual disabilities every year (WHO, 2014e).

Cr toxicity is observed at multiple levels in plants and animals, from reduced yield, through effects on leaf and root growth, to inhibition on enzymatic activities and mutagenesis (Shanker and Venkateswarlu, 2011). Cr(VI) has long been recognized as a carcinogen in human and mammalian systems, and Cr(VI)-containing compounds are genotoxic and can induce gene mutations and DNA lesions (Shanker and Venkateswarlu, 2011). Since chromium is a known mutagen and carcinogen, the prevalent pollution laws in most countries require its complete removal from industrial effluents before discharge (Itankar and Patil, 2014). Cr(VI) inhibits DNA, RNA, and protein syntheses in biological systems (Labra et al., 2003). In addition, Cr is known to induce apoptosis, a process by which cell death is initiated and completed in an orderly manner through activation or synthesis of gene products necessary for cell destruction (Shanker and Venkateswarlu, 2011).

1.3.2.2 Principal organic pollutants

Polycyclic aromatic hydrocarbons (PAHs) are known relatively chemically inert compounds. PAHs have severe mutagenic and carcinogenic effects by binding to DNA (adduct formation) at specific sites known as xenobiotic response elements (XREs), promoting DNA instability and potentially giving rise to strand breakage (Costa et al., 2008). PAHs are capable of covalent interaction with nucleophilic centres of DNA through metabolic activation to electrophilic derivatives (e.g. diolepoxides, quinones, conjugated hydroxyalkyl derivatives). These adducts of PAH to DNA cause base pair substitutions, frameshift mutations, deletions, S-phase arrest, strand breakage and a variety of chromosomal alterations (Piraino et al., 2006). Experimental results on mice showed that when pregnant mice ate high doses of a PAH (benzo(a)pyrene), they had reproductive problems such as birth defects, a decrease in their offspring body weight and other effects including damage to the skin, body fluids, and the immune system (USEPA, 2014b).

Dioxins are highly toxic and can cause reproductive and developmental problems, damage the immune system, interfere with hormones and cause cancer (WHO, 2014b). Once dioxins have entered the body, they remain for a long time because of their chemical stability and their ability to be stored in body fat with their half-life in the body estimated to be 7 to 11 years (WHO, 2014b).

Figure 11: President Viktor Yushchenko of Ukraine before and after dioxin poisoning with 2,3,7,8-TCDD (Schecter et al., 2006)

The Vietnam War ended over 35 years ago, but herbicide residues which contain dioxins are still producing adverse effects on Vietnamese people who lived in the sprayed areas and on the country's ecosystems (Nhu et al., 2009). At least 2.1 million and possibly as many as 4.8 million Vietnamese people would have been exposed to these contaminants (Stellman et al., 2003; Tawara et al., 2011)

1.4 Assessment of soil pollution: chemical and biological approaches

The detection of dangerous compounds in the environment is the first step in the evaluation of the exposure risk for human, which is a very complex condition (risk identification). Although the chemical methodology is the most common and direct approach in determining the xenobiotic presence in the environment, it is important to underline that the dangerousness is related not only to the xenobiotic amount but also to the exposure time (dose) and to its bioavailability, which is the amount of free and biologically active compound available for target structures. In fact the ability of classical chemical analyses to define a pollution level depends on the identification and quantification of the single xenobiotics. Nowadays, that strategy is very limited and is not able to assess the risk. In fact, many compounds inducing diseases such as cancer are still unknown or are active at very low concentration not detectable by analytical instruments. In addition chemical analysis does not allow an integration of

the combined effects produced by the chemical mixture present at a polluted site and total concentrations can overestimate the real risk, as aging processes can strongly reduce the bioavailability and, subsequently, the toxicity of pollutants (Fernandez et al., 2005). For this reason the integration of chemical data with further biological analyses become necessary for a realistic risk assessment for environment and humans. In biological field, bioindication techniques were developed and applied. Exploiting animals, plants and microrganisms sensitive to chemicals, biological analyses enable the qualitative and quantitative determination of the xenobiotic effect on the ecosystems. The actual challenge in biomonitoring consists in the ability to determine the xenobiotic effect at sublethal level, to prevent further environmental and health damage and to allow the application of remediation systems.

Bioindicators are organisms sensitive to contaminants. They are used to determine the presence of environmental contaminants by assessing the effects of pollutant/s on them. For instance white clover is widely used as test plant to assess the presence of genotoxic compounds in both soil and air compartments (Aina et al. 2002; Piraino et al. 2006). In Table 1, examples of organisms used as bioindicators are reported.

Table 1: Examples of organisms used as bioindicators

ORGANISM		BIBLIOGRAPHY
Bacteria	*Bacillus*	Leifer et al., 1981
	Escherichia coli	Brusick et al., 1980
	Salmonella	Ames et al., 1973
Yeast	*S. cerevisiae*	Zimmmerman, 1984; Resnick et al., 1986
Fungi	*Neurospora*	Brockman et al., 1984; Bahnoori and Venkateswerlu, 1998
Algae	*Phormidium*	Wang et al., 1998
	Selenastrum	U.S. EPA, 1978
Piants	*Allium*	Levan, 1949; Sharma, 1995; Fiskesjo, 1995; Rank and Nielsen, 1998, Kipopolou et al., 1999
	Arabidopsis	Conte et al., 1998
	Capsella bursa-pastoris	Aksoy et al. 1999
	Hordeum vulgare	Zhang et al., 1994
	Nerium oleander	Aksov and Ozturk, 1997
	Pisum sativum	Grant and Owens, 2001
	Plantago major	Bakker et al., 2000
	Taraxacum officinalis	Malaska and Wilkormirski, 2000
	Tradescantia	Knasmuller et al., 1998; Fomin et al., 1998
	Trifolium pratense	Micieta and Murin, 1995
	Vicia faba	Kihlman, 1975 ; Grant, 1982a; Kanaya et al. 1994; Koppen, et al. 1996

After exposure to environment, bioindicators are analyzed by considering appropriate markers and specific technologies. Table 2 shows examples of markers.

Table 2: Examples of markers.

CONTAMINANT	MARKER
Heavy metals (Cd, Cr, Cu, Ni, Zn, Hg and Ag)	DNA change
	Metallothionein
	Phytochelatin
	Dry weight
	Enzymes and anti oxidant molecules (i.e. catalase reductase ascorbic acid)
PAH	DNA change
	P450
	Dry weight

Specifically to study DNA changes, Comet test and Random Amplified Polymorphic DNA (RAPD) are two of the principally applied techniques.

The Comet assay method

The comet assay, or single-cell gel electrophoresis (SCGE) has become one of the common methods for assessing DNA damage, with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as fundamental research in DNA damage and repair over the past decade (Collins, 2004). In 1984, Ostling and Johanson introduced the use of a microgelectrophoresis technique to detect increased levels of DNA damage and its repair in individual cells exposed to a genotoxic agent. In 1988, Singh and collaborators introduced an alkaline (pH .13) version of the Comet assay, which greatly expanded possible applications by allowing for the detection in single cells of direct single strand beaks, single strand breaks associated with incomplete DNA repair sites, and alkali-labile DNA damage (Tice, 2010).

In general, the comet assay has many advantages such as relative simplicity, sensitivity, versatility, rapidity and economy. However, although relatively simple to perform, there is not a adequately validated standardized version of the Comet assay and several issues that impact on data interpretation (e.g., optimal cell sampling and

electrophoretic conditions, cell scoring criteria, the impact of cytotoxicity on increased DNA migration, many types of DNA damage are not detected) remain to be resolved.

RAPD: a biomolecular technique to analyse DNA sequence

Biomolecular techniques have been widely applied in biological research for a variety of purposes for many last decades. Since the application of plants in many cases was limited because of their complexity of genome, length, ploidy and difficulties in isolating easily scorable phenotypes, the development of molecular marker technology including RADP has provided new tools for the detection of genetic alteration by looking directly at the level of DNA sequence and structure (Conte et al., 1998).

Among of biomolecular techniques, the RAPD method has been initially used to detect polymorphism in genetic mapping, taxonomy and phylogenetic studies and later in genotoxicity and carcinogenesis studies (Atienzar et al., 1999; Atienzar et al., 2006). In recent years, the RAPD-PCR technique (Random amplified polymorphic DNA-based on polymerase chain reaction) has been used and considered one of the most powerful and useful tools in the assessment of genotoxic effects of organic and inorganic agents on different organisms (Aina et al., 2008; Cansaran-Duman et al., 2011; Cenkci et al., 2009; Liu et al., 2009; Piraino et al., 2006; Salem et al., 2014; Vardar et al, 2014; Wolf et al., 2004). It can be considered extremely efficient for DNA analysis in complex genomes as it could be relatively inexpensive and yields information on a large number of loci (Atienzar et al., 1999; Gupta and Sarin, 2009; Wolf et al., 2004).

The principle of this technique is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels (1.5-2.0%) and visualised by ethidium bromide staining (Kumar and Gurusubramanian, 2011). The comparison of RAPD profiles (the amplified products on gel) obtained

from plants exposed to environment with those obtained from control plants allows the detection of DNA changes induced by genotoxic substances eventually present in the environment. Changes in DNA sequence are related to mutation in primer annealing sequence and in the sequence region between the forward and reverse primer annealing sequences. Profile changes include the appearance of extra amplified bands, the disappearance of amplified bands, and the changes in amplified band fluorescence. New PCR amplification products may reveal a change in the DNA sequence due to mutations (resulting in [a] new annealing event [s]) and/or large deletions (bringing two preexisting annealing sites closer) and/or homologous recombination (juxtaposing two sequences that match the sequence of the primer) shown in Fig 12 and 13.

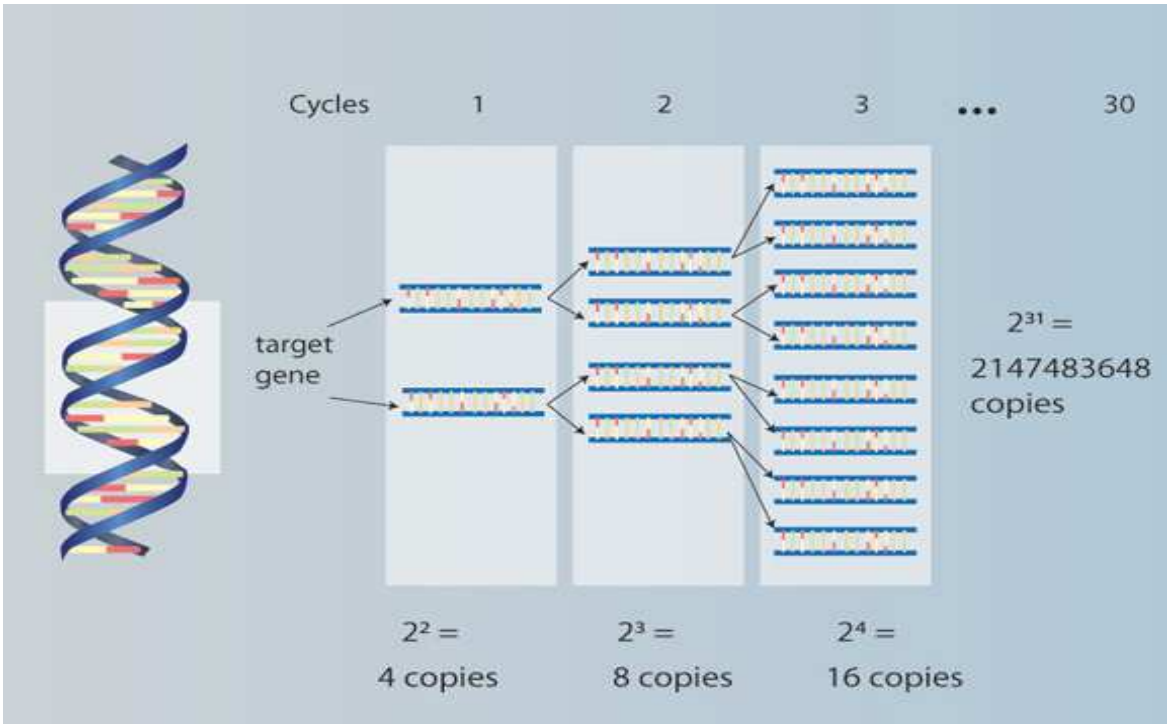

Figure 12: Polymerase chain reaction (PCR) (Allan and Max, 2010): The PCR method begins with total genomic DNA extracted from an organism. The DNA is combined with site-specific primers, Taq polymerase, and other reagents (e.g., MgCl2, buffer, dNTPs) and subjected to repeated cycles, each of which consists of a denaturation phase, annealing phase and extension phase. Denaturation separates double stranded DNA, allowing primers to anneal to specific sites, followed by incorporation of deoxynucleotide triphosphates (dNTPs; A, C, G, T), thereby extending the target site in the 5'-3' direction (on both separated strands). The first cycle is completed when one round of denaturation, annealing and extension is finished, resulting in two new copies of the target site. Subsequent cycles (typically 30-35) repeat the 3-phase process, resulting in many million-fold copies of amplified DNA.

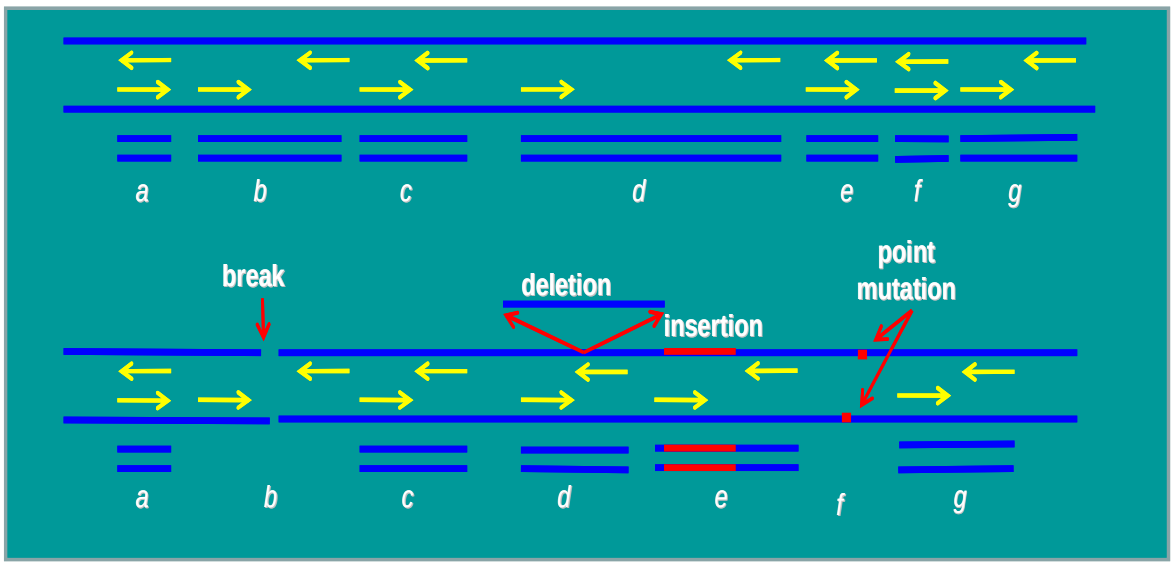

Figure 13: RAPD profiles show changes in the DNA sequence due to break, deletions or insertions, mutations.

2. Aim of the study

The use of efficient early warning bioindication systems represents a powerful approach for assessing and interpreting the impact of natural or anthropogenic perturbations in soil ecosystems preventing environmental alteration and human disease. Living organisms provide information on the cumulative effects of environmental stressors and as such bioindication is complementary to direct physical and chemical measurements (Heger et al., 2012).

Trifolium repens is a pollutant-sensitive plant, suitable for biomonitoring campaigns. Specifically, its environmental exposure followed by a DNA analysis with molecular markers allows the detection of sublethal levels of genotoxic compounds in the environment (Piraino et al., 2006). However, given the limited information available on the joint genotoxic effect of chemicals, the interpretation of biomonitoring results is often difficult. In addition, most environmental risk assessments of contaminated lands are currently based on guideline values derived from the ecotoxicological properties of specific chemicals, whereas it is well known that environmental pollutants interact producing additive, antagonistic or synergistic effects on exposed organisms (Zhou et al., 2006; Liu and Zhang, 2007a; Wang and Fowler, 2008; Huang et al., 2009; Tkalec et al., 2014); it is then evident that there is a clear need to improve the knowledge about the combined effects of stressors on bioindicators.

As mentioned in the introduction, Cd and As are two of the most dangerous pollutants for both environment and human health, as they induce genome alteration to living organisms. However most information regarding Cd and As genotoxicity comes from studies on one or the other of these heavy metals, whereas no data are available on their genotoxic joint action. Starting from these considerations, the objective of the first part of my PhD research was to study the combined toxic and genotoxic effects of soil Cd and As. The study was organized in three successive steps:

(1) assessment of the general toxicity and genotoxicity of soils contaminated with increasing arsenic concentrations;

(2) assessment of the general toxicity and genotoxicity of soils contaminated with increasing cadmium concentrations;

and (3) assessment of the toxicity and genotoxicity of soils simultaneously contaminated with arsenic and cadmium concentrations which were selected on the basis of the results from (1) and (2).

In the second part of my research I used the information and the techniques that I learned during the first period to assess the genotoxicity of soils in Lombardy Region (Italy) performing a biomonitoring experiment in collaboration with Catholic University of Piacenza and European Research Centre of Ispra.

3. Materials and methods

3.1 Experiments with As and Cd contaminated soils -Part 1

3.1.1 Plants used for the experiments

White clover (*Trifolium repens)* cultivar *Ladino* (Ingegnoli Milano) was selected as the test plant. Based on literature data *Trifolium repens* is highly sensitive to organic and inorganic compounds; it also shows a genetic uniformity and is easy to handle and grow (Citterio et al., 2002; Piraino et al., 2006; Aina et al., 2008).

3.1.2 Soil used for control treatments of the experiments

Commercial soil used for control treatments was provided by Compo Company and has the following characteristics:

- pH: 6.2
- Organic matter: 48.5 %
- Components: neutral sphagnum peat, perlite (< 5%), mineral fertilizer
- Total porosity: 91 % v/v
- Density: 135 kg/m^3

Before use, the soil was sifted through a 3 mm mesh sieve.

3.1.3 Sand

Sand used for the preparation of experimental soil was: Ticino Sand (VAGA); granulometry 0,1 mm-0,9 mm

3.1.4 Chemicals

- Agarose, Cadmium Sulfate ($3CdSO_4.8H_2O$) and Sodium Arsenite ($NaAsO_2$) produced by Sigma-Aldrich, USA.
- Trizma base, minimum 99.9% titration (Sigma-Aldrich, USA).
- Boric acid, minimum 99.8% BH_3O_3 (AppliChem, Germany).
- EDTA disodium salt dihydrate, 99% $C_{10}H_{14}N_2Na_2O_8.2H_2O$ (AppliChem, Germany).
- Other chemicals used in this study were bought from QIAGEN.

3.1.5 Soil contamination and plant exposure (As, Cd)

Trifolium repens L. seeds were surface sterilized with 15% bleach solution, for 10 min and then washed with tap water for at least 10 times. After sterilized and washed, seeds were directly sown in 3% organic matter soil for 4 weeks.

The nearly 10-cm high plantlets were transferred to separate pots containing either control soil (uncontaminated soil) and artificially contaminated soil, obtained by

adding different concentrations of metals, for 2 weeks. Cadmium sulfate ($3CdSO_4.8H_2O$) and sodium arsenite ($NaAsO_2$) were dissolved in distilled water to make stock solutions for soil contamination. They were then opportunely diluted and accurately mixed with soil to obtain homogeneous contaminations. The Cd and As concentrations ($mg.kg^{-1}$soil) that were used in this experiment are listed in Table 3.

Table 3: As and Cd concentrations in single and combined treatments

Single Treatments			
As ($mg.kg^{-1}$)	5	10	20
Cd ($mg.kg^{-1}$)	20	40	60
Combined Treatments			
As+Cd ($mg.kg^{-1}$)	5+20	5+40	5+60
As+Cd ($mg.kg^{-1}$)	10+20	10+40	10+60
As+Cd ($mg.kg^{-1}$)	20+20	20+40	20+60

Cd and As concentrations were selected through preliminary experiments (data not shown here) in which the effects of many different concentrations of the two contaminants were tested on plant growth. Each treatment consisted of 3 pots (or 3 repetitions) each containing 18 plantlets for a total of 54 plantlets. Experimental design is shown in the Figure 14A and 14B.

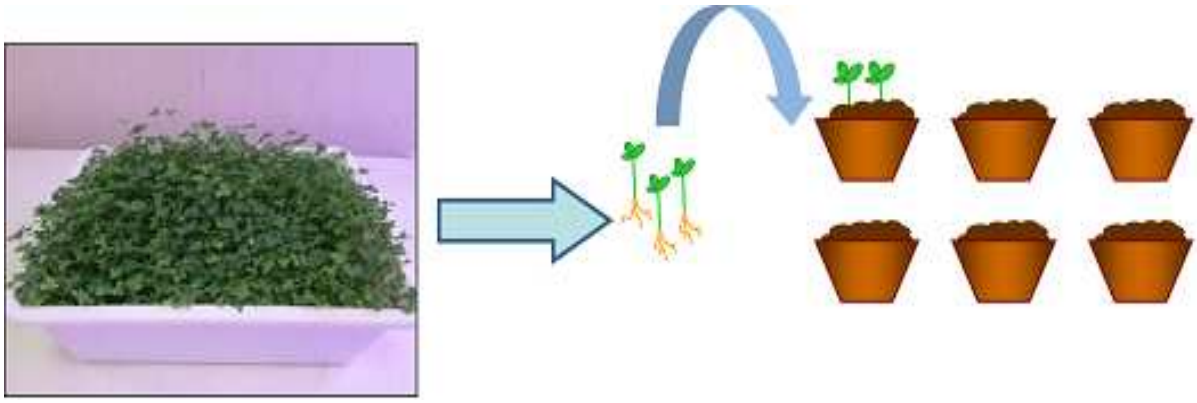

Figure 14A: *Trifolium repens grew* in 3% organic matter soil for 4 weeks, and then they were transferred to the control soil (artificially uncontaminated) and contaminated soils.

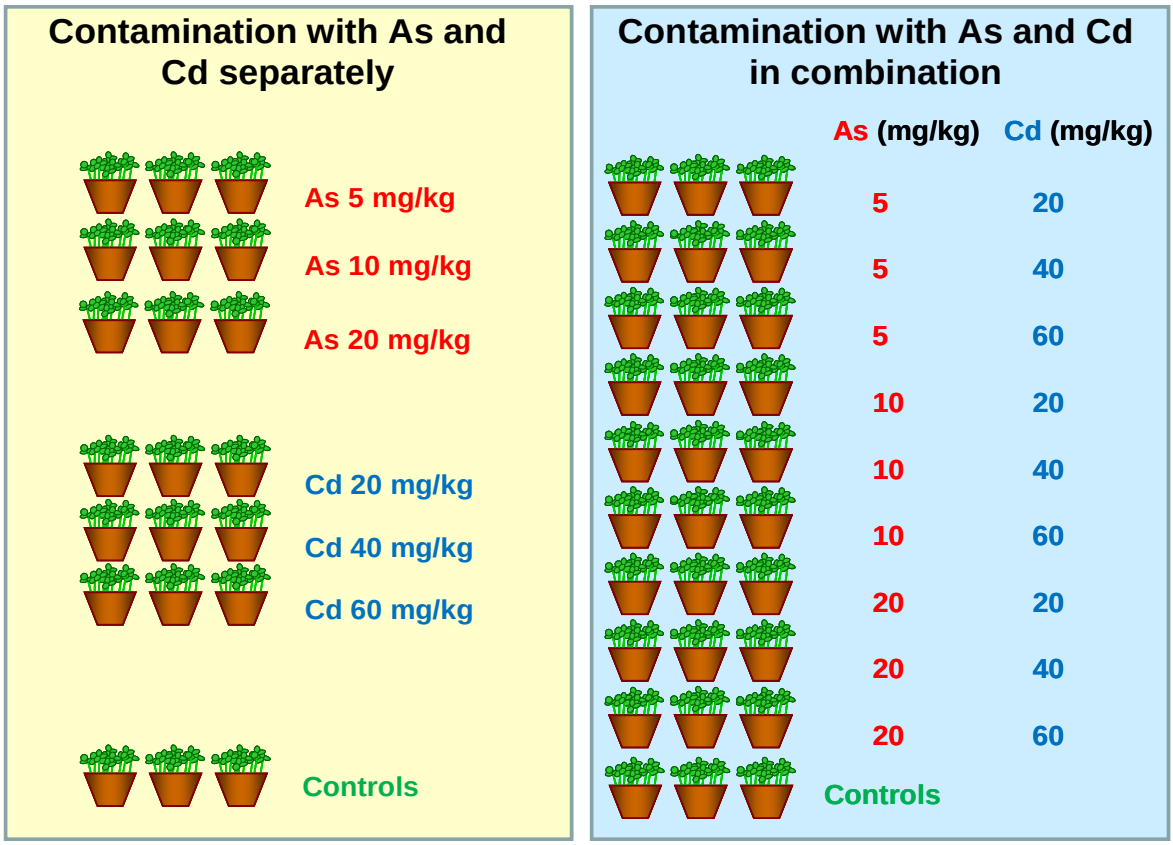

Figure 14B: Experimental design shows *Trifolium repens* were transferred to soil contaminated with As and Cd seperately (on the left); soil contaminated with As and Cd in combination (on the right) and the control (soil not artificially contaminated)

3.1.6 Mortality and dry weight

The survival of plants was assessed during the exposure; at the end the percentage of dead plants was calculated along with the determination of dry weight of plantlets survived.

3.1.7 DNA extraction and quantification

Samples of roots and shoots were ground by mortars and pestles. DNA extraction was performed according to the protocol, DNaesy Plant Handbook (Qiagen). Steps of DNA extraction can be summarized as following:

- Add 400µl Buffer AP1 to 100 g of root (leaf) material which is ground, and then add 4 µlRNase A stock solution (100mg/ml) and vortex vigorusly.
- Incubate the mixture for 10 min at 65^{o}C. Mix 2 or 3 times during incubation by inverting tube.
- Add 130µl Buffer AP2 to the mixture above, mix, and incubate for 5 min on ice.
- Centrifuge the mixture for 5 min at 14000 rpm.

- Pipet liquid in the mixture into the QIAshredder Mini spin column (lilac) placed in a 2ml collection tube, and centrifuge for 2 min at 14000 rpm.
- Transfer the flow-through fraction from the step above into a new tube (not supplied) without disturbing the cell-debris pellet.
- Add 1.5 volumes of Buffer AP3 to the cleared lysate, and mix by pipetting.
- Pipet 650 µl of the mixture from step above, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube (supplied). Centrifuge for 1 min at 8000 rpm and discard the flow-through. Reuse the collection tube in the next step.
- Repeat the step above with remaining sample. Discard flow-through and collection tube.
- Place the DNeasy Mini spin column into a new 2ml collection tube (supplied), add 500 µl Buffer AW, and centrifuge for 1 min at 8000 rpm. Discard the flow-through and reuse the collection tube in the next step.
- Add 500 µl Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 14000 rpm to dry the membrane.
- Repeat this step again, add 500 µl Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 14000 rpm.
- Keep DNeasy Mini spin column to dry the membrane by centrifuge for 2 min at 14000 rpm.
- Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied), and pipet 100 µl sterilized distilled water directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15-25^{o}C), and then centrifuge for 1 min at 8000 rpm to elute.
- Finally, collect DNA for analysis.

The amount of extracted DNA from each sample was estimated by comparing an aliquot of extracted DNA with different concentrations of λ DNA through an electrophoresis on 1% agarose gel in 1xTBE buffer (89mM Tris Base, 89mM Boric Acid, 2mM EDTA). Through the same electrophoretic run the purity and integrity of the extracted DNAs was also evaluated.

3.1.8 PCR-based RAPD profiles

- PCR was performed in a reaction volume of 12.5 µl containing 3 µl of genomic DNA (5ng/µl), 3.25 µl of primer, 6.25 µl of TopTaq Master 2X (Qiagen)

The amplification reaction was carried out in a thermocycler (iCycler^{TM}, Bio-Rad). The PCR program consisted of the following steps:

- Initial denaturation: 94°C for 3 min
- 45 cycles : 94°C for 30 sec
  35°C for 30 sec
  72°C for 30 sec
- Final extention: 72°C for 8 min

- The products of RAPD-based PCR analyses were detected by using agarose gel electrophoresis (2% in 1X TBE buffer) and ethidium bromide (1 µg/ml) staining. GelPilot 1 kb Plus Ladder (Qiagen) was used as DNA marker. Gels were run at 85 V for 3.5 hours. Finally, the products of amplification were examined under UV illuminator and images were acquired with GEL-DOC 2000 (Biorad).

3.1.9 DNA polymorphism assessment

- An initial screening of 20 RAPD primers was performed in order to test amplification profiles for the readability and reproducibility of polymorphism. After this preliminary screening, a total of 12 primers were selected. This selection was based on high polymorphisms and good reproducibility of the fragments generated.

The following are the sequences of primers used in the study:

Primer	Sequence (5'→ 3')
OPA02	TGCCGAGCTG
OPA08	GTGACGTAGG
OPA13	CAGCACCCAC
OPA18	AGGTGACCGT
OPH04	GGAAGTCGCC
OPH08	GAAACACCCC
OPH09	TGTAGCTGGG
OPH12	ACGCGCATGT
OPH18	GAATCGGCCA
OPH19	CTGACCAGCC
OPC06	GAACGGACTC
OPC07	GTCCCCGACGA

- Only reproducible and clear amplification bands were scored for the construction of the data matrix. The marked changes observed in RAPD profiles (disappearance

and/or appearance of bands in comparison with control treatments) were evaluated. Polymorphic bands were scored as present (1) or absent (0) for each primer.

- DNA sequence damage induced by arsenic or cadmium was evaluated as the percentage of polymorphism (P %), which represents the ratio between the number of polymorphic bands and the total detected bands ×100:

$P = [(a+b)/c]*100$

P: polymorphism percentage

a: total number of new appeared bands compared to control

b: total number of disappeared bands compared to control

c: the number of total bands in control sample + (a)

3.1.10 Bioavailable concentrations of As and Cd in soils

Total pollutant concentration (for example Cd or As) in soils is not a good indicator of mobility, availability and the associated environmental risk (Larios et al., 2012) because organisms respond only to the fraction that is biologically available. The bioavailable fractions of contaminants are dependent on soil properties and various processes varying with time and on the behavior or the target organism (Harmsen, 2007). The main difficulty on the study of pollutant availability for plants relies on the evaluation of an appropriate extraction method for soils, since it is desirable that the applied method simulates the real uptake by plants (Larios et al., 2012). Bioavailability may be assessed in two complementary ways: (i) by chemical methods (e.g., extraction methods), which determine a defined available fraction of a well defined class of contaminants; and (ii) by biological methods, which expose organisms to soil or soil eluates to monitor effects (Harmsen, 2007). In this study, for bioavailable As and Cd quantification in soil samples before plant exposure and in control treatments of this experiment, the protocol of Lindsay and Norwell (1969) suitable for metal extraction from non-acid soils was applied. Briefly, 5 g of soil were extracted with 10 ml of 5 mM DTPA (Sigma), 0.1 M trietanolamine (Sigma) and 0.01 M CaCl2 (Sigma), for 2 h at 20 °C under stirring. Samples were then filtered and metal concentrations were determined by graphite furnace atomic absorption spectroscopy (GFAAS; SIMA 6000, Perkin-Elmer).

3.1.11 Concentrations of As and Cd in plant organs (roots and shoots)

To determine the amount of As and Cd in plant organs (roots and shoots), the USEPA 3051a protocol was applied. The harvested plants were carefully washed with tap water and then with distilled water to remove soil debris before analysis. All the samples were dried at 100 °C overnight. For each sample 10 mL of HNO_3 and 2 mL of $HClO_3$ were added to 0.2 g of dry plant matter. The samples were digested by using the ETHOS HPR 100/10 microwave lab station (FKV, Bergamo, Italy) reaching the 180 ^{0}C temperature. After their complete mineralization, they were opportunely diluted and analyzed by graphite furnace atomic absorption spectroscopy (GFAAS; AAnalyst600, Perkin-Elmer). Standards (from ENEA Research Centre, Roma, Italy) and blanks were run with all sample series for quality control.

3.1.12 Statistical analysis

- Statistical analyses were performed using the GraphPad Prism software for Windows (version 4.0 GraphPad Software Inc., San Diego CA): ANOVA and Dunnet or Tukey test were applied to the data when normality and homogeneity of variance were satisfied. Data which did not conform to the assumptions were alternatively transformed into logarithms or were analysed by Kruskal Wallis non-parametric procedures.

- The interaction type existing between Cd and As in each treatment and concerning their joint effect on plant growth and DNA sequence change were evaluated by applying the statistical method reported by Ince et al. (1999). The method was based on testing the null hypothesis of ‘‘additive effect’’ at 95% confidence level and summarized the following:

The interaction of Cd and As in each treatment was assessed by comparing the observed toxicity at the i^{th} test level and at the concentration (x + y)i (where x and y were the concentrations of the first and second element, respectively) with the value of the null hypothesis at that level, defined as ‘‘the sum of the toxicity indices of the two elements, tested previously at x and y’’.

- For the joint effect on plant growth, evaluation of the null hypothesis was based on multiplication of plant dry weigh (PDW) of each element as percentage of control, whereas for the joint effect on DNA sequence changes the null hypothesis was evaluated by the addition of plant damage induced by each element, defined as PP =

polymorphism percentage. Thus, toxic and genotoxic interactions at each binary test level were assessed by statistical testing of the two null hypotheses PDW_H and PP_H, defined by Equation 1 and Equation 2 for growth and DNA damage data, respectively:

(1) H0 Plant growth: $PDW_H(x + y)i = (PDWx)i * (PDWy) i/100$

(2) H0 Plant sequence changes: $PP_H(x+ y)i = (PPx)i + (PPy)i$

where $(x + y)_i$ was the i^{th} combination of Cd and As concentrations in soil, (PDWx)i and (PDWy)i the plant dry weight (as %) for each metal ion, recorded at the xi^{th} and yi^{th} singular concentrations, and (PPx)i , (PPy)i the percentage of polymorphism induced by each element, recorded at the xi^{th} and yi^{th} singular concentrations.

The compound interactions were called ‘‘antagonistic,’’ ‘‘additive,’’ or ‘‘synergistic’’ according to the statistical significance (t student) and the sign of the difference between the tested hypothesis and the value of the observed effect.

Regression and Redundance statistical analyses (RDA) were also applied to investigate the relationships between variables and their relevance to the joint-effects of Cd and As.

3.2 Experiments with soil samples collected in Lombardy region- Part 2

3.2.1 Lombardy Region: an overview

Lombardy is the largest and most wealthy region in Italy; the territory of the province of Lombardy region covers a surface of 24000 square kilometers and the population of approximately 10 million inhabitants (the 3rd most populated region in Europe after Île-de-France and Baden-Württemberg) (Regione Lombardia, 2014).

Gross product pro capita: € 33,648 (Baccini et al., 2011) and the gross domestic product (GDP) of Lombardy amounts to 296 billion euro, representing 20% of the national value (Regione Lombardia, 2014).

The Lombardy region lies in the north of the country, sharing a border with Switzerland. Lombardy region consists of 12 provinces: Bergamo, Brescia, Como, Cremona, Lecco, Lodi, Mantova, Milan, Monza, Pavia, Sondrio, Varese. Milan is the capital city (Figure 15).

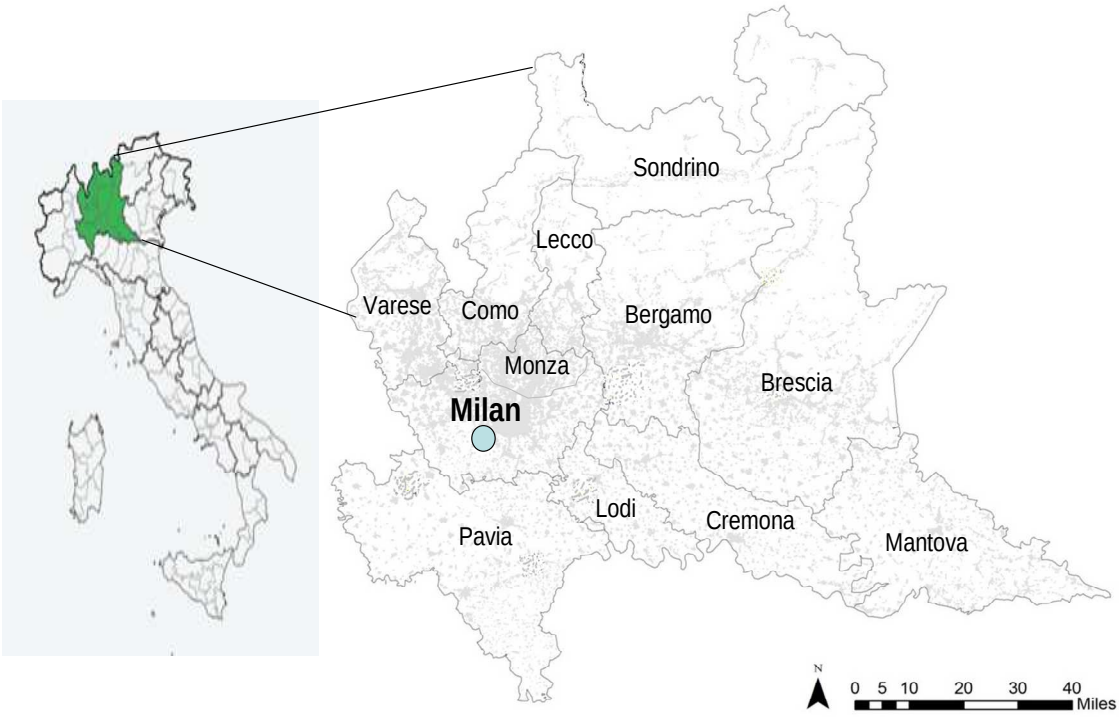

Figure 15: Lombardy region of Italy shows the 12 provinces, Milan is the capital city.

The Lombardy's climate depends on altitude and the presence of inland waters. The temperature shows high annual variations (in Milan, the average temperature is 1.5^0C in January and 24^0C in July), and thick fog is frequent between October and February (Baccini et al., 2011).

Productive activities in this region have developed for many past decades. The region can be geographically and economically divided into 3 zones: the mountain range of the Alps; the sloping foothills; and the immediate facing plains (Baccini et al., 2011). Milan develops mainly service sectors while industrial activities focus in Varese, Como, Lecco, Bergamo and Brescia. Agricultural activities are mainly in Pavia, Sondrio, Cremona, Mantova, Lodi and some parts of Bergamo and Brescia province. Industry and service sectors play an important role in the region. However, agriculture still contributes significantly to the region's economy. Rice (about 600,000 metric tons from nearly 100,000 ha in 2008) as human food and maize (about 3 million metric tons from nearly 250,000 ha in 2008, mainly as cow and pig fodder) are the two main cash crops in the region (Rubino et al., 2012). Italy is the leading rice producer with approximately 50% of the total harvest in European Union, about 41% total rice produced in Lombardy, mainly in Pavia and Milan (Sommella et al., 2013).

The plain area of the Lombardy region is characterized by the combination of unfavorable atmospheric dispersion conditions with a high population density and intensity of industrial, traffic and agricultural activities, which make it one of the most polluted areas in Western Europe (Caserini et al., 2013).

3.2.2 Sampling areas, soil sampling methodology and plant exposure

Soil sampling was carried out in collaboration with AEFORIA, a Catholic University spin-off. Surface soil layer of 0-30 cm in depth was collected. A total of 67 soil samples were collected in 7 agricultural areas of concern in Lombardy region (Figure 16).

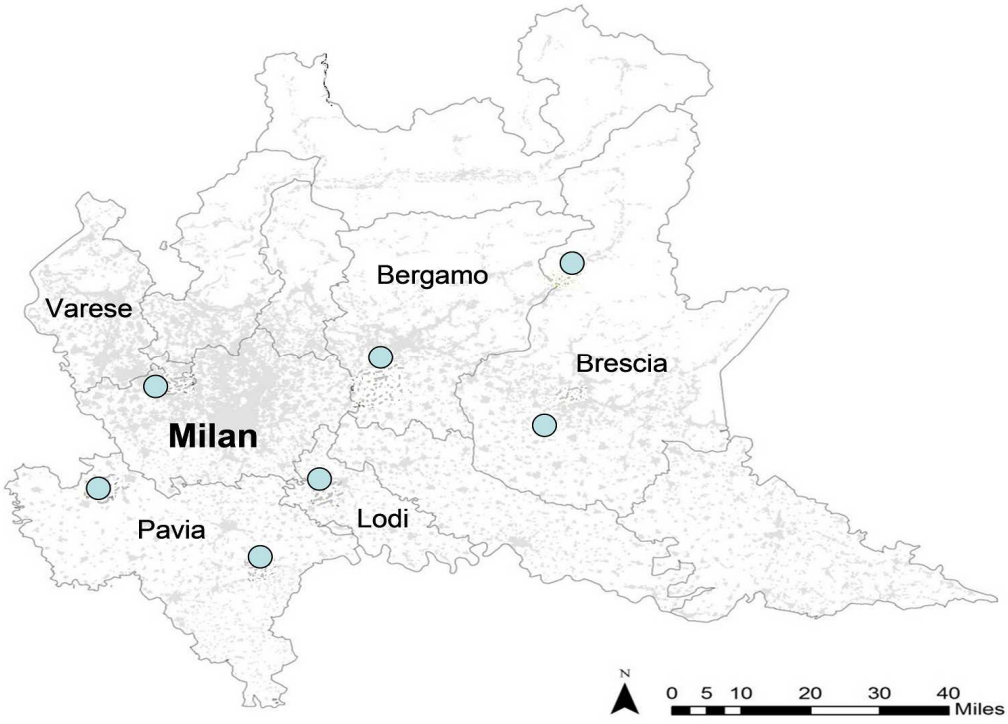

Fig. 16: Localization of the seven areas of concern that were considered in this study.

A brief description of the 7 areas is reported here below:

- Parona area: it is the area surrounding the waste treatment plant located near the town of Parona within Pavia province. The plant occupies an area of about 110,000 m^2 and its total capacity is approximate 200,000 tones of municipal solid waste and non-hazardous waste (Line 1) per year and 180,000 tones of non-hazardous waste per year (Line 2). Twelve samples of soil (P1-P12) were collected in the four cardinal

directions with distance of 500, 1000 and 2000 m respectively from the plant (Figure 17).

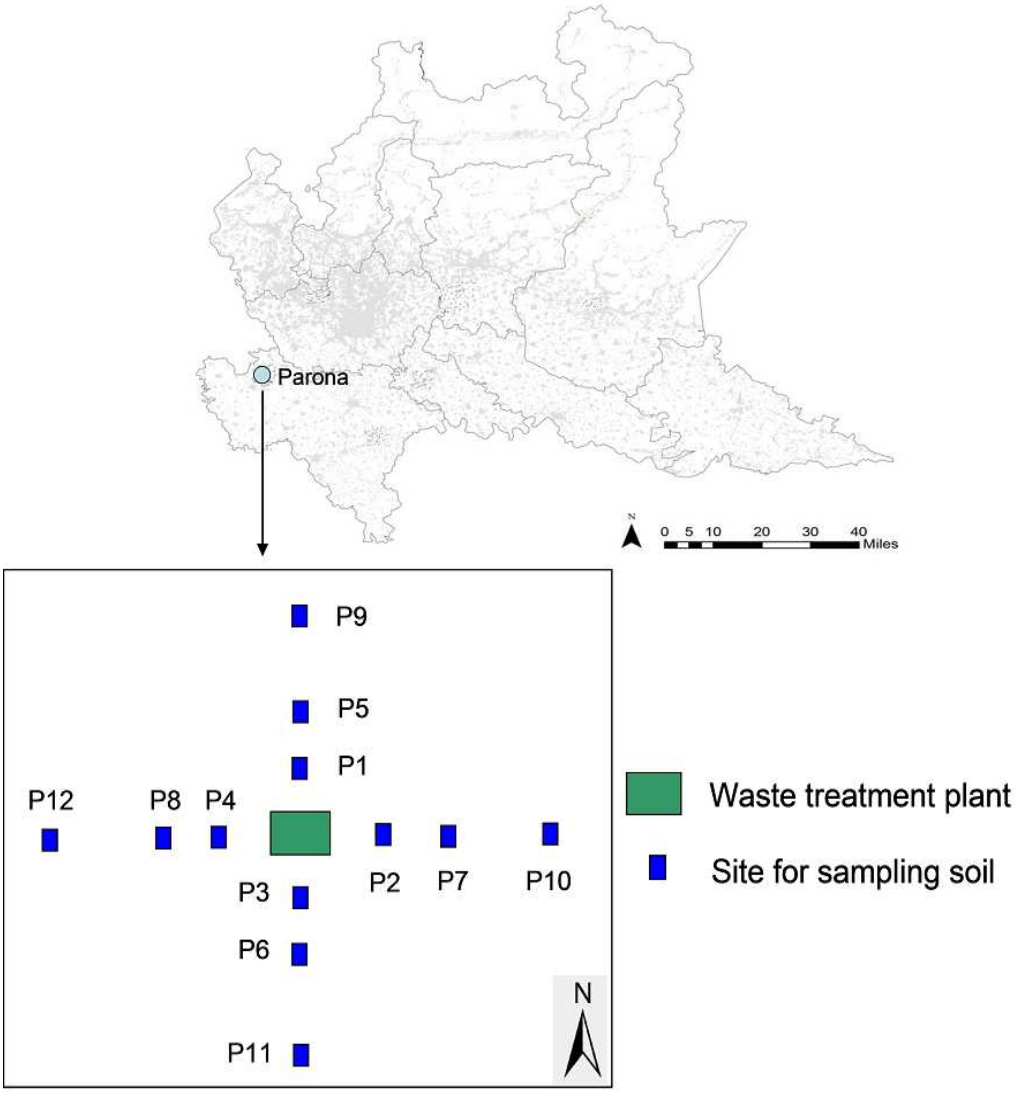

Figure 17: Area for sampling soils around the waste treatment plant in the town of Parona, Pavia province.

- Pieve Fassiraga Viscolube area: at the vicinity of the waste oil refining company of Viscolube which was founded in 1963 in Pieve Fissiraga, within Lodi province. In 2010 this company processed about 130,000 tones of waste oil to produce over 80,000 tons of high quality oils, reducing simultaneously drastic sulfur content. In this area 8 samples of soil (V1-V8) were collected in the four cardinal directions, at a distance of 500 m and 1000 m from the company (Fig 18).

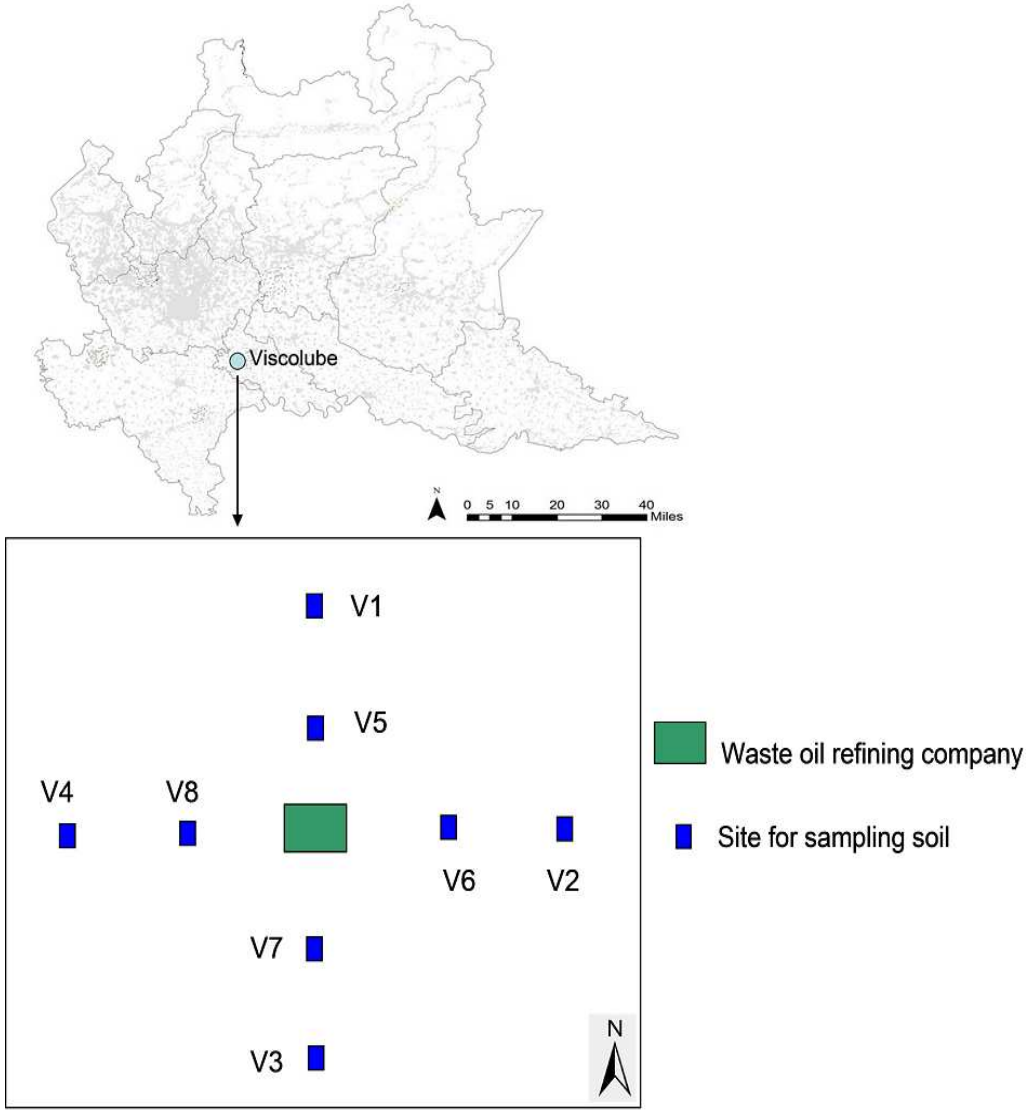

Figure 18: Area for sampling soils around the waste oil refining company in Pieve Fassiraga Viscolube, Lodi province.

- Origgio area: the area adjacent to Milano-Varese Highway near the village of Origgio within Milan province. This area is esposed to the vehicular pollution and was previously investigated for the presence of mercury, palladium, platinum and lead, emitted from catalytic converters, and PAHs. In this area 8 samples of soil (O1-O8) were collected. Sampling points were positioned at 50 m and 150 m from the highway, perpendicularly to it (transet). The distance between two transets was 500 m (Figure 19).

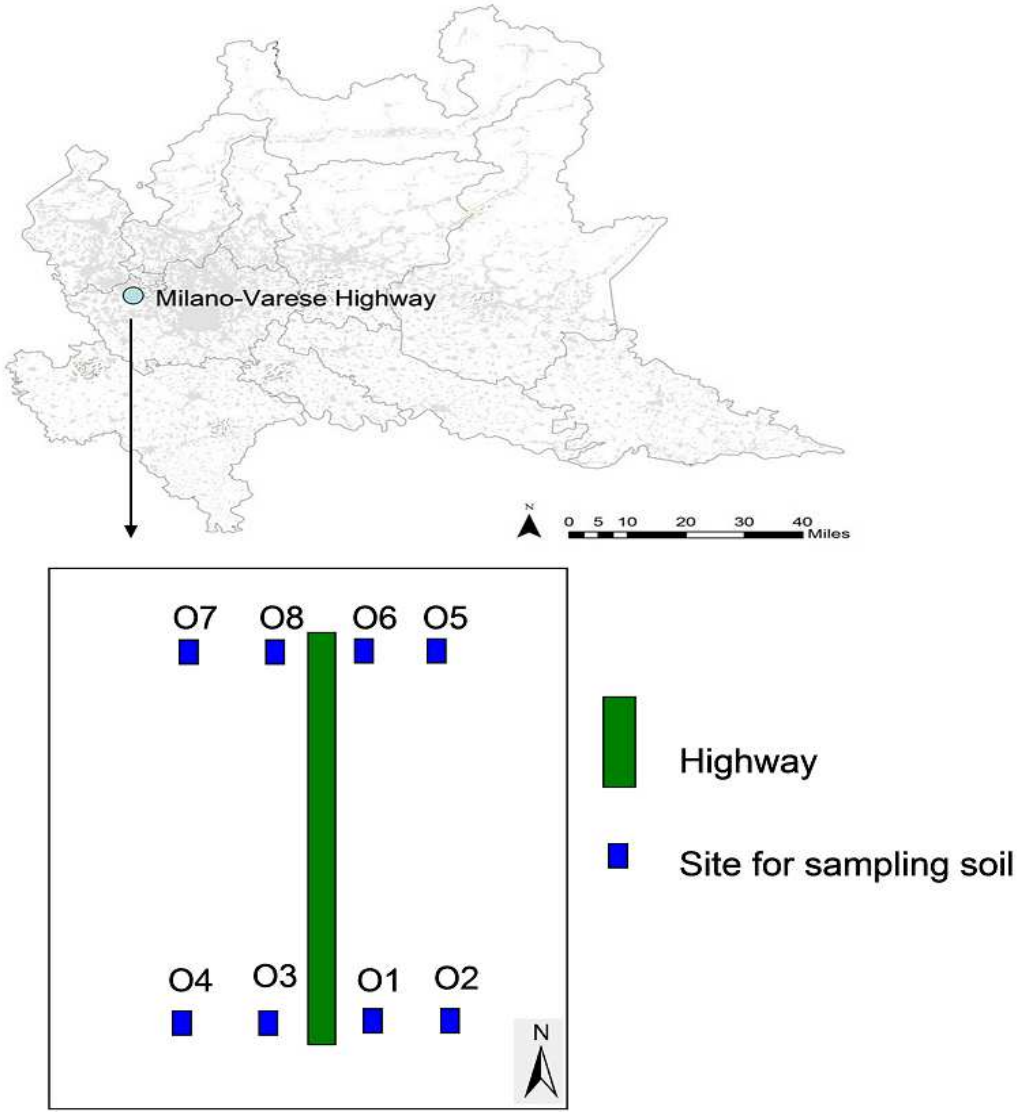

Figure 19: Location of the 8 sampling sites (O1-O8) in the area adjacent to Milano-Varese Highway, Origgio village in Milan, Lombardy

- Brescia area: it is an area affected by both industrial and agricultural activities close to Brescia town. The area belongs to a vast area of national concerns as it is highly contaminated mainly by PCB compounds. Eight soil samples (S1-S8) were collected in 8 sites distant 200 m each other (Figure 20).

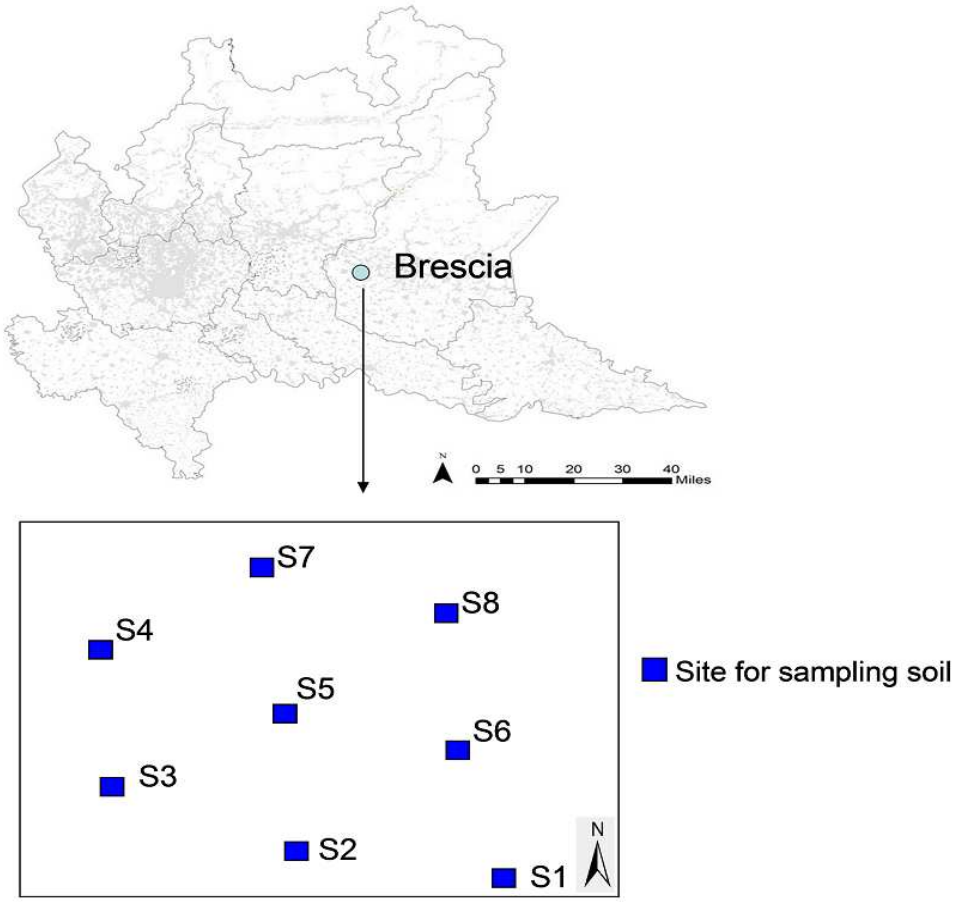

Figure 20: Location of the 8 sampling sites (S1-S8) in Brescia area.

- Treviglio area: This area has been identified in the past as an area contaminated by Cr(VI). The area include five cities (Verdellino, Verdello, Ciserano, Arcene and Pognano) near Treviglio (Bergamo). Fourteen soil samples were collected (Cr1-Cr14) as shown in Figure 21.

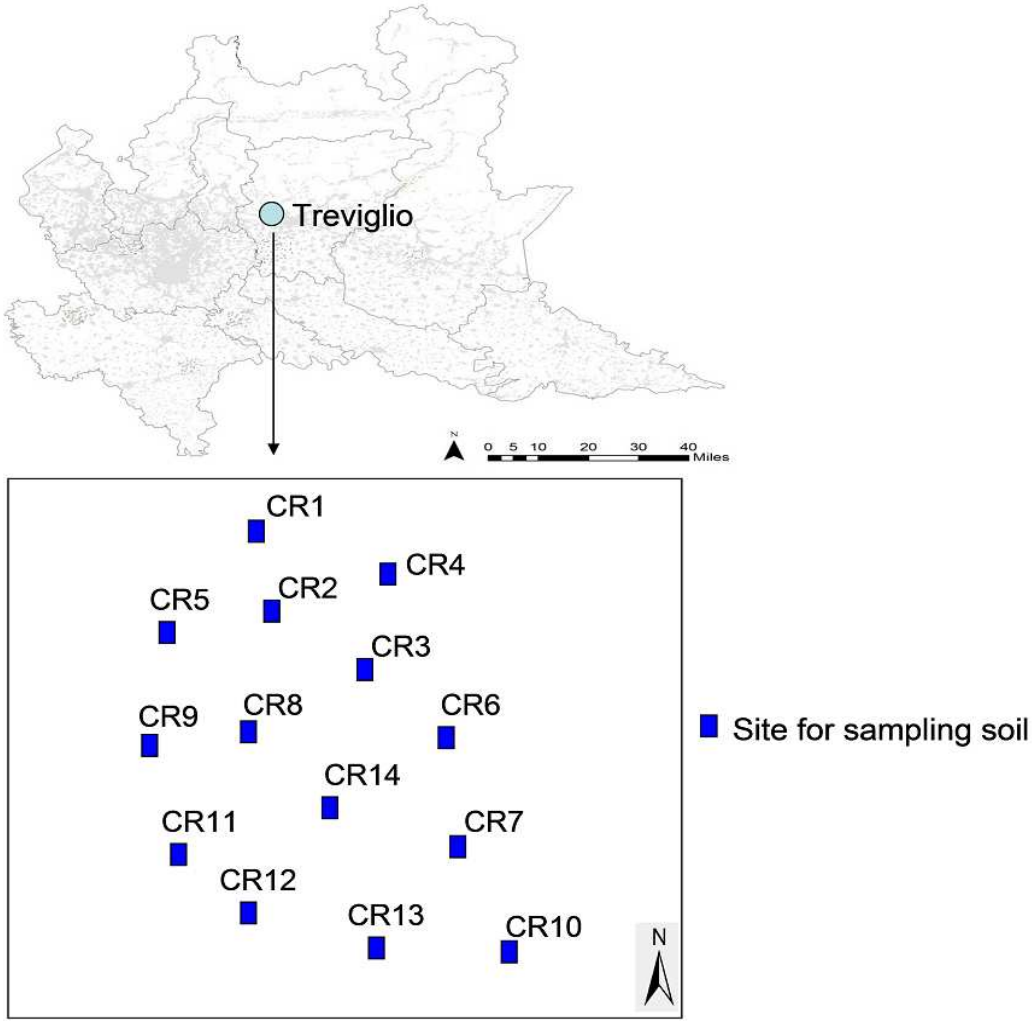

Figure 21: Location of the 14 sampling sites (CR1-CR14) collected in Treviglio area.

- Boario Terme area: it is the area near a steel industry at Boario Terme, within the Brescia province. Eight soil samples were collected (F1-F8) with a distance of 500 and 1000m from the steel industry as shown in Figure 22

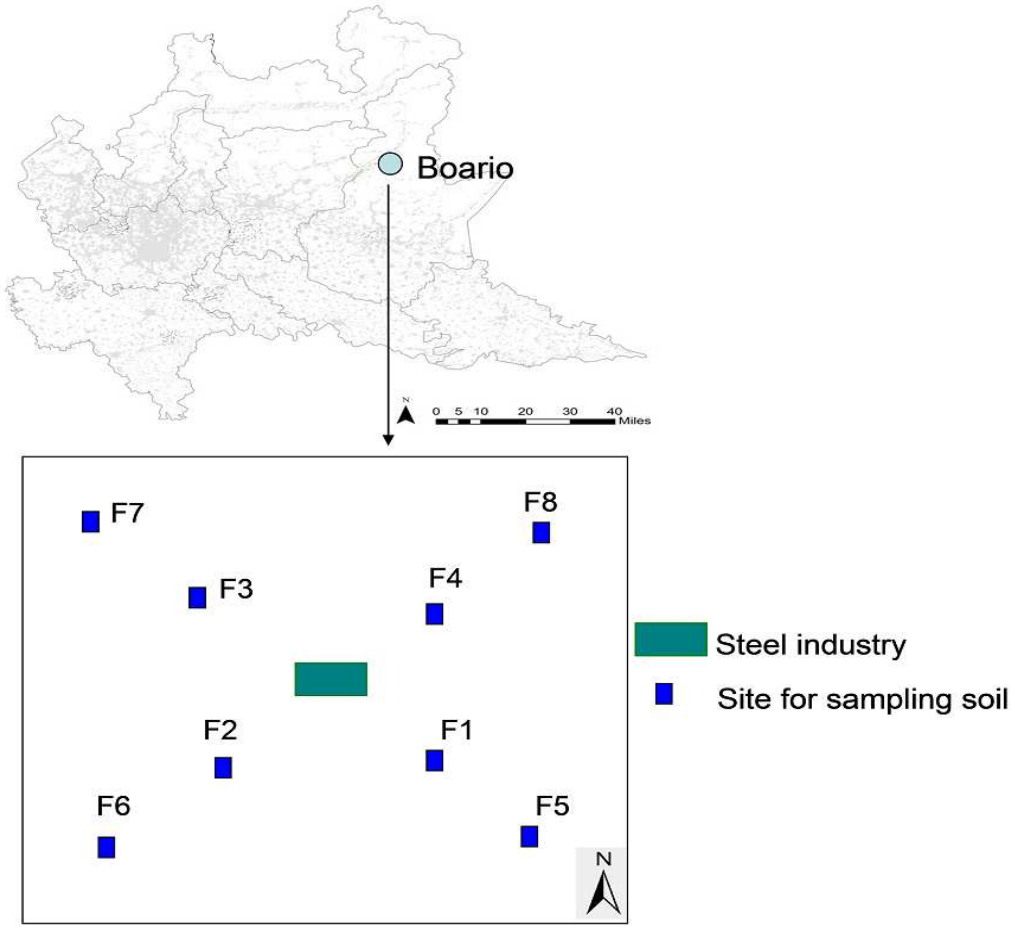

Figure: 22: Location of the 8 sampling sites (F1-F8) in Boario, Brescia province

- Broni area: Nine soil samples (IT1-IT9) were collected within the area surrounding the clinker and cement producing plant of Broni with a distance of 500, 1000 and 1500m from the plant, within Pavia province (Figure 23).

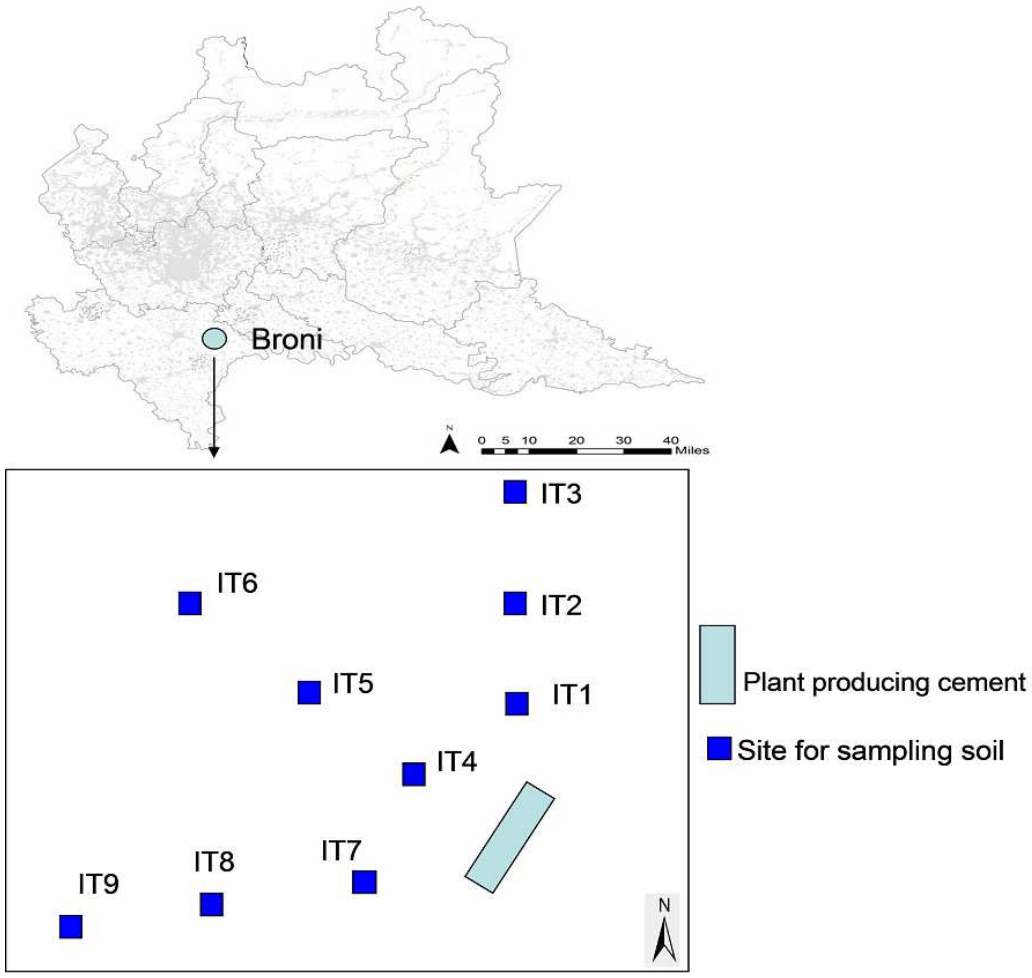

Figure 23: Location of the nine sampling sites (IT1-IT9) in Broni, Pavia province

Soil was collected in these 7 areas by applying a systematic sampling by using a regular grid of 20 x 20 meters divided into 25 subareas was applied. Soil was collected from 15 subareas randomly chosen (Figure 24). The sampling sites were identified through their GPS co-ordinates. If a sampling site fell in inaccessible area, the soil was collected at a new co-ordinate which has a suitably and equivalent distance. The litter, roots, stones and other coarse materials were removed from the field during the sampling procedures.

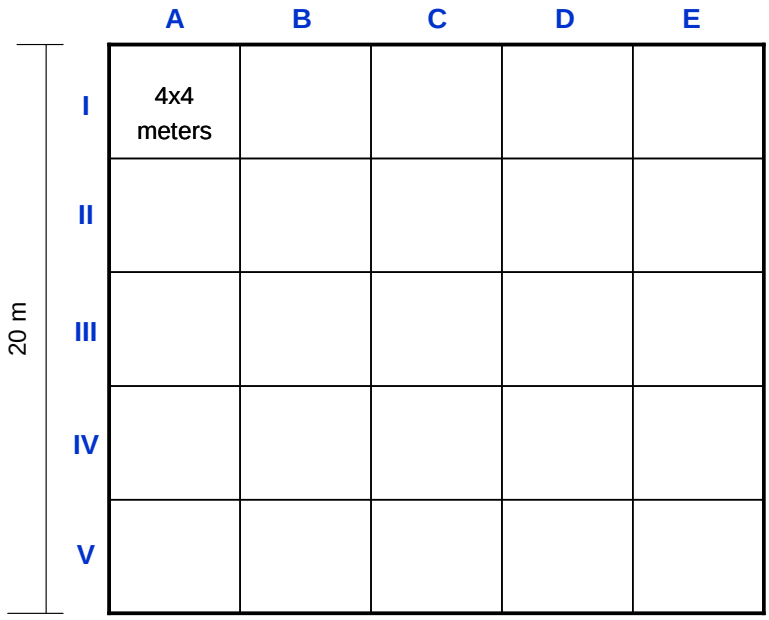

Figure 24: Design for soil sampling

For each site, collected soils were mixed and stored in PVC bags for the successive exposure to *Trifolium repens* and also for analyzing chemical and physical properties. Plant exposure was carried out following the same procedure used for the determination of Cd and As joint effects and described in the chapter 3.1.5. Each soil sample consisted of 3 pots (3 repetitions) each containing 18 plantlets for a total of 54 plantlets.

4. Results

4.1 Results- Experiments with As and Cd contaminated soils

4.1.1 Soil biovailability of As & Cd

The bioavailable amount of Cd and As in artificially contaminated soils was assessed just before white clover exposure. The measured concentrations of DTPA-extractable Cd and As are reported in Table 4. The results showed that Cd was much more bioavailable than As: the percentage of bioavailable As and Cd ranged from 0.016 to 0.055 and from 0.43 to 0.79, respectively. In soils contaminated with single compounds the bioavailable amounts of both Cd and As increased in parallel with the increase of metal concentration added to soil (r^2_{Cd}= 0.99 r^2_{As}= 0.97). A different trend in bioavailability was instead observed in soil simultaneously contaminated with the two elements: the presence of As reduced the amounts of bioavailable Cd, whereas the presence of Cd increased the amounts of bioavailable As.

Table 4: Concentrations of bioavailable As and Cd in control and contaminated soils, evaluated by atomic absorption spectrophotometry (AAS) before plant exposure. The mean values of three different samples for each treatment with standard deviation and the percentage (%) bioavailable are reported. CTR: control soil (soil not artificially contaminated); BDL: below the detection limit of the instrument

Soil sample	pH	Bioavailable As (µg g^{-1})	Bioavailable Cd (µg g^{-1})
CTR	7.9	BLD	BLD
As5	7.8	0.08±0.01	BLD
As10	8.0	0.25±0.04	BLD
As20	7.8	0.80±0.06	BLD
Cd 20	7.8	BLD	15.76±2.72
Cd 40	7.8	BLD	26.81±4.32
Cd 60	7.9	BLD	36.79±5.91
As5+Cd20	8.0	0.13±0.02	9.87±1.59
As5+Cd40	8.0	0.12±0.03	18.65±2.96
As5+Cd60	7.8	0.14±0.02	32.57±5.41
As10+Cd20	7.9	0.33±0.03	8.91±1.49
As10+Cd40	7.9	0.32±0.04	17.41±2.72
As10+Cd60	7.9	0.37±0.03	31.99±5.18
As20+Cd20	7.9	1.11±0.05	9.58±1.55
As20+Cd40	7.9	1.04±0.04	19.83±3.20
As20+Cd60	7.9	0.93±0.05	30.70±4.95

4.1.2 General toxicity through plant survival and growth

Single and joint effects of Cd and As on plant survival and plant development were assessed after 15 days of exposure to treatments. Plant development was evaluated by measuring plant organ dry weight (DW). As expected on the basis of preliminary trials, none of the single Cd or As concentrations negatively affected plant survival and plant DW (Fig.25). Plant survival also was not affected by all the combined treatments. On the contrary, the combination of As5 with the higher Cd concentration (Cd 60) and the combination of As10 with Cd 40 or Cd 60 and of As 20 with all the tested Cd concentrations significantly reduced the shoot development ($p < 0.05$; Fig. 25). Concerning the effect of these combined concentrations on roots, although a growth reduction trend was observed, the results obtained were not statistically significant, given the root very low DW and the consequent difficulty in assessment (Fig.25).

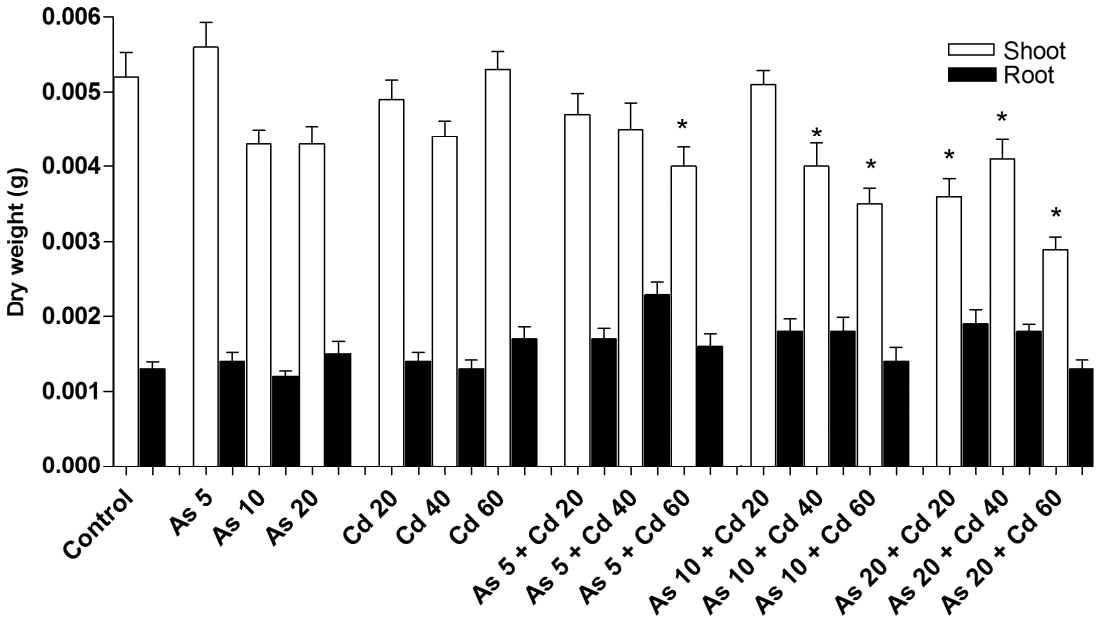

Figure 25: Effect of metal(loid) stress on *T. repens* growth, measured as dry weight (DW). Data are the mean of 30 measurements from single plants per each treatment. The asterisk (*) indicates statistically significant differences with respect to the control (ANOVA and Dunnet test; $P<0.05$).

The statistical approach of Ince et al. (1999) was applied to evaluate the type of interaction existing between As and Cd, responsible for the joint effect on plant growth observed in each treatment. Table 5 shows the results of the analysis. A synergistic effect leading to plant growth reduction was found when the higher tested Cd concentration (Cd 60) was combined with As 5 or As 10 or As 20. An additive effect was instead determined for all the other soil binary mixture.

Table 5: Observed and calculated toxic effects at binary test combinations x:y, and single metal concentrations x, y, respectively (predicted interaction types). PDW: plant dry weight; S: statistically significant; I: statistically insignificant; df: degrees of freedom

	x ($\mu g\ g^{-1}$)	y ($\mu g\ g^{-1}$)	Observed toxicity PDW_{obs}	Calculated toxicity PDW_{calc} (PDW_x * PDW_y /100)	Difference (PDW_{obs} - PDW_{calc})	Difference standard error	t Student (df=34)	Significance (P<0.05)	Interactive Effect
	As	Cd							
SHOOT	5	20	84.3 ± 5.1	101.3 ± 8.1	- 17.0	9.6	- 1.8	I	additive
	5	40	80.0 ± 6.2	91.6 ± 5.9	- 11.6	8.5	- 1.4	I	additive
	5	60	70.1 ± 4.7	110.7 ± 7.8	- 40.6	9.1	- 4.5	S	synergistic
	10	20	90.6 ± 7.6	77.1 ± 6.2	13.5	9.8	1.4	I	additive
	10	40	71.4 ± 5.4	69.7 ± 4.5	1.7	7.0	0.2	I	additive
	10	60	69.0 ± 3.9	84.2 ± 5.9	- 15.2	7.1	- 2.1	S	synergistic
	20	20	64.7 ± 4.1	76.9 ± 6.2	- 12.2	7.4	- 1.7	I	additive
	20	40	73.2 ± 4.8	69.5 ± 4.5	3.7	6.5	0.6	I	additive
	20	60	51.6 ± 3.0	84.0 ± 5.9	- 32.4	6.6	- 4.9	S	synergistic
ROOT	5	20	92.9 ± 7.7	120.8 ± 13.5	- 27.9	15.5	- 1.8	I	additive
	5	40	123.5 ± 8.7	96.8 ± 11.1	- 6.8	14.1	- 0.5	I	additive
	5	60	83.6 ± 8.4	142.8 ± 26.6	- 59.2	27.9	- 2.1	S	synergistic
	10	20	95.2 ± 8.4	81.0 ± 9.0	14.2	13.1	1.1	I	additive
	10	40	98.3 ± 10.4	64.9 ± 7.4	9.1	12.8	0.7	I	additive
	10	60	73.2 ± 9.7	95.8 ± 17.9	- 40.8	20.3	- 2.0	I	synergistic
	20	20	103.6 ± 10.1	97.8 ± 10.9	5.8	14.8	0.4	I	additive
	20	40	97.6 ± 5.6	78.4 ± 9.0	19.3	10.6	1.8	I	additive
	20	60	69.1 ± 6.0	115.6 ± 21.6	- 44.2	22.4	- 2.0	S	synergistic

4.1.3 Accumulation of Cd and As in plant organs

The total amount of Cd and As accumulated in plant organs at the end of the experiment, was calculated by multiplying the element concentration, determined by AAS in root and shoot (Fig.26), with the correspondent organ DW (Fig.25). The obtained results are reported in Fig.27.

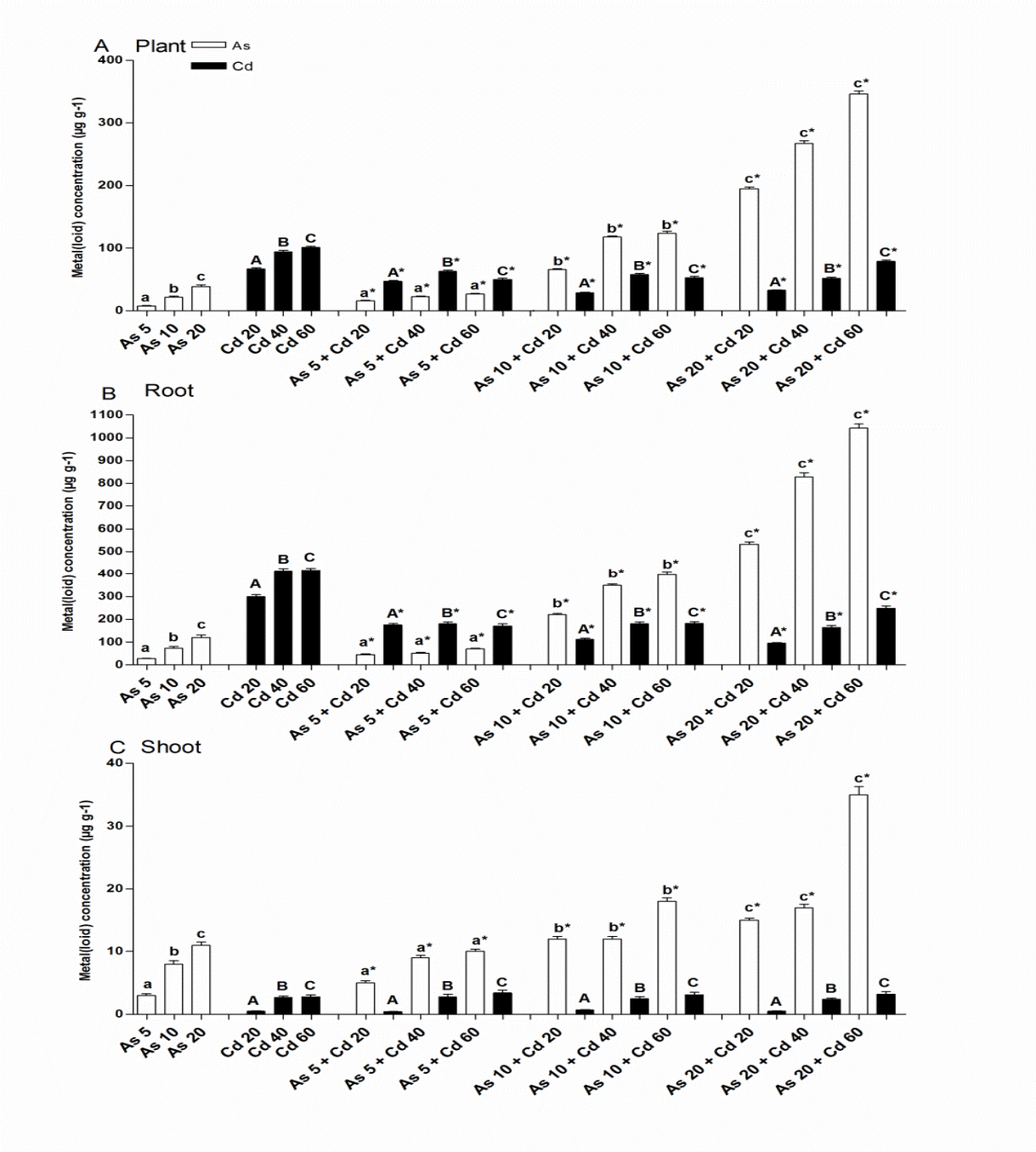

Figure 26: Metal(loid) concentration (µg g^{-1} dry matter) in white clover plants after exposure. The mean concentration obtained by AAS ± standard deviation for each plant organ and for each soil is shown. Uppercase letters represent significant differences with the correspondent concentration of Cd control ($P < 0.05$); Lowercase letters represent significant differences with the correspondent concentration of As control ($P < 0.05$).

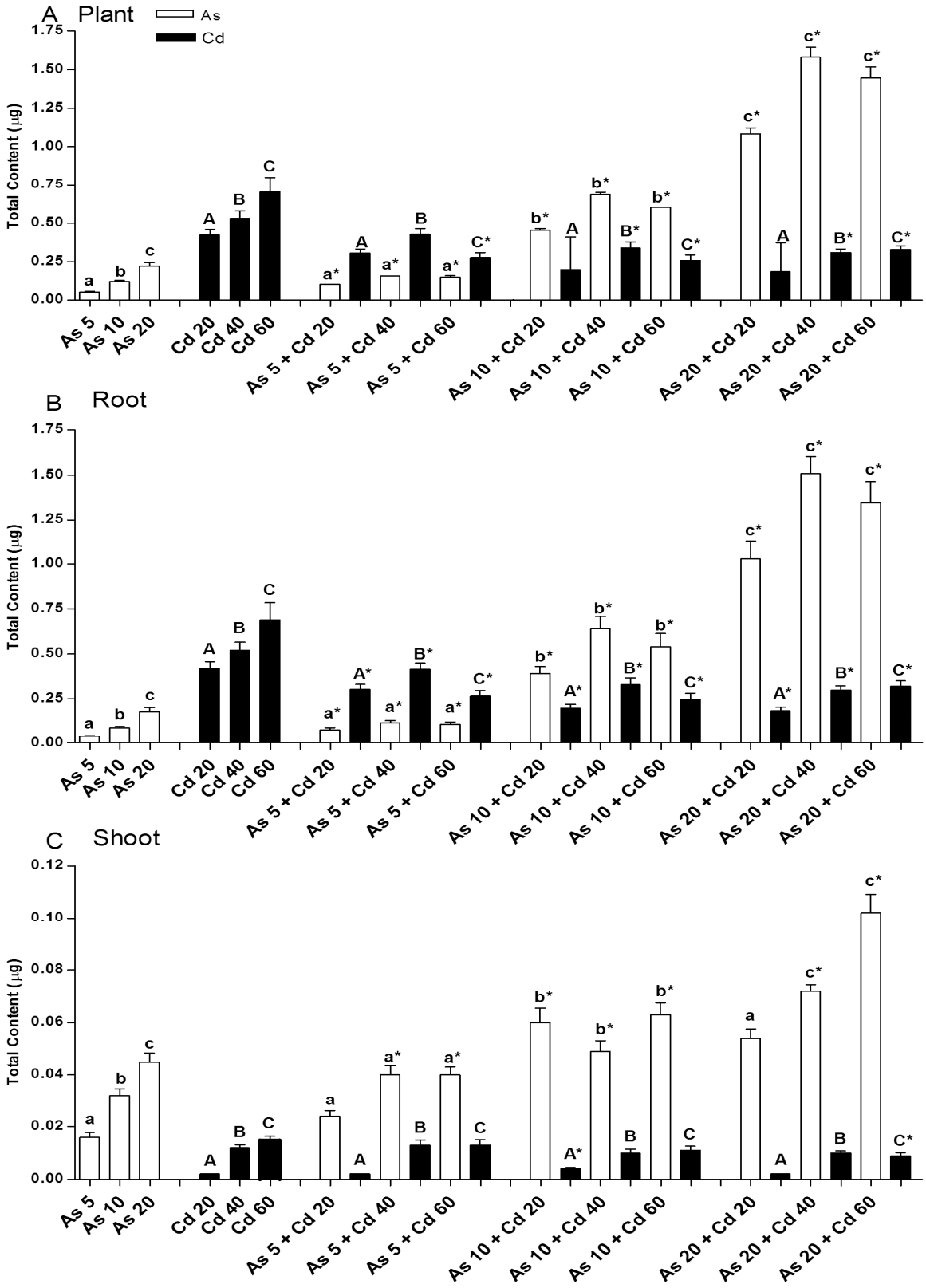

Figure 27: Metal(loid) total content (µg) in *T. repens* plants after exposure. Mean total amount of Cd and As accumulated in plant organs during exposure, was calculated for each treatment by multiplying the metal(loid) concentration, determined by AAS in root and shoot, with the correspondent organ dry weight. Uppercase letters represent significant differences with the correspondent concentration of Cd control ($P<0.05$); Lowercase letters represent significant differences with the correspondent concentration of As control ($P<0.05$).

Arsenic accumulation in plants grown on soil contaminated with As was more or less proportional to its concentration in the soil and to its bioavailability ($r^2_{bioav\text{-}As} = 0.97$, $P<0.05$). For cadmium, there was a slight tendency for increasing Cd accumulation in the plants with higher concentrations in the soil, but it was not significant statistically (Fig.27A). Moreover, with respect to the available amounts of Cd and As, plants accumulated a greater relative amount of As than Cd. Indeed, considering that the available amounts of Cd in each pot containing 2kg of soil were much higher (ranging from about 32 to74 mg) than those of As (ranging from about 0.16 to 1.6 mg), the relative mean amounts of Cd accumulated per plant (ranging from about 0.4 to 0.7 µg) were proportionally lower than those of As (ranging from 0.05 to 0.2 µg;), suggesting different plant absorbtion mechanisms for the two metal(loid)s.

Similarly, in soils contaminated with both As and Cd, As accumulation in plants was related to its bioavailability (multiple $r^2 = 0.90$, $P<0.05$). Furthermore, since the presence of Cd in soil increased the bioavailability of As, concentrations of As in plants grown in the presence of both elements was higher than that measured in the plants grown in presence of As alone. In contrast, Cd accumulation was not proportional to its bioavailability in soil and was lowered by the presence of As (Fig.27A).

Regarding the distribution of Cd and As in plant organs, most of them were accumulated in root (Fig.27B) and the very low amounts translocated to shoot (Fig.27C) were proportional to the amounts accumulated in root ($r^2_{Cd} = 0.51$, $r^2_{As} = 0.69$, $P<0.05$).

A similar trend of Cd and As accumulation and distribution was also observed analyzing the mean metal(loid) concentration measured in plant organs (Fig.26). However it can be observed that, due to the different reduction in plant growth, induced by the different metal(loid) treatments, the mean total amount of Cd and As (calculated multiplying metal concentration for DW), did not always reflect the mean concentration of elements in plant organs. For instance, the mean concentration of Cd measured in roots of plants grown in As20+Cd60 soil was statistically higher than that found in root of plants grown in As20+Cd40 soil whereas the mean total amount of Cd

was not statistically different between the two treatments, due to the higher growth reduction of plants grown in As 20+Cd 60 soil. Thus, in our data elaboration, the total amount of metal(loid)s was calculated to properly correlate the amount of element absorbed by plant with its bioavailable soil quantity, whereas the concentration of elements in plant organs was also taken in to account to better evaluate the observed toxic and genotoxic effects of metal(loid)s

4.1.4 Single and joint genotoxic effects of Cd and As

DNA sequence changes were evaluated by means of RAPD analysis, a technique which detects mutations at the primer annealing sites and also within the amplified DNA fragments (*i.e.*deletions or insertions). Twelve single primers were applied for the shoot and root analysis revealing a total of 130 and of 152 reproducible bands, respectively. Of these bands, 3.52% and 4.62% were polymorphic among the shoot and root controls, respectively. These values were considered as a basal polymorphic level among *T. repens* plants (*i.e.* intra-species variability).

Taking into account all the independent repetitions, DNA sequence damage, induced by Cd and As, was calculated as the percentage of polymorphism (P%) of the treated samples compared to that of the control plants and reported in Figure 28. All tested As and Cd concentrations (alone or in combination) determined a statistically higher percentage of polymorphisms in the shoots and in the roots compared to the control plants. For both Cd and As, induced plant damage was approximately two-three fold higher in the roots than in the shoots, according to the low amounts of both metal(oid)s translocated to shoot. Moreover, DNA damage was related to the concentration of Cd and As accumulated in shoot and in root. Finally, As was more genotoxic than Cd: 5 $\mu g\ g^{-1}$ of As induced a double amount of DNA polymorphisms (14%) than 5 $\mu g\ g^{-1}$of Cd (6%), and 20 $\mu g\ g^{-1}$ of As induced a significant higher amount of DNA polymorphism (32%) than 20 $\mu g\ g^{-1}$ of Cd (25%).

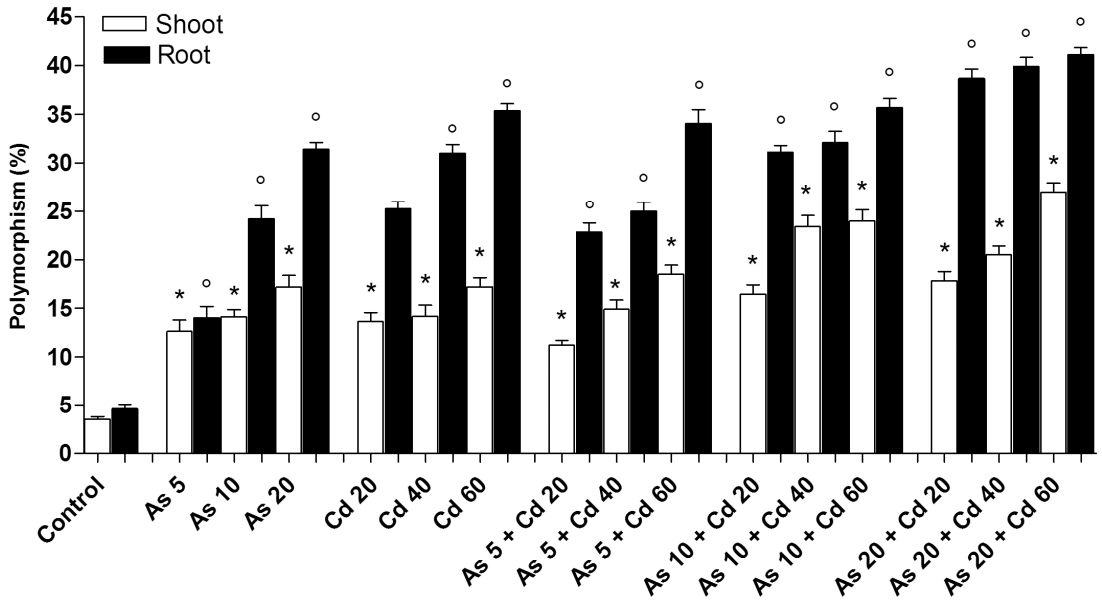

Figure 28: Analysis of the percentage of polymorphism (P% = number of polymorphic loci/number of total loci) detected by RAPD in DNA from *T. repens*plants exposed to increasing concentrations of Cd. Root and Shoot mean percentages ± SD for each treatment are reported. The asterisk and circle show statistically significant differences with respect to the control (ANOVA and Dunnet test; $P<0.05$).

The interactions between Cd and As, responsible for the joint genotoxic effects observed in Fig. 28, were defined applying the statistical analysis of Ince et al. (1999). The results are shown in Table 6. Differently from the interactions responsible for the joint effects on plant development, an antagonistic interaction, leading to a DNA damage reduction, was observed in roots of plants exposed to all the combined concentrations tested. In shoots the interaction was additive except for soils contaminated with the lower Cd concentration (Cd 20) combined with As 5, or As 10, or As 20, which was antagonistic.

Table 6: Observed and calculated genotoxic effects at binary test combinations x:y, and single metal concentrations x, y, respectively (predicted interaction types). PP: percentage of polymorphism; S: statistically significant; I: statistically insignificant; df: degrees of freedom

	x ($\mu g\ g^{-1}$)	y ($\mu g\ g^{-1}$)	Observed genotoxicity PP_{obs}	Calculated genotoxicity PP_{calc} ($PP_x + PP_y$)	Difference ($PP_{obs} - PP_{calc}$)	Difference standard error	t Student (df=4)	Significance (P<0.05)	Interactive Effect
	As	Cd							
S	5	20	11.3 ± 1.2	26.2 ± 3.7	- 15.0	3.9	- 3.9	S	antagonistic
H	5	40	14.9 ± 2.3	26.8 ± 4.1	- 11.9	4.7	- 2.5	I	addidive
	5	60	18.5 ± 2.3	29.8 ± 3.7	- 11.3	4.4	- 2.6	I	additive
O	10	20	16.5 ± 2.3	27.8 ± 2.9	- 11.3	3.7	- 3.1	S	antagonistic
O	10	40	23.5 ± 2.9	28.3 ± 3.4	- 4.8	4.4	- 1.1	I	additive
	10	60	24.0 ± 2.9	31.3 ± 2.9	- 7.3	4.1	- 1.8	I	additive
T	20	20	17.9 ± 2.3	30.8 ± 3.7	- 13.0	4.4	- 3.0	S	antagonistic
	20	40	20.5 ± 2.3	31.3 ± 4.1	- 10.9	4.7	- 2.3	I	additive
	20	60	27.0 ± 2.3	34.4 ± 3.7	- 7.4	4.4	- 1.7	I	additive
	5	20	22.8 ± 2.3	39.3 ± 3.4	-16.4	4.1	-4.0	S	antagonistic
R	5	40	25.0 ± 2.3	45.0 ± 3.7	-20.0	4.4	-4.6	S	antagonistic
	5	60	34.1 ± 3.5	49.4 ± 3.4	-15.3	4.8	-3.2	S	antagonistic
O	10	20	31.1 ± 1.7	49.5 ± 3.9	-18.4	4.2	-4.3	S	antagonistic
O	10	40	32.1 ± 2.9	55.2 ± 4.2	-23.1	5.1	-4.6	S	antagonistic
	10	60	35.7 ± 2.3	59.6 ± 3.9	-23.9	4.5	-5.3	S	antagonistic
T	20	20	38.7 ± 2.3	56.7 ± 2.4	-18.0	3.4	-5.3	S	antagonistic
	20	40	39.9 ± 2.3	62.4 ± 2.9	-22.5	3.7	-6.1	S	antagonistic
	20	60	41.1 ± 1.7	66.8 ± 2.4	-25.6	3.0	-8.5	S	antagonistic

4.1.5 RDA analysis

In order to better understand the correlation among the soil metal(loid) concentrations, their accumulation in plant organs and their effects on plant growth and DNA sequence, a RDA statistical analysis was carried out. Fig. 29 shows that 4 of the 6 variables considered (Cd and As bioavailability, Cd and As concentrations in plant organs) were significant ($P < 0.05$) in determining the toxic and genotoxic effects and that the concentration of As found in plant organs was the most relevant factor (Fig.29)

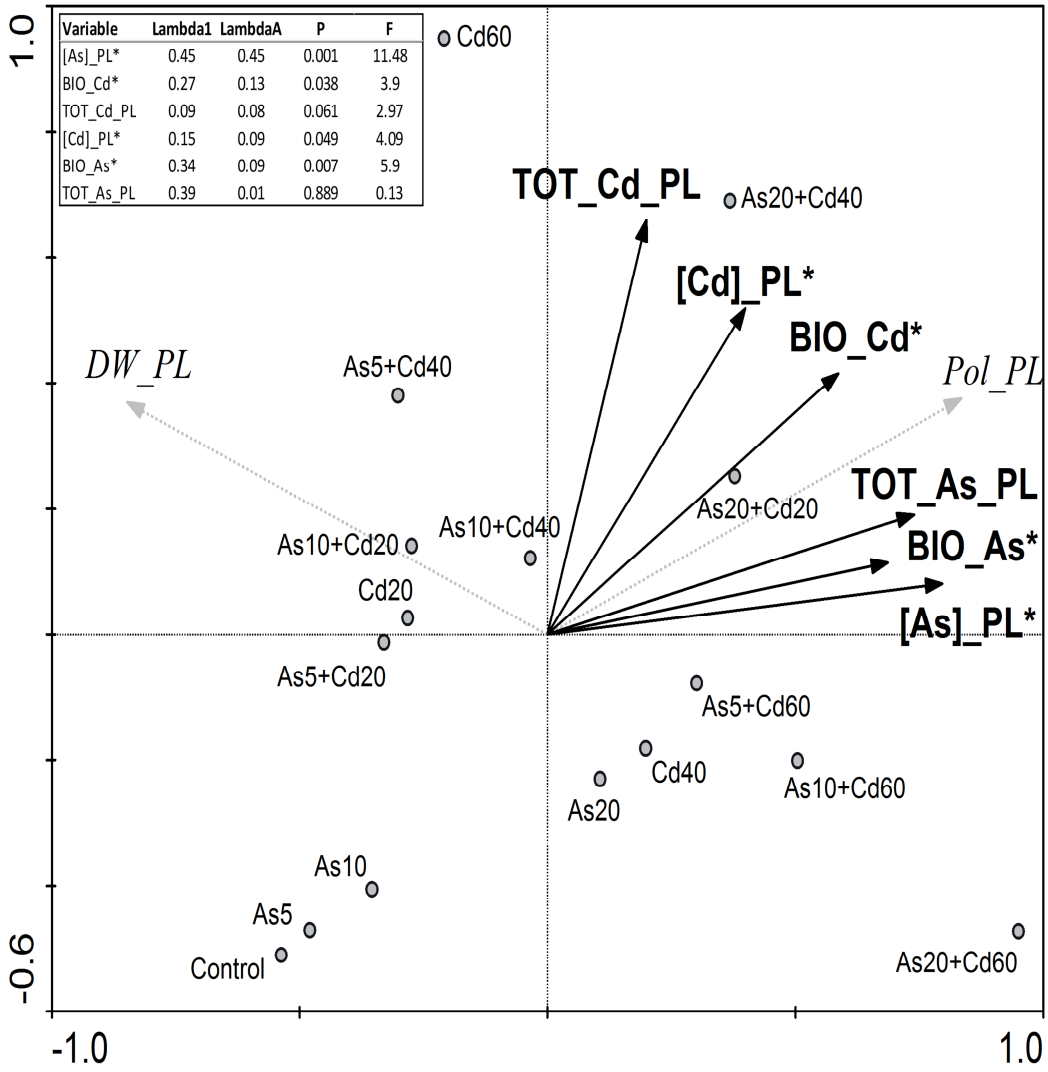

Figure 29: RDA analysis showing the relationship between the metal(loid) effects on plant growth (DW_PL) and DNA sequence (Pol_PL) and the following variables: total content of metal(loid)s in plant (TOT_Cd_PL and TOT_As_PL), concentration of metal(loid)s in plant ([Cd]_PL and [As]_PL). (*) indicates statistically different ($P < 0.05$)

4.2 Results-Experiments with soils collected in Lombardy region

4.2.1 Assessment of general toxicity through mortality and dry weight measurement

The same methodology used in the first part of the thesis was applied to assess the general toxicity of each soil: plant survival and plant development of *Trifolium repens* were assessed after 15 days of exposure to experimental treatments. Plant development was evaluated by measuring plant organ dry weight (DW).

The results obtained from the analysis of samples from each area are reported in the Table 7.

All plants exposed to soil fromt Pieve Fissiraga (Viscolube) and Brescia (agricultural area SIN) survived. For soils from the other locations, a significant number of dead seedlings were observed only for the soil O1/auto/2012 and for the soils CR3 and CR6/plume 2013 from the Origgio and Treviglio areas, respectively. The latter two soils also caused a reduction in the growth of plant shoots. Statistically significant variations in the growth of the shoots of seedlings (measured in terms of dry weight) were also observed for other soils from Treviglio area (CR2 and CR14 / plume / 2013) which did not induce significant plant mortality. Only the soils from the town of Broni (PV) induced a reduction in the growth of the plant roots, but among them only soil IT5 also led to a reduction of shoot growth. This would suggest that in the case of Broni, the variations in the growth can be attributed to the soil characteristics (particularly high presence of clays) more than to the presence of toxic substances in the soil.

Finally, some soils favored the growth of the shoots of the test plants. This phenomenon may be related to hormesis mechanism and/or to the possible presence of a larger amount of organic matter in these soils compared to the control.

In general, these data indicate that the contaminants present in the soils have minor or no effects on short-term survival and growth of the bioindicator. However it is necessary to emphasize that many hazardous substances, such as carcinogenic compounds, while not having immediate effects evident on growth, are responsible for the onset of disease in the long term.

Table 7: Growth parameters (Survival and dry weight) measured for test plants after 15 days of exposure to soils. *: statistical significant (P<0,05, Anova + Dunnet Test) in comparison with the control; in grey the values statistical lower than the control are highlighted.

		Survival		Dry weight g (Mean ± Standard Deviation)			pH
AREA	Sample	Mean	%	Root	Shoot	Entire Plant	
Viscolube Pieve Fissiraga	**V1**/visc/2012	36	100%	0,0044±0,0017	0,0161±0,0046	0,0204±0,0052	5,9
	V2/visc/2012	36	100%	0,0033±0,0019	0,0194±0,0027	0,0227±0,0034	6,3
	V3/visc/2012	36	100%	0,0038±0,0013	0,0186±0,0019	0,0224±0,0022	6,0
	V4/visc/2012	36	100%	0,0037±0,0013	0,0162±0,0031	0,0200±0,0042	6,1
	V5/visc/2012	36	100%	0,0033±0,0016	0,0191±0,0025	0,0223±0,0027	6,6
	V6/visc/2012	36	100%	0,0032±0,0016	0,0211±0,0021 *	0,0244±0,0022 *	6,0
	V7/visc/2012	36	100%	0,0037±0,0012	0,0229±0,0032 *	0,0266±0,0031 *	6,5
	V8/visc/2012	36	100%	0,0034±0,0007	0,0206±0,0036	0,0241±0,0041	6,3
Autostrada Origgio	**O1**/auto/2012	31 *	86%	0,0031±0,0012	0,0105±0,0052	0,0136±0,0049	4,7
	O2/auto/2012	36	100%	0,0048±0,0011	0,0279±0,0053 *	0,0327±0,0062 *	5,2
	O3/auto/2012	36	100%	0,0039±0,0003	0,0303±0,0051 *	0,0342±0,0053 *	5,3
	O4/auto/2012	36	100%	0,0040±0,0014	0,0311±0,0070 *	0,0352±0,0071 *	5,4
	O5/auto/2012	33	92%	0,0036±0,0009	0,0174±0,0032	0,0210±0,0038	4,7
	O6/auto/2012	36	100%	0,0037±0,0009	0,0193±0,0019	0,0229±0,0020	4,9
	O7/auto/2012	36	100%	0,0046±0,0014	0,0223±0,0055	0,0270±0,0065*	5,3
	O8/auto/2012	36	100%	0,0042±0,0008	0,0244±0,0055 *	0,0286±0,0059 *	5,4
Broni Italcementi	**IT1**/cem/2012	34	94%	0,0014±0,0006 *	0,0128±0,0050	0,0143±0,0054	7,5
	IT2/cem/2012	36	100%	0,0016±0,0011 *	0,0151±0,0043	0,0167±0,0044	7,7
	IT3/cem/2012	36	100%	0,0013±0,0011 *	0,0124±0,0020	0,0137±0,0027	7,8
	IT4/cem/2012	36	100%	0,0016±0,0009 *	0,0148±0,0036	0,0164±0,0037	7,9
	IT5/cem/2012	34	94%	0,0023±0,0006 *	0,0094±0,0018 *	0,0116±0,0015 *	7,9
	IT6/cem/2012	36	100%	0,0020±0,0011 *	0,0115±0,0026	0,0136±0,0029	8,1
	IT7/cem/2012	36	100%	0,0019±0,0008 *	0,0148±0,0042	0,0167±0,0044	8,0
	IT8/cem/2012	36	100%	0,0022±0,0006 *	0,0133±0,0017	0,0154±0,0020	8,0
	IT9/cem/2012	36	100%	0,0016±0,0006 *	0,0115±0,0016	0,0132±0,0014	7,8
SIN - Brescia Agricola	**S1**/sin/2012	36	100%	0,0025±0,0010	0,0134±0,0027	0,0158±0,0025	7,5
	S2/sin/2012	36	100%	0,0023±0,0008	0,0146±0,0024	0,0169±0,0030	7,7
	S3/sin/2012	36	100%	0,0035±0,0012	0,0173±0,0031	0,0208±0,0034	7,8
	S4/sin/2012	36	100%	0,0024±0,0005	0,0144±0,0008	0,0168±0,0008	7,8
	S5/sin/2012	36	100%	0,0040±0,0010	0,0195±0,0040	0,0235±0,0049	7,9
	S6/sin/2012	36	100%	0,0033±0,0009	0,0161±0,0014	0,0194±0,0013	7,9
	S7/sin/2012	36	100%	0,0028±0,0006	0,0127±0,0027	0,0155±0,0030	7,9
	S8/sin/2012	36	100%	0,0034±0,0005	0,0161±0,0033	0,0195±0,0036	7,7
Fonderia - Darfo Boario Terme	**F1**/fond/2012	34	94%	0,0023±0,0004	0,0148±0,0041	0,0171±0,0041	5,9
	F2/fond/2012	36	100%	0,0030±0,0008	0,0166±0,0034	0,0196±0,0035	7,3
	F3/fond/2012	36	100%	0,0031±0,0011	0,0309±0,0081 *	0,0341±0,0087 *	7,2
	F4/fond/2012	33	92%	0,0034±0,0015	0,0143±0,0020	0,0176±0,0027	6,3
	F5/fond/2012	36	100%	0,0032±0,0011	0,0159±0,0020	0,0191±0,0015	5,9
	F6/fond/2012	36	100%	0,0030±0,0011	0,0326±0,0063 *	0,0356±0,0059 *	7,6
	F7/fond/2012	34	94%	0,0059±0,0079	0,0169±0,0038	0,0228±0,0058	7,7
	F8/fond/2012	36	100%	0,0042±0,0008	0,0234±0,0052	0,0276±0,0053 *	6,2
Treviglio Plume Cromo esavalente	**CR1**/plume/2013	36	100%	0,0049±0,0023	0,0221±0,0046 *	0,0269±0,0062	7,1
	CR2/plume/2013	33	92%	0,0040±0,0017	0,0132±0,0045 *	0,0172±0,0052	7,5
	CR3/plume/2013	27 *	75%	0,0034±0,0010	0,0106±0,0036 *	0,0137±0,0039	7,0
	CR4/plume/2013	36	100%	0,0038±0,0009	0,0253±0,0043 *	0,0291±0,0044 *	6,2
	CR5/plume/2013	34	94%	0,0045±0,0011	0,0173±0,0029 *	0,0218±0,0031	7,4
	CR6/plume/2013	29 *	81%	0,0044±0,0019	0,0152±0,0039 *	0,0196±0,0056	6,3
	CR7/plume/2013	36	100%	0,0037±0,0009	0,0292±0,0094 *	0,0328±0,0094 *	7,0
	CR8/plume/2013	36	100%	0,0037±0,0006	0,0242±0,0041 *	0,0278±0,0045	7,1
	CR9/plume/2013	36	100%	0,0035±0,0016	0,0212±0,0040 *	0,0247±0,0033	6,8
	CR10/plume/2013	36	100%	0,0046±0,0017	0,0415±0,0087 *	0,0462±0,0102 *	7,1
	CR11/plume/2013	36	100%	0,0042±0,0014	0,0256±0,0056 *	0,0298±0,0053 *	7,7
	CR12/plume/2013	36	100%	0,0039±0,0013	0,0183±0,0042 *	0,0221±0,0050	6,6
	CR13/plume/2013	36	100%	0,0042±0,0008	0,0245±0,0048 *	0,0287±0,0046 *	7,2
	CR14/plume/2013	36	100%	0,0037±0,0019	0,0145±0,0033 *	0,0182±0,0050	6,6
Parona - Inceneritore	**P1**/term/2013	35	97%	0,0024±0,0013	0,0138±0,0027	0,0162±0,0036	4,6
	P2/term/2013	36	100%	0,0033±0,0016	0,0242±0,0059	0,0275±0,0072	5,7
	P3/term/2012	36	100%	0,0045±0,0011	0,0230±0,0044	0,0275±0,0047	7,3
	P4/term/2013	36	100%	0,0031±0,0010	0,0239±0,0067	0,0270±0,0068	5,4
	P5/term/2012	36	100%	0,0038±0,0013	0,0279±0,0052 *	0,0317±0,0045 *	6,3
	P6/term/2013	36	100%	0,0034±0,0011	0,0237±0,0070	0,0271±0,0069	5,1
	P7/term/2013	36	100%	0,0059±0,0014 *	0,0289±0,0085 *	0,0348±0,0089 *	5,5
	P8/term/2013	36	100%	0,0036±0,0012	0,0278±0,0076 *	0,0314±0,0083 *	5,6
	P9/term/2013	36	100%	0,0039±0,0016	0,0252±0,0057	0,0291±0,0067 *	6,1
	P10/term/2012	36	100%	0,0030±0,0005	0,0229±0,0053	0,0259±0,0056	6,5
	P11/term/2013	36	100%	0,0046±0,0005	0,0212±0,0027	0,0258±0,0025	5,4
	P12/term/2013	36	100%	0,0039±0,0011	0,0196±0,0051	0,0234±0,0049	5,2
	CONTROL	36	100%	0,0038±0,0011	0,0148±0,0038	0,0186±0,0035	

4.2.2 Assessment of genotoxicity by PCR-based RAPD profile analysis

DNA sequence damage induced by genotoxic substances eventually present in the soils collected in Lombardy areas, was assessd by RAPD molecular markers and was calculated as the percentage of polymorphism (P%). An example of gel obtained by the RAPD analysis of plants exposed to the soils is reported in Figure 30. It can be observed that in some lanes (soil samples) there is a lack of bands (arrows) which means that soil induced a damage to plant DNA and thus contains one or more genotoxic compounds.

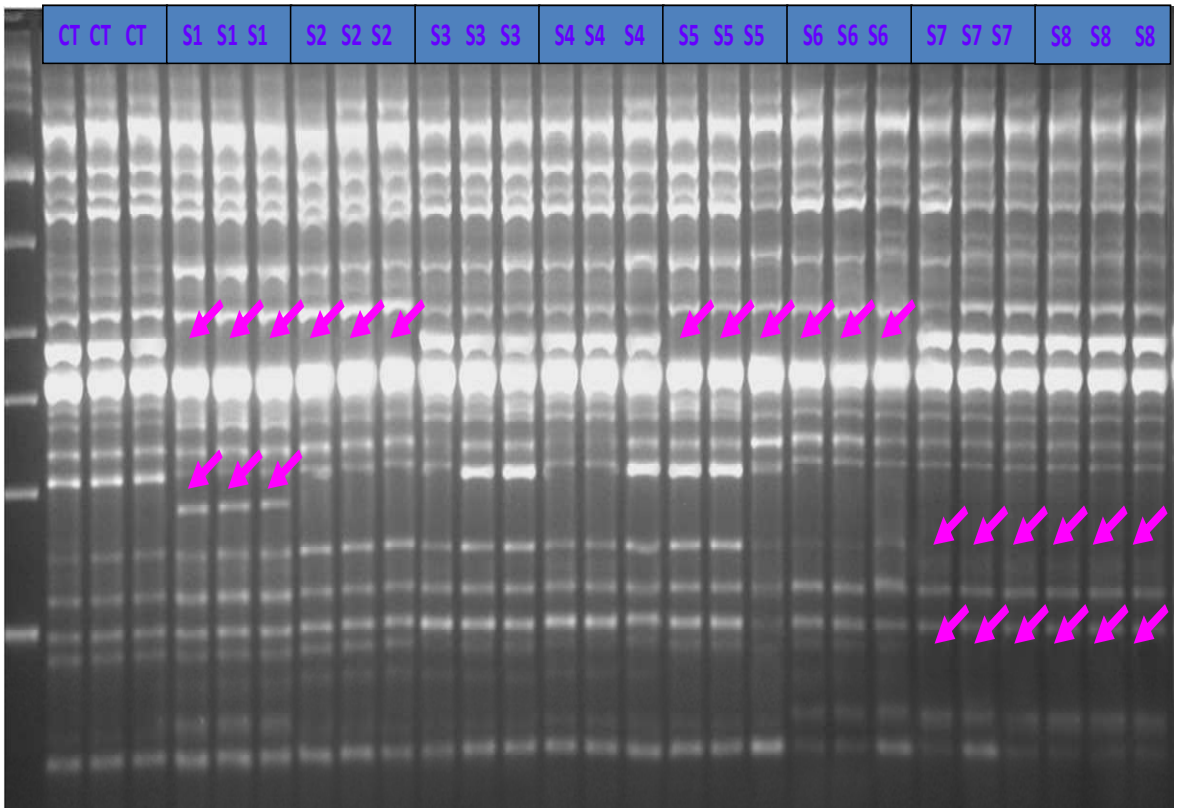

Figure 30: An example of RAPD analysis (primer OPC07) of root DNA from *Trifolium repens* exposed to soils (S1-S8) collected in Brescia province (Lombardy region) and to control (CT). Arrows indicate the principal polymorphic bands.

The results obtained by RAPD analysis are summarized in Figure 31. On the basis of the polymorphic percentage, soils were classified in the 4 classes:

Class	Polymorphism	Explanation
Class1	<6%	Non genotoxic
Class2	6%-20%	Moderately genotoxic
Class3	20%-35%	Genotoxic
Class4	>35%	Highly genotoxic

Area	Sample	Polymorphysm (%)			pH
		Root	Shoot	Plant	
Viscolube Pieve Fissiraga	**V1**/visc/2012	16	5	11	5,9
	V2/visc/2012	14	4	9	6,3
	V3/visc/2012	19	5	12	6,0
	V4/visc/2012	20	5	13	6,1
	V5/visc/2012	21	4	12	6,6
	V6/visc/2012	20	5	12	6,0
	V7/visc/2012	24	5	15	6,5
	V8/visc/2012	16	5	11	6,3
Autostrada Origgio	**O1**/auto/2012	17	6	11	4,7
	O2/auto/2012	14	11	12	5,2
	O3/auto/2012	9	8	8	5,3
	O4/auto/2012	13	17	15	5,4
	O5/auto/2012	17	13	15	4,7
	O6/auto/2012	18	10	14	4,9
	O7/auto/2012	13	19	16	5,3
	O8/auto/2012	12	11	12	5,4
Broni Italcementi	**IT1**/cem/2012	5	2	3	7,5
	IT2/cem/2012	4	2	3	7,7
	IT3/cem/2012	4	3	3	7,8
	IT4/cem/2012	3	1	2	7,9
	IT5/cem/2012	4	1	2	7,9
	IT6/cem/2012	4	2	3	8,1
	IT7/cem/2012	3	3	3	8,0
	IT8/cem/2012	5	1	3	8,0
	IT9/cem/2012	4	2	3	7,8
SIN - Brescia Agricola	**S1**/sin/2012	14	11	12	7,5
	S2/sin/2012	13	6	10	7,7
	S3/sin/2012	14	9	12	7,8
	S4/sin/2012	15	8	12	7,8
	S5/sin/2012	18	7	12	7,9
	S6/sin/2012	16	10	13	7,9
	S7/sin/2012	17	12	14	7,9
	S8/sin/2012	21	9	15	7,7
Fonderia - Darfo Boario Terme	**F1**/fond/2012	19	1	10	5,9
	F2/fond/2012	19	5	12	7,3
	F3/fond/2012	18	4	11	7,2
	F4/fond/2012	18	5	11	6,3
	F5/fond/2012	16	4	10	5,9
	F6/fond/2012	19	3	11	7,6
	F7/fond/2012	16	5	10	7,7
	F8/fond/2012	18	3	10	6,2
Treviglio Plume Cromo esavalente	**CR1**/plume/2013	5	12	9	7,1
	CR2/plume/2013	10	12	11	7,5
	CR3/plume/2013	11	10	10	7,0
	CR4/plume/2013	18	15	16	6,2
	CR5/plume/2013	12	12	12	7,4
	CR6/plume/2013	5	11	8	6,3
	CR7/plume/2013	15	14	15	7,0
	CR8/plume/2013	14	15	14	7,1
	CR9/plume/2013	14	11	12	6,8
	CR10/plume/2013	11	17	14	7,1
	CR11/plume/2013	15	4	10	7,7
	CR12/plume/2013	16	4	10	6,6
	CR13/plume/2013	18	11	15	7,2
	CR14/plume/2013	15	4	9	6,6
Parona - Inceneritore	**P1**/term/2013	20	4	12	4,6
	P2/term/2013	15	3	9	5,7
	P3/term/2012	16	7	12	7,3
	P4/term/2013	16	3	10	5,4
	P5/term/2012	18	7	12	6,3
	P6/term/2013	23	1	12	5,1
	P7/term/2013	22	1	11	5,5
	P8/term/2013	18	1	9	5,6
	P9/term/2013	18	3	10	6,1
	P10/term/2012	16	2	9	6,5
	P11/term/2013	14	1	8	5,4
	P12/term/2013	25	1	13	5,2

Fig 31: DNA damage (Polymorphism %) detected in test plants after 15 days of exposure to soils. Colors indicate the class of polymorphism.

The results reported in Fig 31 indicate that all the soils except those sampled in Broni (PV) induced damage to plant DNA (expressed as % of Polymorphism). However these DNA changes were few, so these soils were classified as moderately genotoxic.

It's interesting to note that in general the soils induced a higher DNA damage to root than to shoot, suggesting the presence of organic pollutants in addition to inorganics in some sites. Exception were soils O4-O7 and CR1-CR2-CR8-CR10 from Origgio and Treviglio, respectively, in which polymorphisms were more consistent in the shoot suggesting in this case the presence of inorganic pollutants which are more easily translocated to the aerial part of the plant.

It is also important to note that the genotoxic potential of a soil is strongly influenced by the bioavailability of contaminants. In fact, only if genotoxic pollutants are bioavailable can they be absorbed by the bioindicator and cause changes to DNA. The bioavailability of contaminants is regulated by several factors of which the most important are the soil pH, the redox potential, the content of organic materials, the presence of humic substances, and adsorbents (including clays). For example all the soils from Broni were alkaline, which the lack of damage to test plants, so these were classified as non genotoxic. In contrast, soils from Pieve Fissiraga and Origgio were acid, which might explain why limited concentrations of pollutant induced DNA changes.

5. Discussion and conclusion

5.1 Experiments with As and Cd contaminated soils

Cd and As are two of the main environmental contaminants, often occurring simultaneously in polluted sites. Although, their individual toxicity and genotoxicity are well known, few data are available on their joint effects; in particular no information is available on their joint genotoxic action. In this study, the effect of combined concentrations of Cd and As on the growth and DNA damage of *Trifolium repens* was investigated, by using a sensitive plant to metals, widely used in biomonitoring campaigns. Plants are efficient bioindicators to get information on the cumulative effects of environmental pollutants. They are used as early warning systems for preventing environment alterations and human diseases. However, given the complexity of the mechanisms causing the final effects, the results obtained through bioindication systems should be better interpreted if the knowledge about the interaction of pollutants had improved.

Individual and joint effects of soil inorganic pollutants on bioindicators depend on different factors. First of all, at soil level, the mobility of chemicals influences the amount of compounds which can be absorbed by test-plant. Nevertheless, the uptake is not only dependent on pollutant bioavailability but it is also dependents on plant uptake mechanisms, which are compound-specific. In addition plants possess detoxification strategies, such as metal exclusion, which influence the final concentration of compounds inside the cells (Verbruggen et al., 2009; Hossain et al., 2012). Finally when two or more compounds are simultaneously present in soil, the toxic final effects depend also on the interaction among pollutants which can occur at all levels.

In this experiment, it was found that all the individual concentrations of Cd and As, selected for the experiment, did not induce any effect on plant survival and growth, whereas they induced a DNA damage related to the metal(loid) concentration measured in plant organs. Moreover, it was also found that some of the tested combined concentrations of Cd and As produced a synergistic effect on plant growth and an antagonistic effect on DNA, suggesting an interaction between the two compounds.

In order to understand the main factors which determined the results, the soil bioavailability of Cd and As and the total amounts and concentrations of metal(loid)s accumulated in plant organs were measured.

Concerning soil Cd and As bioavailability, in keeping with literature, Cd was much more bioavailable than As (Smith et al., 1999; Luan et al., 2008; Sun et al., 2008; Verbruggen et al., 2009). The very low availability of As measured can be ascribed to the form of As that was used to contaminate the soil (arsenite) along with an alkaline soil pH. In fact (Smith et al., 1999) observed that the proportion of arsenite sorbed by soil increased with increasing pH. Specifically they observed that sorption by the soil ranged from approximately 0.80 of added As(III) at low pH, to approximately 0.95 of added As(III) at pH 6 to 7. In addition the low availability of As that we recorded should be related to the DTPA-based method that we used. This method was applied because according to several studies, it provides the prediction of trace elements uptake by plants from soils. In particular, Karak et al. (2011) showed a very high correlation between DTPA-extractable As and the labile pool of As suggesting that the latter is the portion of As most hazardous for human health, due to the possibility of entering the food chain.

Interestingly, for both the metals, bioavailability increased with increasing metal in the soil only when the two compounds were used individually, whereas, when they were simultaneously used to contaminate soil, the presence of Cd increased the amount of bioavailable As and on the contrary the presence of As reduced the Cd bioavailability. The reduction of Cd bioavailability in presence of As was also observed by Sun and collaborators (Sun et al., 2008). This type of result suggests a sort of competition between the two metal(loid)s for binding with soil constituents (clays, Al or Fe or Mn oxides, organic matter etc). Generally, both Cd and As retention in soil is due to their primary association to organic matter and amorphous Fe and Mn oxides (Keil et al., 2011; Karak et al., 2011; Gonzaga et al., 2008). It is then likely that in this experiment the interaction between Cd and As, involved these soil constituents. Anyway, given the different characteristic of As and Cd, it is very difficult to understand the mechanism determining the bioavailability changes that were observed when the two compounds were simultaneously present in a soil and further work beyond the aim of the present study is needed to clarify the Cd and As sorption-desorption processes.

In any case in the experiment, bioavailability was a very important factor for As accumulation, given the linear correlation found between the total As in plant and soil As bioavailability.

The result was consistent with previous works (Luan et al., 2008; Sun et al., 2008; Fayiga and Ma, 2005) showing a significant ($p < 0.01$) correlation between As uptake by plants in various treatments and total soil As. On the contrary, regression analysis indicated that Cd accumulation was not linearly correlated to soil bioavailability. This is also in agreement with previous studies which showed that the uptake of Cd by plant increases proportionally to increasing soil Cd only up to about 20 mg kg^{-1} above which the trend becomes curvilinear (Smolders, 2001). The different behavior of the two metal(loid)s could be explained by considering their absorption mechanisms. The uptake of Cd from the soil occurs mainly via Ca^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} transporters (Clemens, 2006), whereas that of As(III) (the form that we used for contamination which likely represents the main form in our soils) occurs mainly by diffusion across membrane through members of the NIP (nodulin 26-like intrinsic protein) subfamily of aquaporins (Bienert et al., 2008; Isayenkov and Maathuis, 2008). Thus it can be assumed that in conditions of this experiment, the main factor determining As accumulation in white clover was bioavailability, whereas the limiting factor for Cd accumulation was related to the uptake system. Moreover, the possible combination of that fraction of arsenate [As (V)], likely formed in soil from [As (III)], with Cd ($Cd^{2+} + AsO_4^{3-} \rightleftharpoons Cd_3(AsO_4)_2$) could have decreased the ion activity on the root surfaces playing a role in the depression of Cd uptake, as demonstrated by Liu and Zhang (2007a) and explaining the reduction of Cd accumulation that was observed in plants grown in presence of both the metal(loid)s.

Interestingly, as shown by RDA analysis, in this study the accumulated total amounts of Cd and As in plant organs were not statistically significant to explain the observed toxic and genotoxic effects. This because some treatments induced a plant organ reduction, so that the effects were related to the concentration of metal(loid)s measured in plant organs and not to the total absorbed amounts. Specifically As concentration was the most important variable due to both its intrinsic toxicity, that was higher than that of Cd at equal concentration (in agreement with Luan and collaborators, 2008), and to its chemical characteristics allowing a plant uptake

proportional to soil bioavailability which was also increased by the presence of Cd in soil. Moreover, although the concentration of Cd was also important in determining the observed effects, it should be considered that, differently from As(III) which is chemically neutral, a fraction of the total amount of Cd^{2+} accumulated in plant organs was likely stored in cell walls, as the negative charges of the cell wall bind and retain heavy metals (Polle and Schützendübel, 2003; Lux et al., 2010). It is one of the several mechanisms evolved by plants to cope with Cd^{2+}, limiting intracellular internalization and associated toxicity (Clemens, 2006; Zhu et al., 2013).

Concerning the observed toxic effect, a reduction of plant growth was induced by most of the combined concentrations of Cd and As tested. The type of interaction between the two metal(loid)s was additive except for the combinations of the higher Cd concentration (Cd 60) with any As concentration, which were synergistic. Joint Cd and As toxicity on plant growth was previously investigated with contrasting results. For instance, Luan and collaborators (2008) reported a synergistic effect on soybean plants. On the contrary, Liu and Zhang (2007) and Sun et al (2008) observed an antagonistic effect on wheat and rice biomass production. The divergent results are probably due to the different experimental conditions and to the plant mechanisms of response to metal stress which are species-specific and even development stage and organ specific (Tkalec et al., 2014). White clover is a pollutants-sensitive plant and lack of consistent tolerance mechanisms. For this reason it cannot tolerate high concentrations of metal(loid)s, whose effect can be exacerbated when they acts simultaneously. Accordingly, in this experiment a synergistic effect on plant growth was observed in those plants showing a higher total concentration of metal(loid)s. Likely a consistent inhibition of enzymes due to the high Cd and As reactivity to sulfhydryl groups (–SH) along with oxidative stress and deregulation of homeostasis of essential element or their displacement from protein, primarily due to Cd chemical similarity to Zn Cu and Fe, led to the inhibition of cellular functions and growth.

In addition the observed plant growth reduction could be associated to an arrest of cell cycle specifically induced by plant in response to high DNA damage caused by high concentrations of metal(loid)s. The temporary inhibition of cell cycle progression and DNA synthesis would provide a longer time for DNA repair and for the production of free radical scavengers. In support of this hypothesis it was found an antagonistic

genotoxic effect in most of the combined treatments. The antagonism could be also related to the similar genotoxic mechanisms of Cd and As involving the induction of ROS and the inhibition of DNA repair enzymes which could be reach a maximum in presence of a defined concentration of metal(loid)s beyond which it does not increase. Anyway, further investigations are needed to clarify the cellular molecular mechanisms involved in the interaction between Cd and As.

In conclusion, the results of this experiment showed that Cd and As can interact at different levels producing additive, synergistic or antagonistic effects. In this experimental condition, in soil the Cd presence increased As bioavailability whereas As presence reduced Cd bioavailability. Nevertheless bioavailability determined the absorption of As but not that of Cd which was likely limited by its uptake mechanisms. Toxicity and genotoxicity were related to the total concentration of Cd and As in plant organs and As concentration was the most significant variable. Joint effects on plant growth were additive or synergistic, whereas joint genotoxic effects were additive or antagonists. It was supposed that growth reduction was due to both toxic effects of Cd and As and plant response to high DNA damage, which has led to a temporary arrest of cell cycle providing a longer time for DNA repair and for the production of free radical scavengers. This hypothesis is consistent with the antagonistic genotoxic effect observed in most of the combined treatments. Nevertheless the antagonistic interaction of Cd and As could be also associated to the similar genotoxic mechanisms own of the two metal(loid)s.

5.2 Experiments with soils collected in Lombardy region

Soil pollution is a very important environmental problem which has been attracting considerable public attention over the last decades. Sewage sludges, fertilizers, manure and pesticides applied from agricultural activities are distributed on the soil. Pollutants dispersed in the atmosphere from industrial and traffic activities could settle on the soil. These can cause a negative impact over time. Soil is considered as a sink of environmental pollutants, both inorganic and organic pollutants, non-genotoxics and genotoxics. Many genotoxic pollutants have been introduced into soils through anthropogenic pathways such as improper disposable of industrial wastes, wastewater irrigation, pesticide application and accidental leakage/spoilage occurring during

transport and storage of industrial materials with increasing industrial production and organic waste release (Ansari and Malik, 2009). Furthermore the physiochemical and biological reactions of organic and inorganic pollutants with naturally occurring inorganic compounds in soil might lead to the formation of by-products which are mutagenic or genotoxic (Song et al., 2006). The complexity of contaminant composition can make difficulty the evaluation of genotoxic potential through conventional chemical and physical analysis because standard chemical and pedological analyses are limited in their ability to characterize the chemical composition of genotoxicants in soil (Alam et al., 2009). Soil pollutants can induce additive, antagonistic or synergistic effects and soil microflora can convert non-genotoxic compounds to genotoxic derivatives (Piraino et al., 2008). Moreover, the interaction of genotoxics is affected by living species, types of genotoxics and environmental factors (temperature, humidity, light, soil pH, CEC, Eh, organic matter content, etc). Bioassays provide a means of assessing the genotoxicity of complex mixtures without the need for precise chemical characterization (Alam et al., 2009). The use of efficient early warning bioindication systems represents a powerful approach for assessing and interpreting the impact of natural or anthropogenic perturbations in soil ecosystems preventing environmental alteration and human disease. Living organisms provide information on the cumulative effects of environmental stressors and as such bioindication is complementary to direct physical and chemical measurements (Heger et al., 2012). In part 2 of this study, a strategy which is based on plant biomonitors was applied to evaluate the genotoxic potential of the soil environment in Lombardy region. Many previous studies were carried out and have demonstrated that plants growing in or close to environment polluted by genotoxics from agricultural, industrial and traffic activities showed the significant DNA damage compared to that growing in unpolluted environment (Sriussadaporn et al., 2003; Piraino et al., 2006; Aina et al., 2008; Cansaran-Duman et al., 2011). Recently, Salem et al (2014) reported that fishes living in surface water body polluted with heavy metals from municipal leachates also showed high DNA damage compared to that living in unpolluted water environment.

There are several limitations for biological tests. The first is the different reactions of various organisms to the same environmental factor. A second limitation is that

bioassays depend on environmental conditions, and not only on weather or season, but also on different micro-conditions in the test sites (Čėsnienė et al., 2010). However, in this study experimental plants in the control treatments and tested treatments (soils) grew in the same growing chamber. So, artificial environmental conditions for plant growing (temperature, humidity, light density, water and so on) were equal to both treatments. Another disadvantage for bioassays in the case of polymorphism test is that polymorphic bands can occur between plants within a species. To minimize these limitations and standardize results achieved from analysis of polymorphic bands, polymorphism evaluation was also performed within the plant individuals of the control. Aina et al. (2006) reported that approximately 4.8% and 3.9% of reproducible bands were polymorphic among the control shoot and root of *Trifolium repens* L, respectively. These values were considered as a basal polymorphic level among *Trifolium repens* L. plants, representing the intraspecies variability. Based on DNA damage levels induced by genotoxics in *Trifolium repens* DNA carried out by Citterio and collaborators (2002), polymorphism values can be divided into four levels (%): 0–6, no genotoxicity; 6–20, low genotoxicity; 20–35, medium genotoxicity and above 35, high genotoxicity.

In the present study soil samples were collected in 7 areas of concerns within Lombardy Region. The potential toxicity and genotoxicity of the soils were assessed by using the bioindication system set up by Citterio et al. (2002) and based on the use of white clover as plant bioindicator and molecular markers as tool to determine DNA damage.

Potential toxicity was assessed by measuring growth parameters (plant surviving and dry weight).

In the following pages the results obtained for each of the seven areas will be discussed considering the mean values of toxicity and genotoxicity parameters and the characteristics of the areas.

Table 8: The mean values of toxicity and genotoxicity parameters and the soil characteristics of the PIEVE FISSIRAGA area

PIEVE FISSIRAGA AREA		Mean	SD	Law limit
Survival (%)		100	0	-
Dry weight (g)	Root	0,0036	0,0013	-
	Shoot	0,0192	0,0029	-
	Entire plant	0,0228	0,0034	-
Polymorphism (%)	Root	19	3	-
	Shoot	5	1	-
	Entire plant	12	2	-
pH		6,2	0,3	-
C organic (%)		1,53	0,30	-
Inorganic elements higher than law limits	Sn mg/kg	1,4	0,99	A(152/06)

The results of the biological tests indicate that in the soils collected in the Pieve Fissiraga area (Table 8) there are compounds (inorganic and/or organic) that do not affect the growth of bioindicator but have a moderate genotoxic activity. As the genotoxic damage involved only the root system it is likely that the cause has to be found among the organic compounds that are unlikely translocated to the shoot.

This observation is supported by the features of the Pieve Fissiraga area, which is characterized by the presence of a waste oil refining company (Viscolube) founded in 1963. In 2010 this company processed about 130,000 tones of waste oil to produce over 80,000 tons of high quality oils, reducing simultaneously drastic sulfur content. Oils from Viscolube plant can be then responsible for the soil genotoxicity observed in this area.

Table 9: The mean values of toxicity and genotoxicity parameters and the soil characteristics of the ORIGGIO area

ORIGGIO AREA		Mean	SD	Law limit
Survival %		97	5	-
Dry weight (g)	Root	0,0040	0,001	-
	Shoot	0,0229 *	0,0048	-
	Entire plant	0,0269 *	0,0052	-
Polymorphism (%)	Root	14	3	-
	Shoot	11	5	-
	Entire plant	12	3	-
pH		5,1	0,3	-
C organic (%)		1,4	0,17	-
Inorganic elements higher than law limits	Sn mg/kg	1,6	0,84	A (152/06)

The soils of this area (Table 9) induced a moderate DNA damage in both shoot and root, suggesting the presence of bioavailable (due to the acidity of the soil) genotoxic inorganic substances, which were translocated to the shoot. This is also supported by the hormetic effect (biomass increase due to the presence of low concentrations of inorganic) induced by the soil on the growth of bioindicator. The cause of the observed genotoxicity could be then due to the presence of inorganics, which, even if present in limited concentration, may act in an additive/synergistic way. The presence of a heavily trafficked highway is likely the source of this kind of pollutants.

Table 10: The mean values of toxicity and genotoxicity parameters and the soil characteristics of the BRONI area

BRONI AREA		Media	SD	152/06
Survival %		99	2	-
Dry weight (g)	Root	0,0017 *	0,0009	-
	Shoot	0,0128	0,0032	-
	Entire plant	0,0146	0,0033	-
Polymorphism (%)	Root	4	1	-
	Shoot	2	1	-
	Entire plant	3	0	-
pH		7,9	0,2	-
Texture	Clay %	39,5	5,5	-
	Silt %	43,9	4,6	-
	Sand %	16,6	6,4	-
C organic %		1,42	0,30	-
Inorganic elements higher than law limits	Cu mg/kg	192,3	117,98	A
	Sn mg/kg	2,0	1,26	A

On average, the soils from Broni area did not induce any toxicity/genotoxicity in the bioindicator. The decrease in root growth compared to the control was probably due to the texture of the soil (high clay percentage). It must be also noted that the alkaline pH of the soil limited the bioavailability of inorganic elements (Table 10).

Broni area is characterized by the presence of a cement factory. The lack of negative effects that I found analyzing the test plants could be explained taking into account that, although the cement plant opened in 1962, occupies an area of 4.6 hectares and produces 240,000 tonnes of clinker per year and 380,000 tones of cement per year, the plant's activity has committed to reduce and prevent the risk of soil contamination. For example, with regard to the emission of dust, sulfur dioxides and nitrogen oxides, the plant has used filtering systems and their values are constantly monitored 24 hours everyday. The plant air quality is also tested through the analysis of the honey produced by bees specifically placed inside the perimeter of the plant. So, pollutants emission may have been controlled strictly to prevent their release into the surrounding area.

Table 11: The mean values of toxicity and genotoxicity parameters and the soil characteristics of the BRESCIA area

BRESCIA AREA		Media	SD	152/06
Survival %		100	0	-
Dry weight (g)	Root	0,0030	0,0008	-
	Shoot	0,0155	0,0025	-
	Entire plant	0,0185	0,0028	-
Polymorphism (%)	Root	16	3	-
	Shoot	9	2	-
	Entire plant	13	2	-
pH		7,7	0,1	-
Texture	Clay %	9,7	4,0	-
	Silt %	46,4	4,9	-
	Sand %	43,9	5,8	-
C organic %		2,7	0,39	-
Inorganic elements higher than law limits	As mg/kg	71,0	58,62	B
	Pb mg/kg	203,5	185,34	A
	Cu mg/kg	137,5	62,76	A
	Zn mg/kg	276,0	159,40	A
	Hg mg/kg	5,9	6,59	B
	Sn mg/kg	4,6	2,58	A

Overall, the results obtained in the Brescia area (Table 11) indicate the presence of potentially genotoxic substances in the soil, which did not affect the survival and growth of the bioindicator because they probably were not highly bioavailable. These soils are known to be contaminated with PCBs, mercury and arsenic, which together would induce high genotoxic damage. The moderate damage found can be probably attributed to the alkaline pH of soils which surely restricted the inorganic contaminant availability and to the presence of PCBs congeners with a high number of chlorine atoms, which are less bioavailable than the low chlorinated PCBs (Anyasi and Atagana, 2011).

Table 12: The mean values of toxicity and genotoxicity parameters and the soil characteristics of the BOARIO TERME area

BOARIO TERME AREA		Media	SD	152/06
Survival %		98	4	-
Dry weight (g)	Root	0,0035	0,0011	-
	Shoot	0,0207	0,0044	-
	Entire plant	0,0242	0,0047	-
Polymorphism (%)	Root	18	1	-
	Shoot	4	1	-
	Entire plant	11	1	-
pH		6,8	0,8	-
C organic %		2,18	0,64	-
Inorganic elements higher than law limits	As mg/kg	58,9	32,82	B
	Sn mg/kg	1,6	0,84	A

On average, the results show no evidence of toxicity (mortality and reduced growth) of soils from the Boario Terme area (Table 12). Nevertheless the analysis of genotoxicity allowed us to classify theses soils as moderately genotoxics. Polymorphism percentages suggest the presence of bioavailable genotoxic substances in the soil that are not translocated to shoot. The presence of arsenic could explain the results. In fact thanks to the experience and data that I acquired during the first part of my thesis I can state that As is low translocated to clover shoot and that the presence of the “sole” As in the soil, although inducing genotoxicity to the bioindicator, cannot reduce its growth.

The steel industry present in this area can be the source of arsenic.

Table 13: The mean values of toxicity and genotoxicity parameters and the soil characteristics of the TREVIGLIO area

TREVIGLIO AREA		Media	SD	152/06
Survival %		96	8	-
Dry weight (g)	Root	0,00404	0,00139	-
	Shoot	0,02160 *	0,00485	-
	Entire plant	0,02562 *	0,00541	-
Polymorphism (%)	Root	13	4	-
	Shoot	11	4	-
	Entire plant	12	3	-
pH		7	0,4	-
C organic %		1,78	0,52	-
Inorganic elements higher than law limits	Zn mg/kg	175,1	165,9	A
	Sn mg/kg	2,2	1,52	A

The data obtained indicate the presence of bioavailable genotoxic contaminants in the soil, which can be translocated to the shoot. As in the case of the Origgio area the induction by the soil of a hormetic effect is in agreement with the presence of low concentrations of inorganic bioavailable compounds that, even when present in very low concentrations, may act in an additive/synergistic way. This area was considered in this study because in the past it was identified as an area contaminated by Cr(VI). Low bioavailable concentration of Cr, which were no more found in the soil by chemical analysis, and/or other inorganic compounds such as Zn can be the cause of the observed negative effects on the bioindicator.

Table 14: The mean values of toxicity and genotoxicity parameters and the soil characteristics of the PARONA area

PARONA AREA		Media	SD	152/06
Survival %		100	0	-
Dry weight (gr)	Root	0,0038	0,0011	-
	Shoot	0,0235 *	0,0056	-
	Entire plant	0,0273 *	0,0059	-
Polymorphism (%)	Root	18	3	-
	Shoot	3	2	-
	Entire plant	11	2	-
pH		5,7	0,7	-
C organic %		1,0	0,34	-
Inorganic elements higher than law limits	Sn mg/kg	1,2	0,75	A

The results suggest the presence of inorganic and/or organic substances in the soil (Table 14), which were bioavailable, potentially genotoxic, but that were not translocated to the clover shoot. Given the absence of inorganic elements exceeding lawful limits (othern than Sn whose limit is not reliable) the genotoxic activity can be ascribed to additive/synergic effects of individual elements (present at low concentration) and/or organic substances. Their source could be the waste treatment plant located in the area.

Overall the results from the seven areas examined showed that the quality of most of these soils is poor and that remedial actions should be started as soon as possible. In fact the potential risk due to the contaminant bioaccumulation and transfer to the food chain, that has humans as ultimate consumers, must not be underestimated.

GENERAL CONCLUSION

Results from this study showed that *Trifolium repens* is a sensitive plant not only to organic genotoxics but also to inorganic genotoxics confirming the data reported by other authors (Citterio et al, 2002; Piraino et al., 2006; Aina et al., 2008). In addition they showed that RAPD technique is a powerful and useful tool for detecting DNA damage induced by organic and inorganic genotoxic compounds, especially non-lethal levels of contaminants. Since there were many kinds of contaminants in the soils, which can induce DNA damage and since their co-exposure may also cause genotoxic effects, even if the concentration of individual contaminant is very low (Feng et al., 2007), it is clear that soil genotoxicity assay is a valuable complement to chemical analyses not only in supplying useful information of soil containing multi-genotoxics but also in identifying the potential ecological risks of pollutants brought in to the soil ecosystem (Song et al., 2006).

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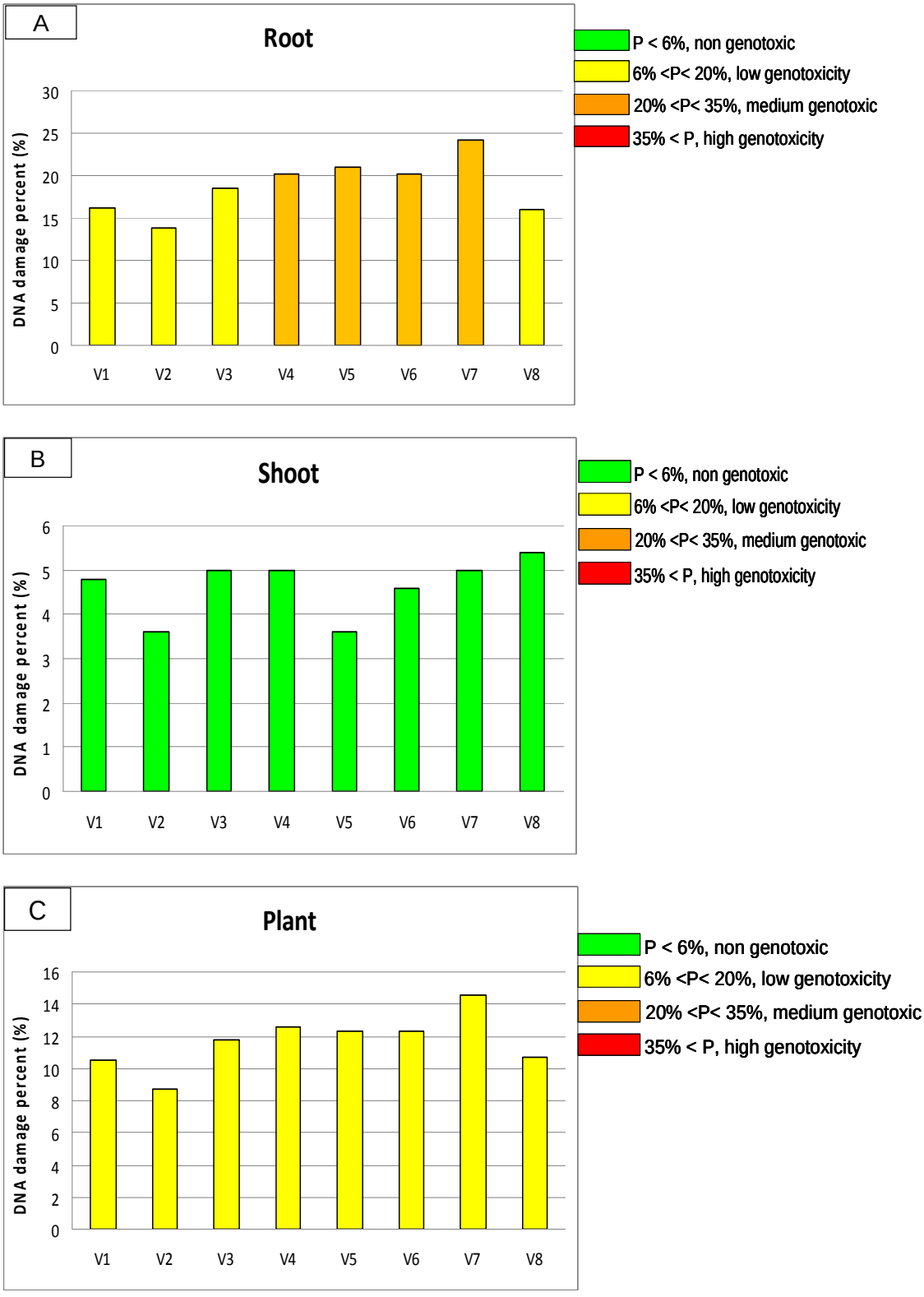
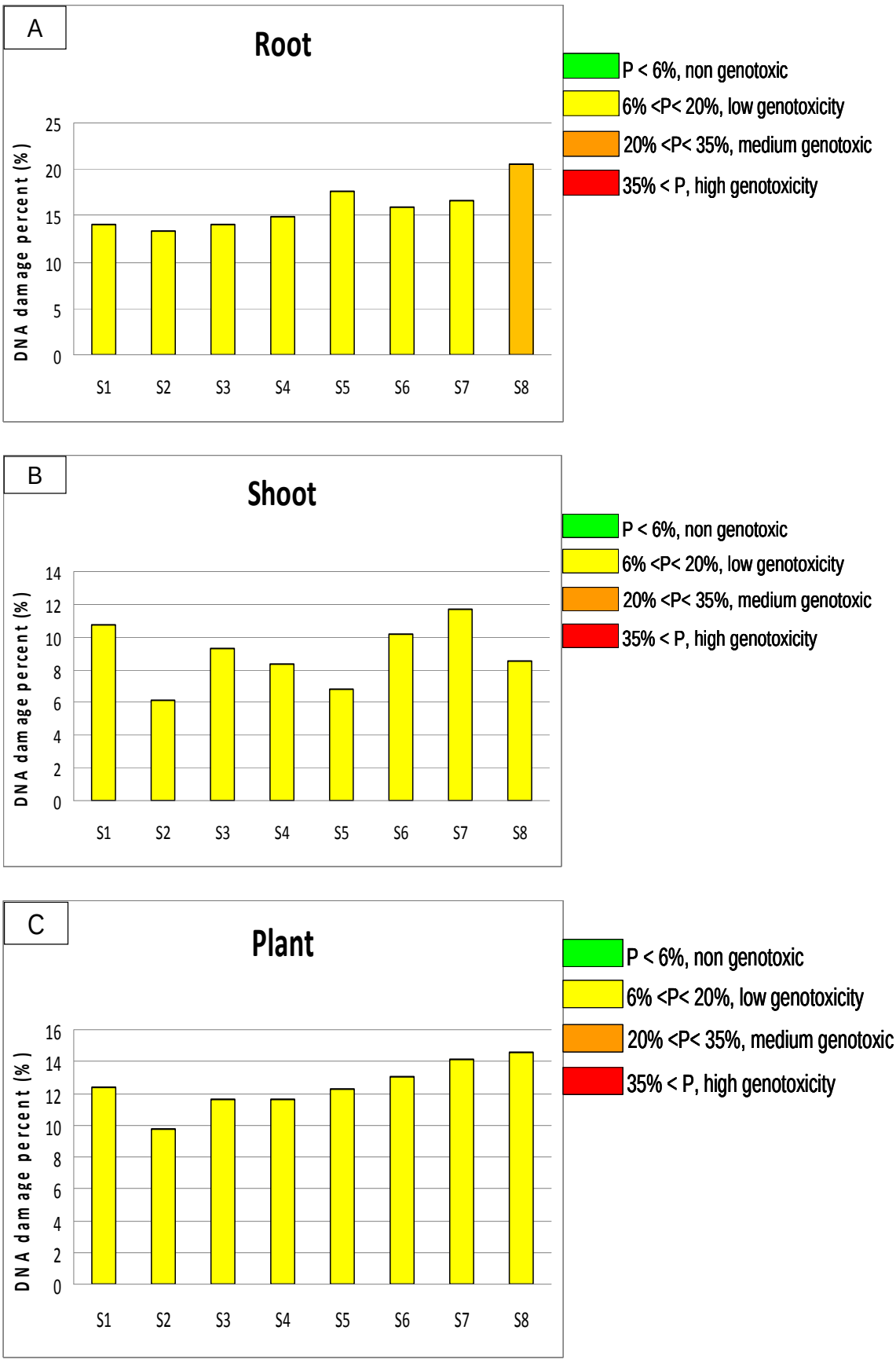
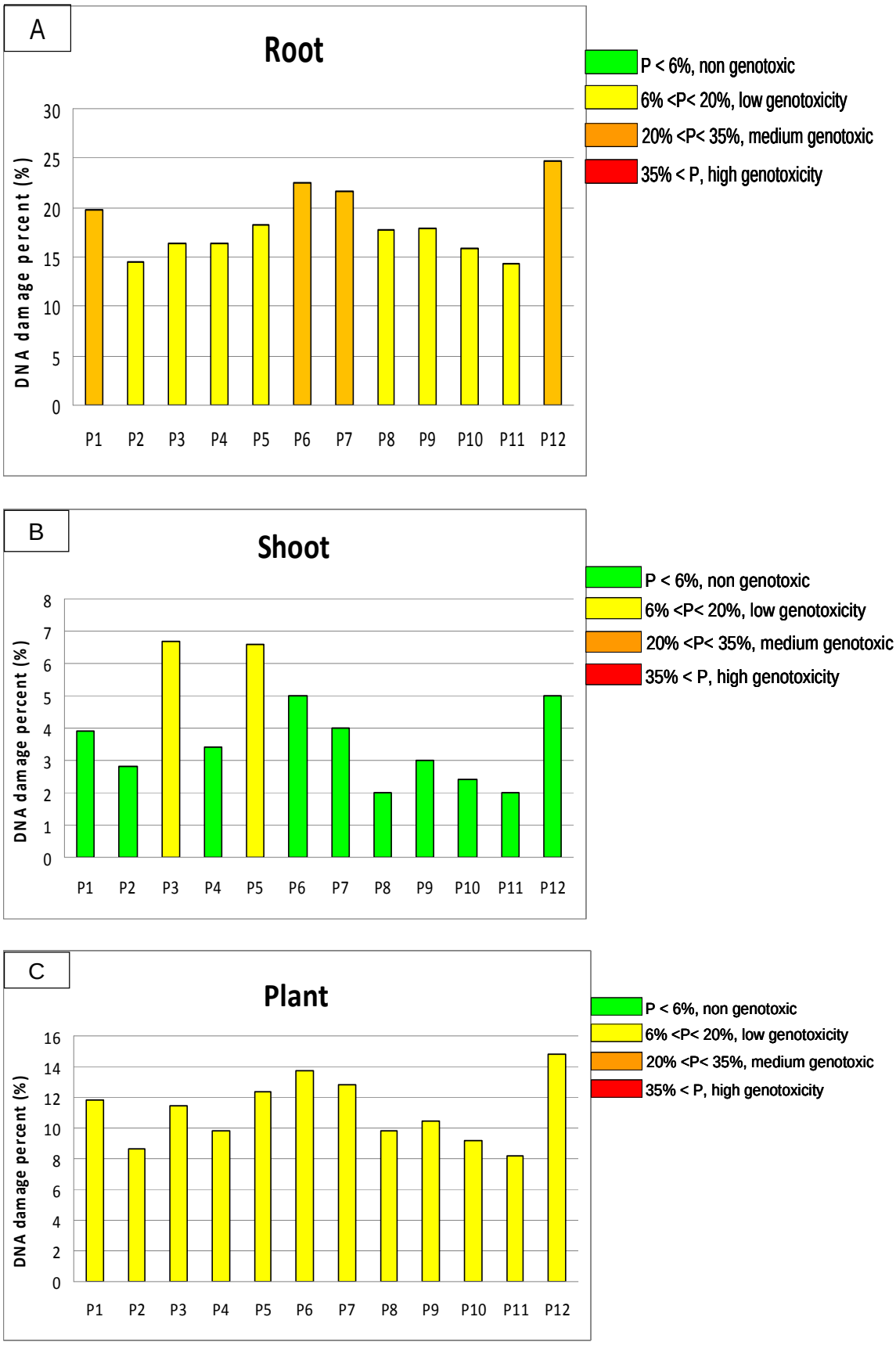
APPENDICES



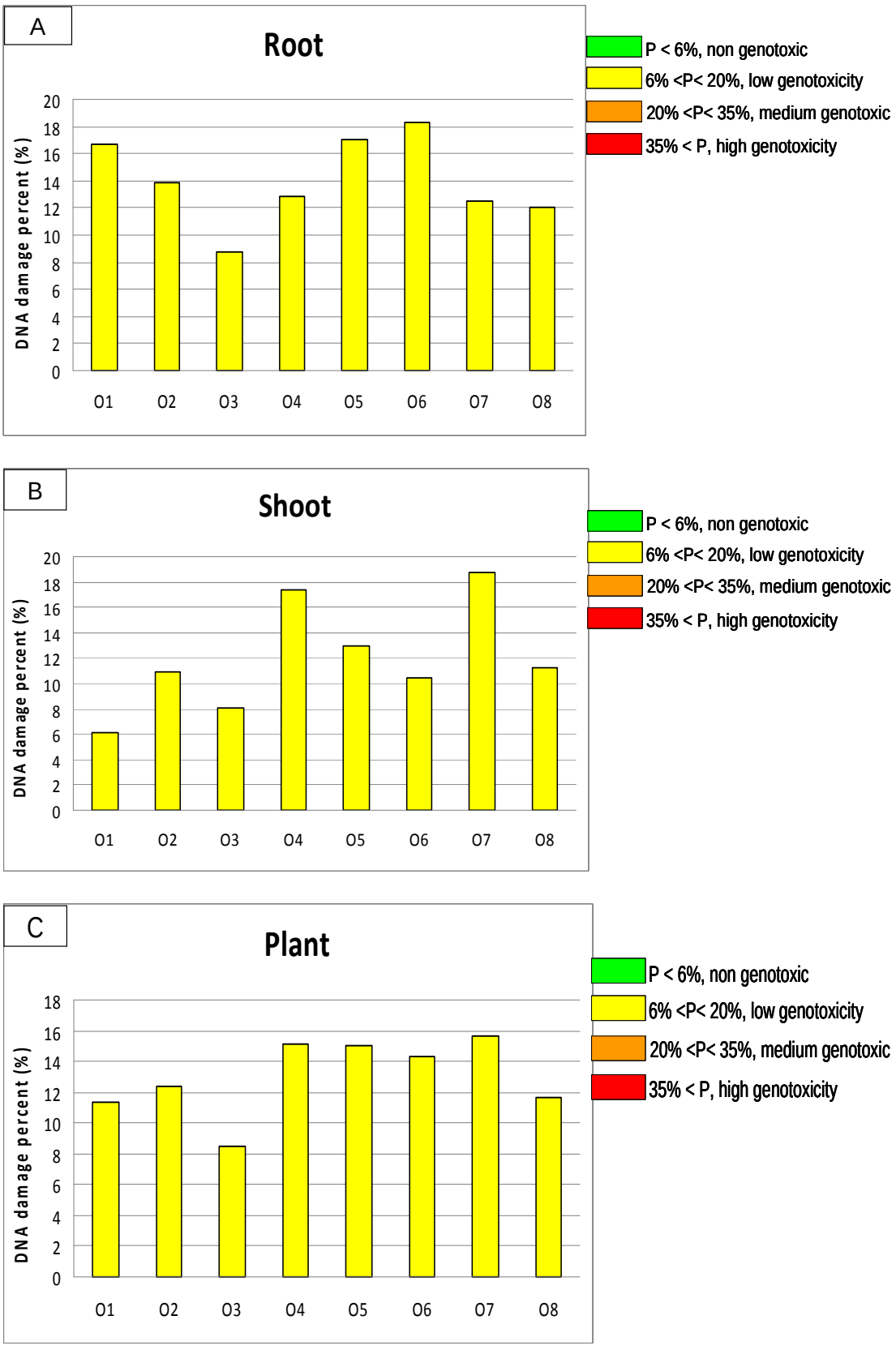
Appendix 1: DNA damage (Polymorphism %) detected in root, shoot and plant of *Trifolium repens* after 15 days of exposure to soils collected in Pieve area, Lodi province, Lombardy region.



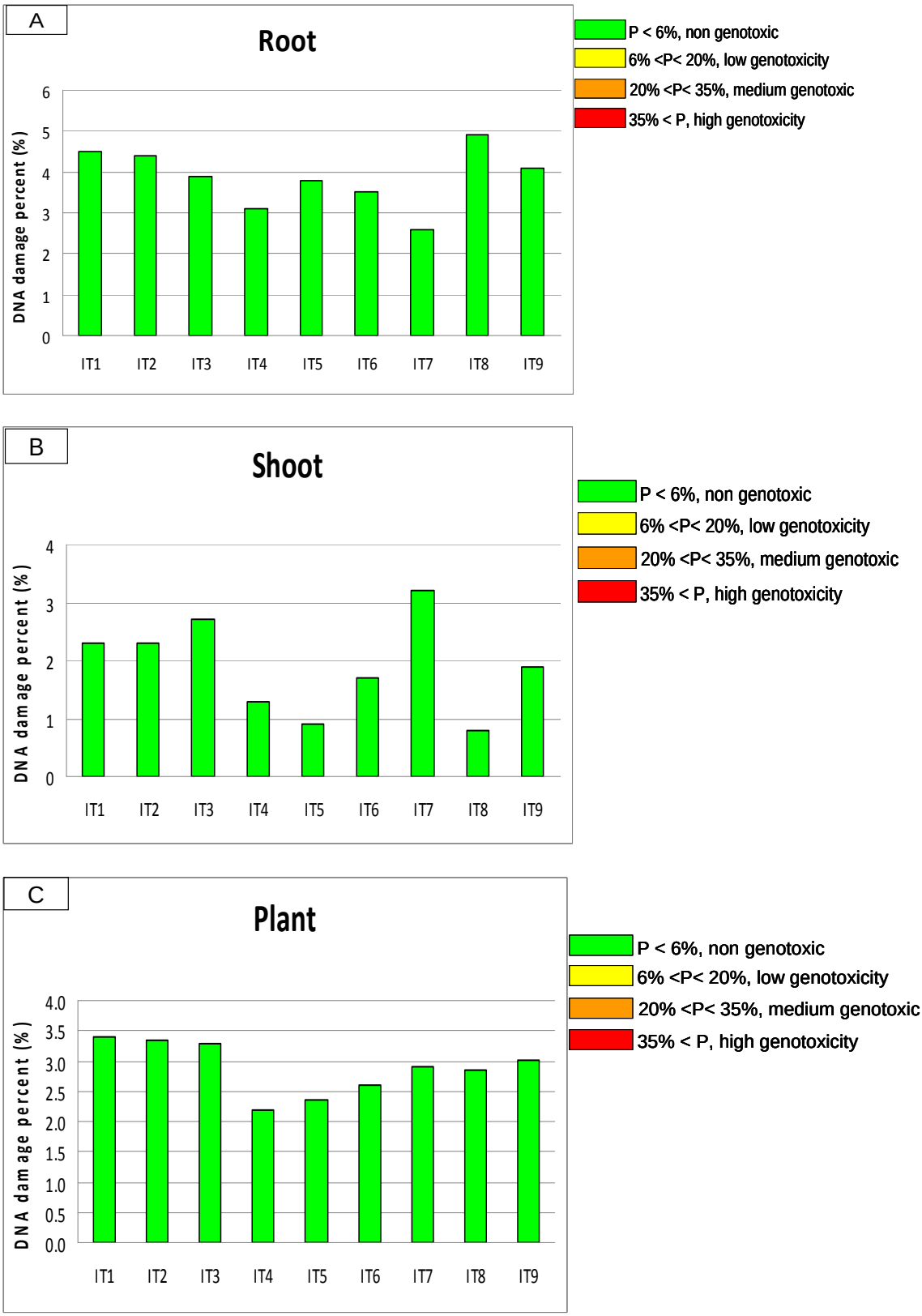
Appendix 2: DNA damage (Polymorphism %) detected in root, shoot and plant of *Trifolium repens* after 15 days of exposure to soils collected in Brescia Agricola area, Brescia province, Lombardy region.



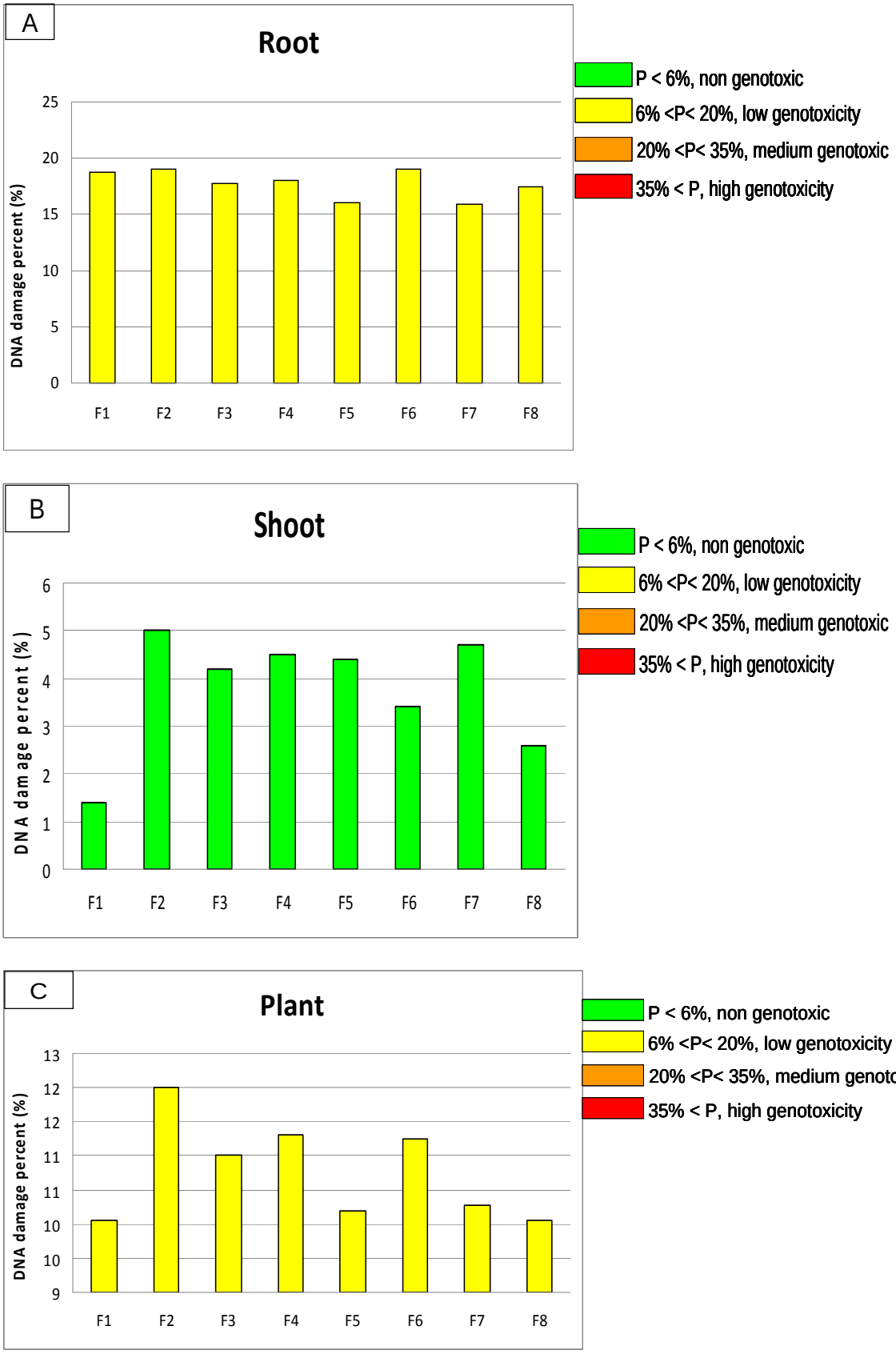
Appendix 3: DNA damage (Polymorphism %) detected in root, shoot and plant of *Trifolium repens* after 15 days of exposure to soils collected in Parona area, Pavia province, Lombardy region



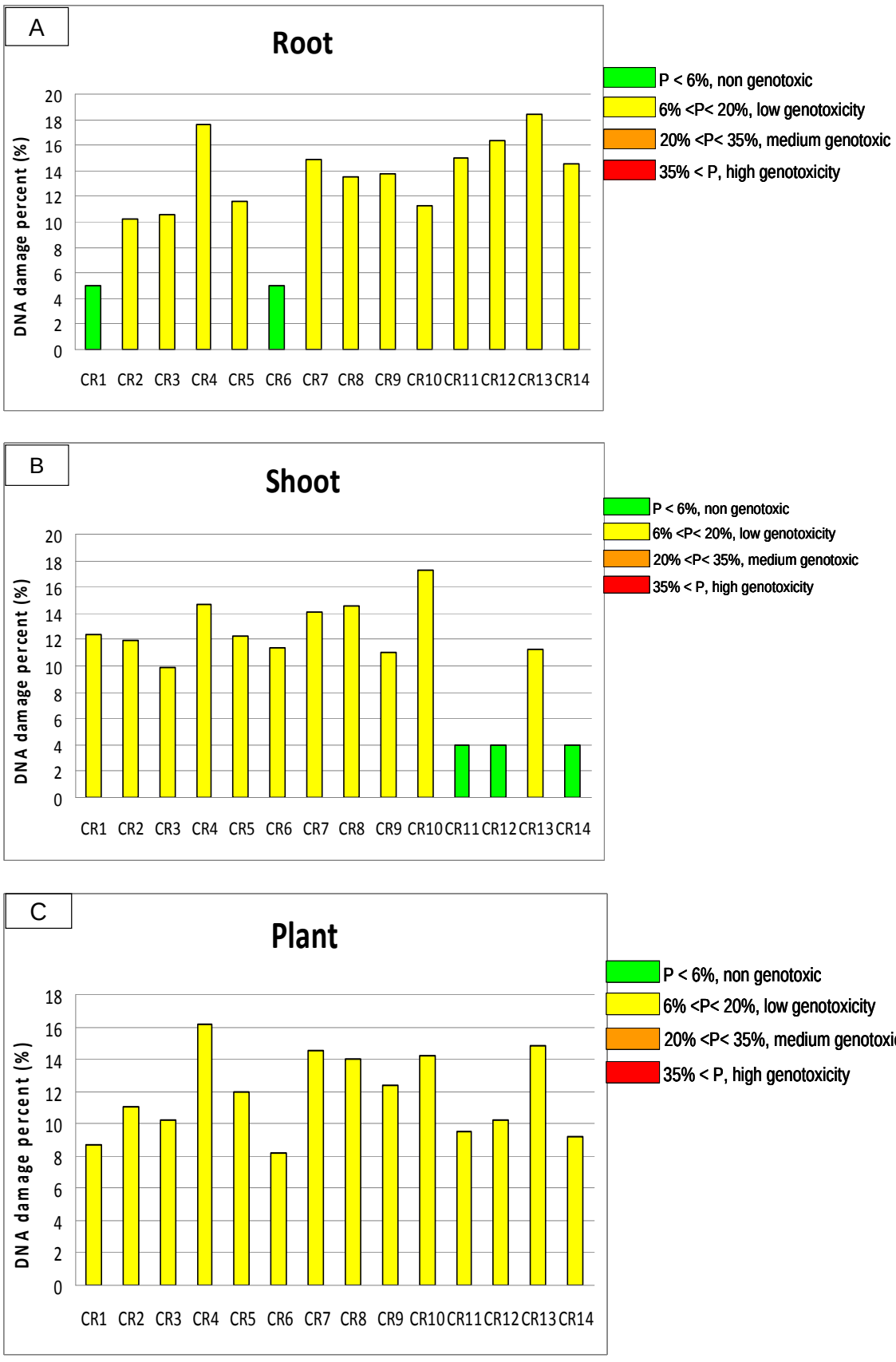
Appendix 4: DNA damage (Polymorphism %) detected in root, shoot and plant of *Trifolium repens* after 15 days of exposure to soils collected in Autostrada Origgio area adjacent to the Milano-Varese Highway, Milan province, Lombardy region



Appendix 5: DNA damage (Polymorphism %) detected in root, shoot and plant of *Trifolium repens* after 15 days of exposure to soils collected in Broni area, Pavia province, Lombardy region



Appendix 6: DNA damage (Polymorphism %) detected in root, shoot and plant of *Trifolium repens* after 15 days of exposure to soils collected in Boario Terme area, Brescia province, Lombardy region



Appendix 7: DNA damage (Polymorphism %) detected in root, shoot and plant of *Trifolium repens* after 15 days of exposure to soils collected in Treviglio area, Bergamo province, Lombardy region