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PLANTS DEALING WITH HEAVY METALS: BIOINDICATION POTENTIAL, PHYSIOLOGICAL RESPONSES AND STRESS ASSESSMENT TECHNIQUES

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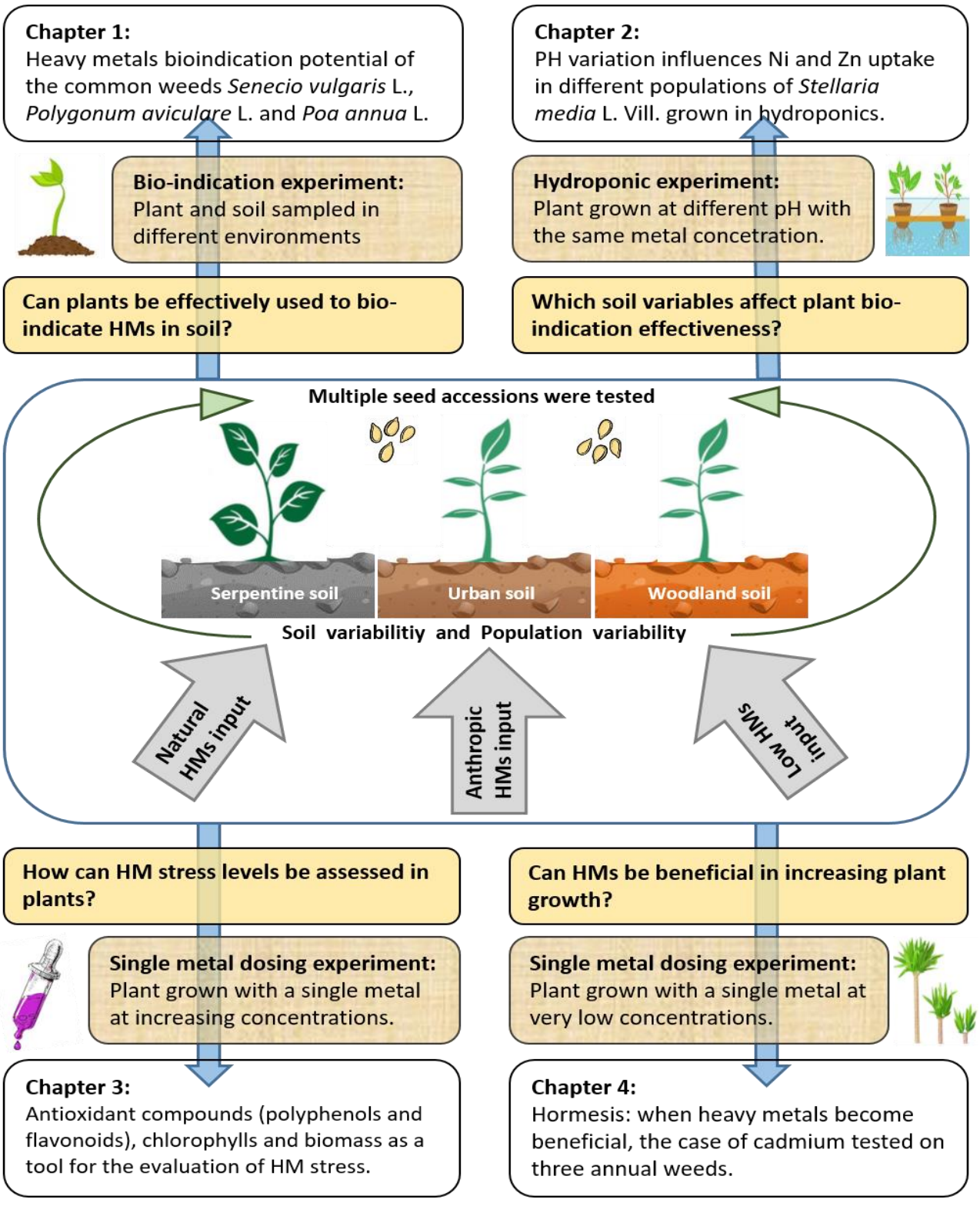
CONCEPTUAL MAP OF THE STUDY

This thesis aims at showing the multiple aspects related to plants dealing with heavy metals (HMs), from the presence of these metals in soil, to their uptake by plants, to the physiological and phenological responses. The thesis is composed by 4 chapters in the form of scientific articles that discuss different topics all deeply interconnected. The conceptual map, with the scientific questions that guided the research is presented below.



1. INTRODUCTION

Since the beginning of the industrialization era, the impact of man on the biosphere has been so important that it has become necessary to indicate as *anthroposphere* the sphere of man's settlement and activity. This term can be applied to any part of the biosphere that has been deeply changed under the influence of technical civilization (Kabata-Pendias, 2011). The always growing changes caused by human activities, are becoming more and more impactful on all planet ecosystems.

One of the global issues connected to human activities is the release of heavy metals (HMs) in the air, water and soil. Several processes are responsible for HM pollution, among which the most important are: combustion of oil and carbon, mining and smelting activities, use of chemical fertilizers and sludge in agriculture.

In recent years, the overwhelming amount of studies related to HMs contributed to a better understanding of the biogeochemical processes that control trace element cycling and their permanence in the environment. This knowledge will be at the basis of our future possibility to manage and reduce trace elements release in the environment, and a prerequisite for a sustainable land use. This is a fundamental objective to achieve, since the concentration of most HMs in plants (i.e. food crops), is often positively correlated with the abundance of these elements in soils. It is therefore our priority the maintenance of soil productivity and safety, avoiding the spreading of anthropogenic pollutants along the food chain (Kabata-Pendias, 2011).

1.1. What is an "heavy metal" ?

The term "heavy metal" refers to metallic elements with a density greater than 5 g/cm^3 (Nies, 1999). HMs usually behave like cations when they are free ions in water solutions. They have an ionic diameter between 138 to 160 picometers, are mostly divalent elements (Mn^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, etc) and are quite reactive (Weast, 1984). Most HMs are transition elements with incompletely filled *d* orbitals, this characteristic makes them redox-active and able to form complexes with organic ligands (Nies, 1999). In general, in the literature, the term "heavy metal" has always been used with a negative meaning connected with environmental pollution, biological hazard and toxicity. Heavy metals are also called "trace elements", and this definition, having a more neutral meaning, indicates their low concentration (< 0.1%) in biological tissues, since they are mostly micronutrient for all living organisms. The term trace elements only relates to ions abundance and also includes

elements having various chemical properties (Kabata-Pendias, 2011). Regardless of the term used, a list of HMs of great environmental concern has been summarized by Kabata-Pendias (2011) and among them are worthy of being cited: As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Se, Tl, V, Zn.

In the present thesis, six of them have been studied and a brief description of each element is provided below.

1.1.1. Cadmium (Cd)

Figure 1. Native Cadmium. *Image source: Wikipedia*

The average Cd (Fig. 1) content for the Earth's crust is 0.1 mg/kg, with similar abundance in both igneous and sedimentary rocks. The metal occurs rarely in nature in pure form, and its common minerals are greenockite (CdS), octavite (CdSe) and monteponite (CdO). Cd is especially associated with Zn and Pb ore deposits. Cd and Zn have similar ionic structures, electronegativities and chemical properties, therefore their behaviour during absorption and transportation by living organisms is similar.

During weathering processes, Cd forms simple compounds, such as CdO, $Cd(OH)_2$, that are easily mobile in the soil, especially at acidic pH (Alloway, 1995). According to Taylor and Percival (2001), between 55% and 90% of Cd in soil pore water is present as free metal ion Cd^{2+} and is readily available to plants. Moreover, Cd in the soil solution occurs in complexes with various organic acids and its availability is significantly correlated mainly with pH (Basta et al., 2001). Cadmium is considered one of the most ecotoxic metals that exhibit adverse effects on all biological processes. The global production of Cd was 20.800 tons in 2008 (USDI, 2009), but its production is not dependent on actual Cd demand but mainly on Zn production. Generally, 3 kg of Cd is produced from one ton of Zn ores. The main use of Cd is in the sector of Ni–Cd and Ag–Cd battery production. Relatively high amounts of Cd are used as yellow pigments and as stabilizers for various plastics.

1.1.2. Chromium (Cr)

Figure 2. Native Chromium.
Image source: Wikipedia

The abundance of Cr (Fig. 2) in the Earth's upper crust is on averages 100 mg/kg, while in ultramafic rocks its content can be over 3000 mg/kg. Cr-rich minerals are likely to be associated with pyroxenes, amphibolites and micas in intrusive rocks.

The geochemistry of Cr is complex because of its easy conversion from +3 to +6 oxidation state, with the second much more toxic than the first (Bartlett and Kimble, 1976; Bartlett, 1997). Since Cr^{3+} is slightly mobile only in very acidic media, its compounds are considered to be very stable in soils. On the other hand, Cr^{6+} is very unstable in soils and is easily mobilized in both acid and alkaline soils. Global production of Cr in 2008 was reported at 21.5 million tons (USDI, 2009).

The major proportion of Cr is used for stainless steel and chromate plating. In the chemical industry, Cr (both +3 and +6) is used primarily in pigments, metal galvanizing and as wood preservatives. The main source of Cr pollution are considered to be the dyeing and leather tanning process wastes that are discharged directly into waste streams. Thus, chromite ore processing residue is of the greatest environmental risk in some regions.

1.1.3. Copper (Cu)

Figure 3. Native Copper.
Image source: Wikipedia

Copper (Fig. 3) occurs in the Earth's crust at concentrations between 25 and 75 mg/kg, it is particularly abundant in mafic igneous rocks and in argillaceous sediments. Copper reveals a strong affinity for sulphur, hence its principal minerals are chalcopyrite ($CuFeS_2$), bornite (Cu_5FeS_4), chalcocite (Cu_2S) and covellite (CuS)(Kabata-Pendias, 2011). World Cu production was 15.7 million tons in 2008 (USDI, 2009). Due to its versatile properties, Cu has a wide range of applications, such as in the production of various conductor materials, it is added in fertilizers, pesticides and animal fodder.

Generally, Cu is accumulated in the upper layer of soils due to its tendency to be adsorbed by organic matter (Logan et al., 1997). Cu is a rather immobile element in soils; the only process that

significantly contributes to increase soil Cu availability, is desorption due to the mineralization of organic matter.

1.1.4. Lead (Pb)

Figure 4. Galena mineral (PbS). *Image source: Wikipedia*

The average Pb content is the Earth's crust is estimated as 15 mg/kg. Its terrestrial abundance indicates a tendency for a concentration in the acid series of igneous rocks and argillaceous sediments. In the environment, two kinds of Pb are known: primary and secondary. Primary Pb is of a geogenic origin and was incorporated into minerals at the time of their formation, while secondary Pb is of a radiogenic origin from the decay of U and Th. The most common Pb mineral (Fig. 4) is galena (PbS). The global production of Pb in 2008 was 3.8 million tons (USDI, 2009) which was obtained mainly from galena deposits. However, in the United States, above 90% of all Pb was produced from secondary sources, like Pb scraps from spent lead-acid batteries.

The largest worldwide use of Pb is in fact for lead-acid batteries and, until 1990s, as an additive in petrol in most developed countries. Lead is the least mobile respect to other trace metals in soils, because it easily forms insoluble precipitates or is strongly bound to clay minerals (Vega et al., 2007). In soils, primary Pb is mostly localized in surface layers given its affinity to organic matter, but also as consequence of its deposition form atmospheric particles (Blum et al., 1997).

1.1.5. Nickel (Ni)

Figure 5. Nickel ores after smelting. *Image source: Wikipedia*

In the Earth's crust, the mean Ni (Fig. 5) abundance has been estimated around 20 mg/kg, whereas in the ultramafic rocks Ni ranges from 1400 to 2000 mg/kg.

In rocks, Ni occurs primarily as sulphides and arsenides and is associated with several Fe minerals. After weathering, most Ni precipitates with Fe and Mn oxides, and becomes included in goethite, limonite, serpentinite, as well as in other Fe minerals. Organic matter exhibits a strong ability to absorb Ni, thus it is highly

concentrated in coal and oil. For this reason, a significant proportion of Ni emissions in the environment are from fossil fuel combustion. Global Ni production was estimated to be 1.6 million tons in 2008 (USDI, 2009). The 68% of this metal is used for stainless steels. It is also widely used for magnetic components and electrical equipment. Its compounds are utilized as dyes, in ceramic and glass manufactures, and in batteries containing Ni–Cd compounds (Reck et al., 2008).

1.1.6. Zinc (Zn)

Figure 6. Zinc ores after smelting. *Image source: Wikipedia*

Average Zn (Fig. 6) content of the Earth's crust is estimated at 70 mg/kg, Zn is quite uniformly distributed in magmatic rocks, whereas in sedimentary rocks it is likely to be concentrated in argillaceous sediments.

This element is very mobile during weathering processes and its compounds are readily precipitated by reactions with carbonates. Global production of Zn in 2008 was 11.3 million tons (USDI, 2009). The principal Zn ores are sphalerite, wurzite and smithsonite, all containing about 50% of Zn. Zinc ores often contain other trace metals, such as Pb, Cu, Ag and Cd.

Zinc is used in many industrial productions, mainly as corrosion protector of steel. It is an important component of various alloys and is widely used as catalyst in different chemical production (e.g. rubber vulcanization, pigments, and plastic). It is also used in batteries, pipes, and electronic devices. Agricultural fertilization is known to increase Zn contents of surface soils since the deficiency of this element is quite common (Huang and Jin, 2008). In natural environments, Zn leaching is counterbalanced by its atmospheric input that, in last years, exceeded its output due to the significant contribution of anthropic emissions.

1.2. Heavy metals in plant nutrition: essential and non-essential elements for plants

Plants are autotrophic organisms in which nutritive processes are based on the conversion of inorganic carbon (CO_2) to organic compounds through photosynthesis combined with the uptake of other essential nutrients. Some of these nutrients are necessary for the plant survival at high concentration, while others only at low concentration, and are therefore defined respectively "macronutrients" and "micronutrients" (Fig. 7). Macronutrients (C, H, O, N, P, K, S, Ca, Mg) represent the main constituent elements of proteins and DNA, besides covering also a fundamental structural role. (Manahan, 2000; Clemens, 2001). Micronutrients instead are mostly structural components of some enzymes and act as enzymatic activators or regulators (Clemens, 2001). At present, 17 micronutrients (Al, B, Br, Cl, Co, Cu, F, Fe, I, Mn, Mo, Ni, Rb, Si, Ti, V, Zn) are known to be essential for plants; some are proved to be necessary for few species only, and others are known to have stimulating effects on plant growth, but their functions are not yet recognized (Kabata-Pendias, 2011). When micronutrients are present at higher concentrations than necessary, they can easily cause toxicity, conversely when their concentration is too low, plants show deficiency symptoms (Clemens, 2001). In addition to these elements, plants are able to absorb a great variety of other "non-essential" elements (e.g. Cd, Cr, Pb) present in soils. Plants' average concentration of the metals object of study in the present thesis are summarized in Table 1.

Figure 7. Label of a commercial fertilizer with micronutrients. *Image source: Amazon*

Table 1. Plants' average concentration of heavy metals found in plants. Only the six elements studied in the present thesis are shown. Deficiency and toxicity limits are reported when possible. All values are expressed in ppm on plant FW basis. *Adapted from: Kabata-Pendias (2011).*

Element	Essentiality	Deficiency limit (ppm)	Average concentration (ppm)	Toxicity limit (ppm)
Cd	no	no	0.03–5	> 5–30
Cr	no	no	1-30	> 10–30
Cu	yes	< 2-5	5-25	> 20-100

Ni	yes	< 0.05	5-100	> 50-100
Pb	no	no	0.5-10	> 30-300
Zn	yes	< 10-20	20-400	> 200-400

The chemical composition of plants therefore reflects the elemental composition of the growing media (e.g. soil). The extent to which this relationship exists, however, is highly variable and is governed by many different factors (see chapter 3). For example Cr, Pb are slightly soluble and strongly absorbed by soil particles, similarly Cu is mainly bound to organic matter, therefore they are not easily taken up by plants. Ni, Cd, Zn are mobile in soil and readily taken up by plants (Kabata-Pendias, 2011).

Since this study is focused on the effects of six HMs (Cu, Zn, Ni, Cd, Pb, Cr), a summarised description of their specific uptake mechanisms and functions in plant cells is reported below.

1.2.1. Physiology of metal uptake and transportation

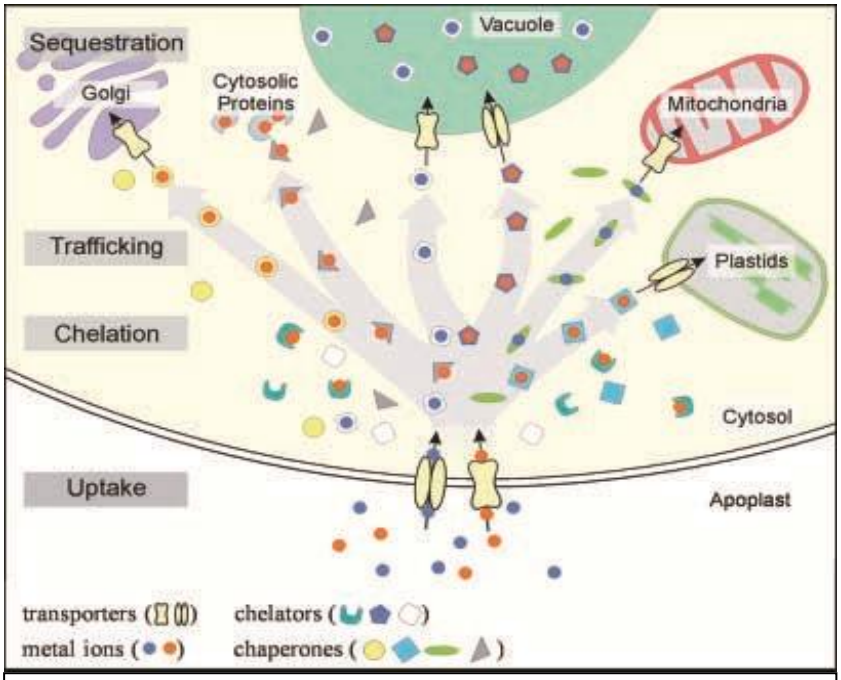


Figure 8. Simplified scheme of the metal homeostasis in a plant cell. Following the uptake through transporters, metal ions are bound by chelators and chaperones. Chelators buffer cytosolic metal concentrations; chaperones are involved in metal trafficking. Uptake into the organelles is catalyzed by metal-ion pumps that directly interact with specific chaperones. Detoxification and storage of excess metal is achieved by sequestration in the vacuole. *Adapted from Clemens (2001).*

Most of the current knowledge on metal accumulation process comes from the in-depth study of few species like *Arabidopsis thaliana* (non-accumulator), *Arabidopsis halleri* (Cd hyperaccumulator) and *Thlaspi caerulescens* (Zn, Ni, Cd hyperaccumulator). Thanks to the complete *A. thaliana* genome sequence (The Arabidopsis Genome Initiative, 2000) and a relatively high gene sequence conservation among Brassicaceae species (94% similarity between *A. thaliana* and *A. halleri* and 88% similarity between *A. thaliana* and *T.*

caerulescens), many gene comparisons have been made to better understand those involved in metal homeostasis and accumulation (Talke et al., 2006; van de Mortel et al., 2006). These candidate genes are mostly involved in metal transport, metal chelation and metal-induced oxidative stress response. Despite the overwhelming amount of studies, connections between metals and specific transporters seem hard to find (Fig. 8). One of the reasons is probably the low specificity of some mechanisms involved in metal transportation and chelation, thus allowing some species to tolerate and accumulate several metals simultaneously (Van der Ent et al., 2017b). To make the situation furtherly complicated the mechanisms of metal uptake are also influenced by soil metal concentration. Morel (1997), described that at low cation concentrations (both HMs and nutrients) of the soil solution (<0.5 µM), active absorption predominates, whereas at higher concentrations (>0.1 mM) the absorption is dominated by passive (diffusion) processes.

1.2.2. Zinc (Zn) and Cadmium (Cd)

Zn and Cd have high chemical affinity and many studies (e.g. Lu et al., 2010, Meyer and Verbruggen, 2012) demonstrated that the uptake of these two ions follows the same biological pathway. Zn is an important micronutrient for plants while for Cd, with the exception of the recently described Cd-carbonic anhydrase of marine diatoms (Lane & Morel, 2000), no biological functions are known (Kabata-Pendias, 2011; Van der Ent et al., 2017b).

Prior to uptake, these metals are actively mobilized from the soil by acidification or by chelating secretion from roots (Clemens et al., 2002). Nicotianamine (NA) is a common chelator excreted by plants that binds metals including Zn, Fe and Cd. After mobilization, divalent metal transporters of the ZIP family (Zrt-Irt-like Protein) present on the root surface, pump the metal inside the cells. It is still not clear if Cd uptake is determined by specific or via Zn-Fe transport mechanisms, but these are likely to be partially overlapped in most non specialized species (Meyer and Verbruggen, 2012). Once in the roots these metals are often complexed with NA; for example the complex Zn-NA provides Zn with a high symplastic mobility towards the xylem (Deinlein et al., 2012; Cornu et al., 2015). The Zn and Cd present in root tissues is then actively loaded into the xylem by the HMA4 proteins (ATPase pumps), as observed in *A. halleri* (Talke et al., 2006; Courbot et al., 2007; Hanikenne et al., 2008). It is supposed that HMA4 also acts as a physiological regulator: while it depletes the metal pool from roots, it triggers a Zn-deficiency response resulting in high expression of several ZIP genes (Hanikenne et al., 2008). Once in the xylem, metals are transported to the shoots thanks to the evapo-transpiration negative pressure. In this compartment, Zn is mainly

bound to organic acids such as malate and citrate (Lu et al., 2013; Cornu et al., 2015). Eventually, the metal reaches the leaves and it is suggested that HMA4 and ZIP transporters again play an important role in unloading and distributing metallic ions to shoot tissues (Krämer et al., 2007; Hanikenne and Nouet, 2011). The metal is then stored in the vacuole, and this function is most likely ensured by MTP1 (Metal Tolerance Protein 1) as suggested for *A. halleri* and *T. caerulescens* (Dräger et al., 2004; Talke et al., 2006; Shahzad et al., 2010) event tough the role of this protein has to be further confirmed.

1.2.3. Chromium (Cr)

Cr can be absorbed both as Cr +3 or Cr +6, and no specific mechanism for its uptake is up to date known. This metal is generally uptaken by other non-specific carriers together with other essential elements and water (Shanker et al., 2005). Members of the Brassicaceae family that are sulphur-loving plants, have been found to accumulate high Cr amounts (Zayed et al., 1998), thereby suggesting that Cr is translocated in the plants via S uptake mechanism, such as sulphate carriers (Barceló and Poschenrieder, 1997). Due to chemical similarity between these two elements, the presence of high S in the growing medium reduces the uptake of Cr in the plants as both compete for the same transport channel (Skeffington et al., 1976; Singh et al., 2013). Cr interacts positively with plant Fe nutrition (Bonet et al., 1991). In fact, it has been observed that Fe-loving plants, such as spinach (*Spinacia oleracea*) and turnip (*Brassica rapa* subsp. *rapa*), are the most effective in translocating Cr to aerial tissues compared to lettuce (*Lactuca sativa*) and cabbage (*Brassica oleracea var. capitata*) that do not accumulate Fe and are thus less effective in Cr translocation (Cary et al., 1977). Cr is generally accumulated in the roots, which in can accumulate 100-fold higher Cr than the shoots (Zayed et al., 1998). The poor translocation of this element to the aerial parts of the plant is probably due to formation of insoluble Cr compounds inside the roots vacuole.

1.2.4. Copper (Cu)

Cu is a micronutrient for plants but, despite its essentiality, it becomes extremely toxic at levels slightly above the plant needs. Cu uptake and homeostasis is therefore strictly regulated by specific transporters located in the plasma membrane (Kampfenkel et al., 1995). Eukaryotic cells utilize copper transporter (CTR family) proteins to transport Cu^{2+} ions into the cytosol (Penarrubia et al., 2010). The CTR-like transporters in plants are called COPT (Copper Transporter) (Kampfenkel et al.,

1995). Until recently, the only functionally characterized COPT transporter was COPT1 (Sancenon et al., 2004) which was involved in Cu transport, but was never detected in roots. Conversely, the production of COPT5 has been detected throughout the plant (except in pollen), with clearly elevated values in roots, confirming its function in Cu uptake from soil (Jaquinod et al., 2007; Garcia-Molina et al., 2011). Inside the plants Cu ions are complexed with phytochelatins and metallothioneins (Maitani et al., 1996). These proteins constitute one of the cytosolic Cu storage and contribute to copper detoxification in plant cells (Hamer et al., 1985). The importance of metallothioneins was demonstrated by Murphy and Taiz (1995), in an experiment of metallothionein induction by Cu treatment, on different *Arabidopsis* ecotypes. During the transport of Cu in the xylem and phloem, nicotianamine has been demonstrated to act as main chelator (von Wirén et al., 1999), its physiological role has been mainly studied in a NA-deficient tomato mutant, which exhibits severe growth limitation and intercostal chlorosis due to the lack of Cu (Pich and Scholz, 1996). Vacuolar sequestration of Cu is poorly documented, mainly because Cu is immediately utilized in protein production. This thesis, in supported by the presence of a Cu fast recycling mechanisms, for instance during leaf senescence when Cu is re-mobilized to other plant growing parts in order to minimize the loss of valuable nutrients (Himelblau and Amasino, 2000).

1.2.5. Lead (Pb)

No biological function is known to date for Pb. This metal, which is toxic even at low concentration, is usually poorly available to plants and immobilized in soil in non-soluble forms. It is therefore unlikely that transporters with specificities for this metal cation exist. Instead, this non-essential metal is able to enter cells through cation transporters with a broad substrate specificity. For example, it is well documented that iron-deficiency leads to an enhanced uptake of other metal ions including Pb (Cohen et al., 1998). The pathways of Pb uptake have been poorly investigated and only few studies are available on this topic. Arazi et al. (1999) investigated a transporter (*NtCBP4*) in transgenic tobacco plants that demonstrated to have high specificity for Pb. This transporter is involved in metal uptake across the root plasma membranes. Transegenic plants that overexpressed *NtCBP4*, have higher Pb accumulation both in roots and shoots, and show Pb toxicity when compared to the wild types. Once in the plant, Pb is probably bound to phytochelatins and then transported towards shoots (Clemens, 2001). Storage of Pb in the vacuole was found to be correlated with high levels of histidine in cells, suggesting a role of this amino acid in Pb chelation.

However, the histidine response during vacuolar metal uptake has been found for several other metal ions suggesting a low specificity of this mechanisms (Krämer et al., 1996).

1.2.6. Nickel (Ni)

In *A. thaliana*, the mechanisms involved in Ni homeostasis are strongly linked to Fe homeostasis (Schaaf et al., 2006; Morrissey et al., 2009; Nishida et al., 2011), so that the metal transporter IRT1 (ZIP family) required for the Fe uptake from the soil was also shown to be involved in Ni uptake (Vert et al., 2002; Nishida et al., 2012). Interestingly, the over-expression of IRT1 in the roots of *N. caerulescens* (now *T. caerulescens*) is also correlated with Ni hyperaccumulation in the same plant accession located in Monte Prinzera, an Italian serpentine site with soil/rocks characterized by high Ni concentrations (Halimaa et al., 2014).

Once in the roots, Ni requires metal chelators (citrate and malate) that are able to stabilize this metal at different pH allowing its transport to the aerial parts of the plant (Callahan et al., 2006; Sarret et al., 2013). The load in the xylematic flow of Ni-citrate and Ni-malate is probably carried out by the MATE transporter protein family (Multidrug And Toxic compound Extrusion). Those transporters are more expressed in the hyperaccumulator *N. caerulescens* than in the related non-accumulator *A. thaliana* (van de Mortel et al., 2006). Again, nicotianamine has a strong affinity for Ni over a wide pH range and is proposed to bind Ni in more neutral compartments such as cytoplasm or phloem (Callahan et al., 2006; Rellan-Alvarez et al., 2008; Alvarez-Fernandez et al., 2014). When Ni reaches the leaves, several evidences indicate that ferroportin (FPN) and iron-regulated (IREG) transporters play an essential role in the sequestration of Ni in vacuoles. It was in fact demonstrated that an over-expression of *AtIREG2* in transgenic *Arabidopsis* plants significantly increases Ni tolerance and accumulation (Schaaf et al., 2006; Merlot et al., 2014).

1.3. Environmental sources of heavy metals

One of the most dramatic aspects that man will be facing in the immediate future is the pollution of soil determined by the growing release of HMs.

These elements are naturally present in the environment, but in the last decades the exponential growth of human activities responsible for the production of these pollutants, arouse concerns due to the potential risk of widespread contamination of soils (Fusco et al., 2005).

HM soil pollution is particularly severe around large urban areas as a result of vehicular traffic, where high concentration of typical anthropogenic metals like Zn, Cu and Pb, can be detected in soil and dust (Li et al., 2003).

In the last 25 years, a number of new metals have been incorporated into automotive technologies,

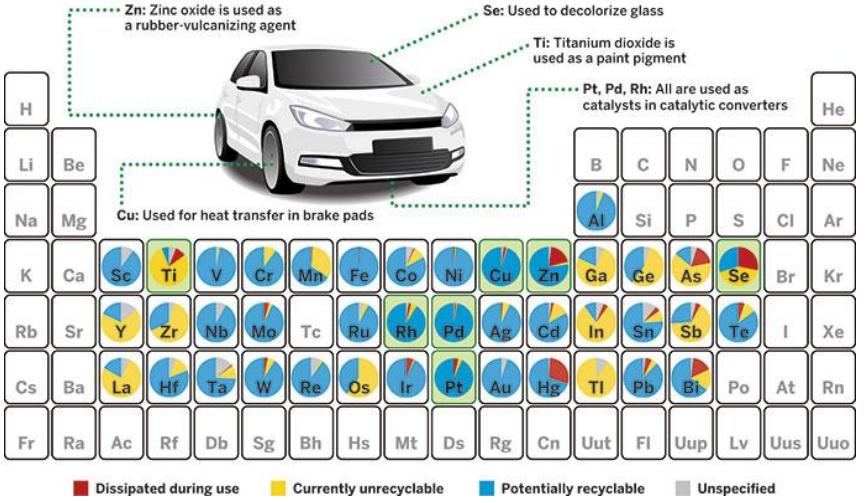


Figure 9. An analysis of the fate of elements used in commercial products, including automobile parts, illustrates the unsustainability of current metal use. *Source:* https://cen.acs.org/articles/93/i14/Digging-Through-New-Types-Waste.html

(Fig. 9) which are now being dispersed into the environment (De Silva et al., 2016). These metals are in fuels (As, Cd, Cr, Mn, Ni, Se and Zn), engine oil (Cd, Cr, Ni, Zn and W), tires (Cd, Co, Cu, Cr, Pb, Ni, Se and Zn), brakes (As, Cd, Cu, Cr, Ni, Pb and Zn) and vehicular exhaust catalysts (Pt, Pd, Rh) (Hjortenkrans et al., 2006; Li et al. 2001). Large amounts of trace metals such as Cu, Pb, Zn, Pt after the release are deposited on the surface of roads and eventually transported into waterbodies by storm water runoff (Camponelli et al., 2010). Water transport coupled with wind transport is the primary source of diffuse HM pollution around big urban centres. It has been demonstrated that elevated levels of Zn in surface soil and sediments are always associated with anthropogenic sources (Varrica et al., 2003; Yuen et al., 2012). Ho et al. (2003) also found high concentration of Zn (mean = 5150 µg/g) in aero-dispersed PM10 collected in Hong Kong, confirming the previous hypothesis. The contamination of agricultural soils is often the final consequence of anthropogenic activities (McLaughlin et al., 1999). To make the situation more serious, in addition to cities, there are many important sources of HMs related to agriculture practises such as: mining and smelting industries, addition of fertilizers, spreading sewage sludge,

use of pesticides (Singh, 2001). Soil contamination is not only a social and sanitary issue, but is also an economic concern since it implies elevated monetary costs related to the decreased agricultural productivity and to the eventual remediation (Bini, 2010).

1.3.1. Anthropic environments polluted by heavy metals: cities

In cities, heavy metals may originate from various types of sources, nonetheless vehicular emissions are considered one of the main sources of HM contamination, especially Pb (Duong and Lee, 2011, WSDE, 2011). This metal is present in gasoline and many other vehicle parts including batteries, wheel balancing weights and metallic paints. Pb emissions from gasoline combustion reached their peak in the early 1970s and then started to declined, especially after the EU ban of leaded gasoline in 2000 (UNEP, 2015). Although leaded gasoline was phased out decades ago, Pb concentrations in road dust (Fig. 10) are still much higher than background levels, primarily due to wheel balancing weigh (Hwanga et al., 2016).

Figure 10. Cleaning operation of road dust deposit. *Image source: Alamy stock photos.*

Another typical metal of urban areas is Cu. This element is highly present in brake pads, to make smooth braking and to prevent brakes from squeaking. Cu content in metallic brake pads varies between 1% and 15% (Hulskotte et al., 2007; McKenzie et al., 2009; Straffelini et al., 2015). To reduce HM pollution originating form brakes wearing, the automotive industry signed an agreement to reduce the use of Cu and other metals in vehicle brake pads to less than 0.5% by 2025 and other constituents such as Cr, Pb and Hg to less than 0.1% (USEPA, 2015).

Tire and galvanized metals are the two largest sources of Zn in urban areas (CASQA, 2015; Vos and Janssen, 2008). The tire industry remains the largest single market for zinc oxide, consuming more than half of the total worldwide demand of 1.2 million tons (Walter, 2009). Each tire may contain as much as 1.5% of Zn by weight (Councell et al., 2004) and thus tire wear particles add significant amount of Zn to the environment.

Another important class of heavy metals in cities are the one called platinum group elements (PGEs). PGEs, which include platinum (Pt), rhodium (Rh) and palladium (Pd), that have been used as catalyst converters since the early 1970s (Palacios et al., 2000). Global catalyst emissions contribute an

estimated dispersion of 6 tons of Pt annually (Rauch et al., 2005). Despite adverse effects have never been observed on the environment, PGEs in road dust are up to three orders of magnitude higher than in background soil. For example, concentrations of 2000 ng/g Pt, 1000 ng/g Pd, and 100 ng/g Rh have been detected in Shieffield (UK) road dust (Jackson et al., 2007).

1.3.2. Natural environments polluted by heavy metals: serpentine soils

HMs are released in the environment also trough natural processes, like the weathering of metal enriched rocks as in the case of serpentine soils. Serpentine soils (Fig. 11) occupy a very small part of the land surface of the earth, less than 1% according to Brooks (1987), but are highly valuable areas renowned for their particular vegetation and ore extraction. Serpentine soils derive by the weathering of ultramafic rocks that contain serpentinite mineral (Oze et al., 2004; McGahan et al., 2008). Serpentinite weathering originates soils characterized by altered chemical and physical properties that reduce plant productivity and induce stress and toxicity to non-adapted species (Jenny, 1980). Several factors are thought to be responsible of this low productivity, such as a low Ca:Mg ratio, caused by the high amounts of Mg released from the parent material, and abundant HMs (in particular Ni, Cr, Co). In addition, these soils often have low macronutrient (N, P, K) concentrations because of their paucity in the rock and the presence of scarce vegetation (Alexander et al., 2007).

Figure 11. *Stellaria media* growing on serpentine soil at Monte Prinzera (Parma, Italy). *Photo: M. Salinitro.*

Mafic and ultramafic ones are richer in Cr and Ni (up to 3400 mg/kg of Cr and 3600 mg/kg of Ni) if compared to average concentrations of Cr and Ni in normal rocks (about 84 and 34 mg/kg, of Cr and Ni respectively) (McGrath, 1995).

Because of their particular pedogenesis, serpentine soils often host a specialized flora, therefore these areas have been identified as hotspost of suitable species for phytomining, phytoremediation and phytostabilization of HMs (Bini et al., 2017).

Serpentine rocks and soils are particularly abundant in the ophiolite belts and are typically found within regions of the Circum-Pacific margin and Mediterranean sea (Oze et al., 2004). Pedogenesis of serpentine soils results to be different among locations, because of the wide distribution of

ultramafic substrates in different climate, topography and biota (Lee et al., 2004; Hseu, 2006). Nonetheless, the release of Cr and Ni into ecosystems during serpentine mineral weathering is a common trait of serpentine pedogenesis. These processes are source of non-anthropogenic metal contamination, however, if compared with HMs of anthropogenic origin, those of lithogenic-derived ones are less mobile in soil and hardly available in the soil solution (Becquer et al., 2003; Garnier et al., 2006).

1.4. Plant strategies in dealing with metals

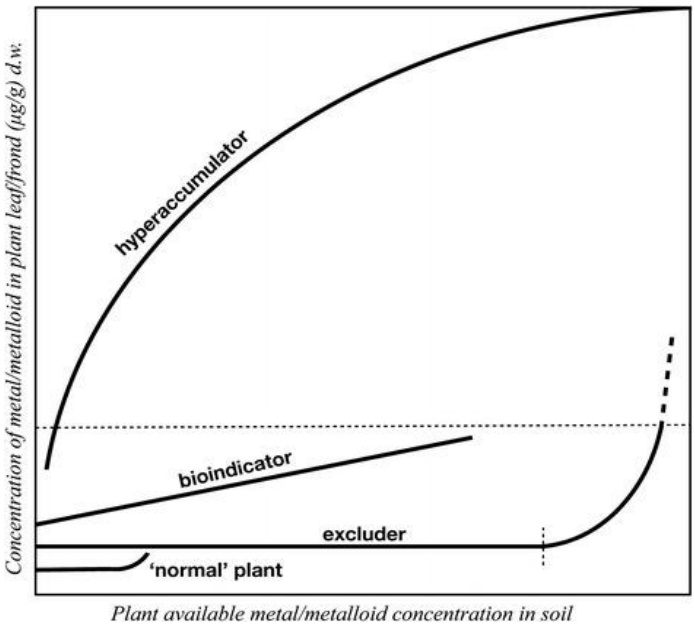


Figure 12. Conceptual response of different plant categories: normal plants, excluders, bioindicators and hyperaccumulators. *Adapted from Baker (1981).*

When plants end up growing in metal contaminated soil, they cannot prevent metal uptake due to their concomitant absorption together with other essential nutrients. However, plant are able to tolerate/accumulate these toxic ions present at various amounts in their leaves and shoots, showing four different types of behaviour (Baker, 1981) (Fig. 12). *Normal plants* can only tolerate low concentrations of bioavailable metals in soil, before they die due to acute phytotoxicity. *Hyperaccumulators* are able to withstand much higher concentrations of bioavailable metals than all the other categories but, because of competitive disadvantages and greater sensitivity to fungal and pathogen infections (in absence of metals), most do not occur over non-metal-enriched soils. *Excluders* can grow over a wide range of available metals before physiological mechanisms breaks down and allow unregulated uptake, resulting in death of the plant. *Bioindicators* absorb metals linearly, over a wider range of metal concentrations until phytotoxicity prevents further growth and causes the death of the plant.

1.4.1. Hyperaccumulators

Plants that are capable of extracting heavy metals from soils and to concentrate them into their above-ground tissues at levels higher than those present in the soil are widely called

hyperaccumulators (Mganga et al., 2011; Baker, 1981). Hyperaccumulators can be further divided in '*obligate*' and '*facultative*' hyperaccumulators. The obligate hyperaccumulator species are endemic to some type of metalliferous soil and always exhibit metal uptake. Facultative hyperaccumulators, on the other hand, are species in which some populations exhibit hyperaccumulation and some other not (Pollard et al., 2014). It has been proposed (Proctor, 1993; Horger et al., 2013) that plants showing this behaviour have an advantage in the protection against pathogens and herbivores because of their toxicity.

To define a plant as hyperaccumulator several authors (i.e. Baker and Brooks, 1989; Broadley et al., 2007; Krämer, 2010) proposed metal thresholds based on unusual metal concentrations that were sensibly above the average in some species. Normal concentration ranges in plants have been tabulated for major, minor and trace elements in many reviews (i.e. Raskin and Ensley, 2000) and a recent discussion regarding appropriate criteria for defining hyperaccumulation thresholds of many elements can be found in van der Ent et al. (2013) (Table 2).

Table 2. Reference metal concentrations for normal and hyperaccumulating plants. *Adapted from van der Ent et al. (2013).*

Element	Normal leaves concentration (ppm)	Hyperaccumulation threshold (ppm)
Zn	20 - 400	>3000
Cu	5 - 25	>300
Cd	0.03 - 5	>100
Ni	5 - 100	>1000
Pb	0.05 - 10	>1000
Cr	1 - 30	>300

However, nominal thresholds should be applied sensibly and not as absolute cut-off and other factors should be considered when accounting a hyperaccumulator. According to van der Ent et al. (2013) metals have to be present with 2–3 orders of magnitude higher in plant leaves than in than in the soil (for normal soils) and at least one order of magnitude greater for metalliferous soils. Plants have to be cultivated in natural soil, in order to reproduce natural growth conditions or possibly collected in their natural habitat. In fact, is known that in hydroponics condition or artificially spiked soil, high concentration of metals can be reached even by non-accumulator plants. Moreover, when regarding to a hyperaccumulator, a bioconcentration factor >1 (but often >50), a shoot to-root metal concentration quotient >1 and extreme metal tolerance, must be present (Baker and Whiting, 2002). If adopting the previous criteria, about 500 plant taxa have been cited in the literature as hyperaccumulators of one or more elements (As, Cd, Co, Cu, Mn, Ni, Pb, Se, Tl, Zn).

These numbers are subject to change and may increase with further exploration and analysis. Nonetheless, many doubts remain about records regarding Pb, Cu and Cr. For example, Pb root uptake is severely restricted root cell membrane while and Cu levels in plant tissues are strictly regulated specific transporters and chelators even in enriched soils. It is therefore possible that many records are a consequence of the accidental contamination of the samples due to soil dust, as demonstrated for most of the Cu hyperaccumulator by Faucon et al. (2007). Conversely, hyperaccumulation for Ni, Zn, Cd have been confirmed experimentally beyond any doubt in a range of plant species (van der Ent et al., 2013) among which the most known are *Noccaea caerulescens* for Zn (Fig. 13), *Alyssum murale* for Ni, *Arabidopsis halleri* for Cd.

Figure 13. *Noccaea caerulscens. Image source:* www.lurigaltervista.com

1.4.2. Excluders

When exposed to an excess of metals, most plant species adopt the so-called excluder strategy to prevent metal accumulation in photosynthetically active shoot tissues (Krämer, 2010). This can be achieved by limiting metal absorption by roots, increasing metal excretion from root tissues or increasing metal storage in root cell walls and vacuoles (van der Ent et al., 2017). By convention, a plant which has high levels of heavy metals in the roots but with shoot/root quotient < 1, is classified as a heavy metal excluder (Mganga et al., 2011). Excluders are capable to limit the internal levels of HMs translocation, preventing further absorption by the radical system, however they can still contain large amounts of metals in their roots (Mganga et al., 2011). When the tolerance limits of excluder species are exceeded, it is common to observe nonspecific breakthrough of metals into the shoot, yet this is not hyperaccumulation if the metal uptake results in the death of the plant (Baker, 1981).

1.4.3. Indicators

Indicators (Fig. 14) are those plants in which the metal concentration inside their above-ground tissues is directly proportional to the external concentration of the soil (Baker and Walker, 1990). These species are generally characterized by slow and reduced biomass production and a linear significant soil/plant correlation. However, exposed to continued uptake of heavy metals, these plant species are possible pollutant indicators and are also useful in soil phytostabilization (Mganga et al., 2011).

Figure 14. The dandelion (*Taraxacum officinale*) has been discovered to be a good indicator of HMs in soil. *Photo: Mirko Salinitro.*

1.5. Global experimental design

The aim of this section is to give an overview of the criteria used in the selection of plant species, sampling locations, and on seed collection and sample processing methods. The goal is to provide an overall view of the entire sampling design that cross-links all the experiments presented in this thesis.

Following, in each chapter, the methods related to the described specific study will be discussed in detail.

1.5.1. Selection of plant species

During the present research, a total of five plant species were studied, with the aim of investigating different aspects all related to HMs uptake by plants. The plants species were selected on the basis of the following features:

- Herbaceous
- Cosmopolite
- Present in a wide variety of environments
- Annual life cycle
- Fast-growing
- Several generations per year
- High production of seeds

- High viability of seeds
- Easy to grow
- Small size

The use of herbaceous annual plants is certainly convenient because most of these species can complete their life cycle (from germination to fruiting) in less than two months. Therefore, lab experiments can be carried out in a reasonable amount of time, observing the plant at all its phenological stages. Cosmopolite plants can be found in worldwide making it possible to extend in the future applied methodologies to other areas. Moreover, the presence of these plants in several environments makes them common, easy to find and to identify. These plants are generally resistant and adaptable to a wide range of conditions, so that it is possible to find their populations adapted to polluted areas (like urban environments), agroecosystems, woodland areas and ultramafic outcrops. The high production and viability of seeds make the germination stage easier in lab experiments, in addition seeds of ruderal plants can keep high germination rates for years even without specific conservation protocols. Finally, the choice of species characterized by small size and easy to grow, makes their cultivation possible in restricted spaces and artificial conditions as in the case of hydroponics, one of the most common methods of lab plant cultivation.

After a screening of several urban species, all possessing the above mentioned characteristics, five species were chosen belonging to five different botanical families (Table 3).

Table 3. List of plant species used for the experiments. For each species is reported the chapter of the present thesis, in which it was used.

Species	**Botanical family**	**Common name**	**Studied in chapter**	**Fig.**
Polygonum aviculare L.	*Polygonaceae*	common knotgrass	1, 3	15
Senecio vulgaris L.	*Asteraceae*	groundsel	1, 3	16
Cardamine hirsuta L.	*Brassicaceae*	hairy bittercress	4	17
Poa annua L.	*Poaceae*	annual bluegrass	1, 4	18
Stellaria media (L.) Vill.	*Caryophyllaceae*	chickweed	2, 4	19

1.5.2. Polygonum aviculare L.

Figure 15. *Polygonum aviculare* L. from the urban station of Porta Garibaldi in Milan city centre. *Photo: M. Salinitro.*

The name *Polygonum* is derived from a Greek word meaning "many knees", because of the conspicuous enlarged nodes of the plant, while *aviculare* means "related to birds" as these animals feed on this plant seeds.

P. aviculare (Fig. 15) is an annual herb with semi-erect stems that may grow up to 40 cm long. The leaves are hairless, elliptical with short stalks, 25 to 35 mm long and 10 to 15 mm wide. The flowering period is summer to autumn, the plant produce small green-white flowers inserted in the leaf axils. The fruit is a dark brown, three-edged nut. The root is a deep taproot with few ramifications.

1.5.3. Senecio vulgaris L.

Figure 16. *Senecio vulgaris* L. from the ultramafic station of Mount Prinzera, Parma. *Photo: Mirko Salinitro*

The name *Senecio* means "old man", in reference to the plant becoming grey and hairy when fruiting, while *vulgaris* means "common" as the plant grows in many habitats. *S. vulgaris* (Fig. 16) is an erect herbaceous annual plant growing up 45 cm tall. Leaves are sessile, lobed, around 61 mm long and 25 mm wide, smaller towards the top of the plant. Leaves are sparsely covered with soft, smooth, fine hairs. Yellow inflorescences appear at the top of plant in spring, carried by several small branches. The seeds are achenes with a pappus of about 1 cm, useful for wind dissemination. The root system consists of a shallow, well branched taproot.

1.5.4. Cardamine hirsuta L.

Figure 17. *Cardamine hirsuta* L. from the woodland station Ticino Park close to Milan. *Photo: Mirko Salinitro.*

The name *Cardamine* derives from the Greek word to indicate "cress", while *hirsuta* means "hairy" in reference to the short hairs that cover plant leaves. *C. hirsuta* (Fig. 17) is an annual erect plant that grow up to 30 cm tall. The floral stem could be branched or unbranched and leaf-less and it emerges from a leaf rosette at the base. The leaves in this rosette are pinnately divided into 7 to 15 leaflets, 3.5 to 15 cm long. The stems, petioles, and upper surfaces of the leaves are sparsely hairy. The small white flowers are appear in spring and have white petals. The seeds are hold in upright pointing siliquae, which are 1.5 to 2.5 cm long and around 1mm in diameter. When the fruit is ripe, the valves of the siliquae burst explosively, sending the seeds far away from the parent plant. The root system is composed by a shallow, poorly branched taproot.

1.5.5. Poa annua L.

Figure 18. *Poa annua* L. from the urban station of Porta (door) San Donato in Bologna city centre. *Photo: Mirko Salinitro*

The name *Poa* is derived from the Greek word that stands for "fodder grass", while *annua* means "one year" in reference to the life cycle of the plant. *P. annua* (Fig. 18) is a widespread meadow grass, stems grow up to 25 cm high and are slightly flattened. The leaves are bright green, 4 to 15 cm long, blunt at the end and soft. The leaves are covered by thin hairs on both sides. The ligula is truncated and silvery. It blooms throughout the year except for the coldest periods. The panicle is open and triangular shaped, 5 to 7.5 cm long, sometimes

they is tinged purple. The seeds are small brown caryopsis. The root system is composed by thin, shallow, collated roots.

Figure 19. *Stellaria media* (L.) Vill from the woodland station of Park Talon close to Bologna. *Photo: Mirko Salinitro.*

1.5.6. Stellaria media (L.) Vill.

The name *Stellaria* is derived from a Latin word meaning "star", which is a reference to the shape of its flowers; *media* is derived from Latin and means, "intermediate" because of its mid-size. *S. media* is annual plant, with weak and generally creeping stems that could reach a length up to 40 cm. The plant germinates in autumn, then forms large mats of foliage during winter. The leaves are oval and opposite, 20 to 25 mm long and 10 to 15 mm wide. Lower leaves have stalks while the upper ones are sessile. Blooming season starts in early spring, flowers are white and small with 5 deeply lobed petals. The whole plant is sparsely hairy, especially on leaf stalks and flower calix. The fruit consists of an oval capsule with inside several flattened, brown, kidney-shaped seeds, 0.8 to 1.3 mm big. The root system is composed by thin roots that also emerge from the nodes of the creeping branches.

1.5.7. Selection of sampling stations

Several sampling stations were chosen for the collection of soil and plant material in three different environments: urban, woodland and ultramafic (Fig. 20).

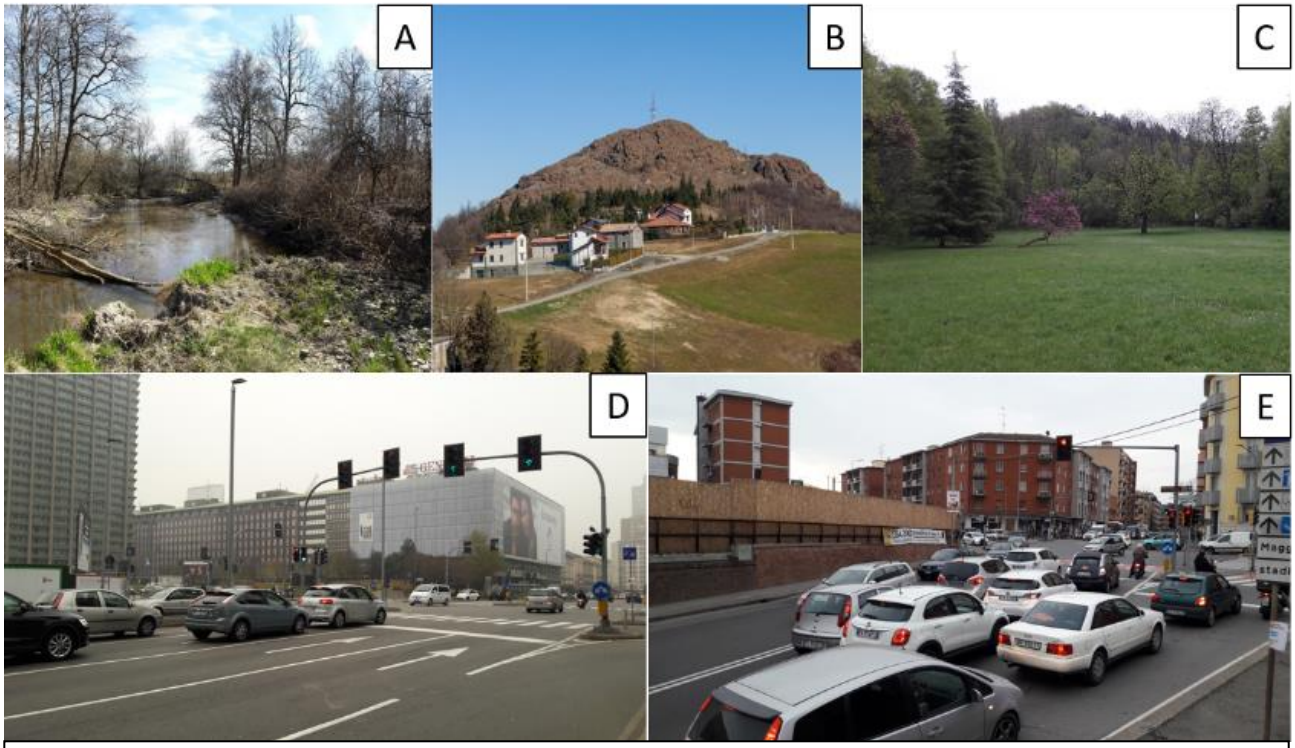


Figure 20. Examples of Sampling stations in the different areas. A) Ticino river (NAT3). B) Mount Prinzera (whole area). C) Talon park meadow (NAT4). D) crossing: via Melchiorre Gioia, viale della Liberazione (MI4), Milan. E) Crossing: via Zanardi, via Bovicampeggi (BO7), Bologna. *Photo: M. Salinitro.*

Urban environment was represented by two sampling areas (Bologna and Milan city centres), woodland environment was represented by one park at the outskirt of Milan (Ticino River natural park) and several stations at the outskirt of Bologna (such as parks and woodlands). Finally, the ultramafic environment was represented by the serpentine outcrop of Mount Prinzera (Parma). The heterogeneity of the sampling areas, allowed us to maximise the variability of soils, HM levels and plant growth conditions. In each sampling area, five to 10 sampling stations were selected and in every place soil, plants and seeds were collected. The list of all sampling stations is shown in Table 4. Urban stations of Milan and Bologna were chosen among the busiest street crossings, preferably in presence of traffic lights. In fact, high vehicular traffic and frequent car braking make these crossings particularly polluted by HMs. Conversely, woodland stations of Milan and Bologna outskirts were located far from diffuse pollution sources (i.e. roads), usually reachable only by feet and surrounded by trees. Ultramafic stations were all located at Mount Prinzera, an important

Table 4. Detailed list of sampling stations. In each of the 5 sampling areas (identified by the 5 colours) several stations were sampled. Seeds collected in every station were pulled together to form a single bulk accession for each area, instead, soil and plant material were kept separated.

Milan (urban)		**Bologna (urban)**	
Name of the sampling station	**Code**	**Name of the sampling station**	**Code**
Porta Magenta	MI1	Porta San Felice	BO1
Porta Garibaldi	Mi2	Porta Santo Stefano	BO2
Porta Genova	MI3	Porta Castiglione	BO3
Crossing: via Melchiorre Gioa, viale della Liberazione	MI4	Porta San Vitale	BO4
Piazza della Repubblica	MI5	Porta San Donato	BO5
Monumental Cemetery	MI6	Porta Galliera	BO6
Crossing: via Beatrice D'Este, via Isabella D'Aragona	MI7	Crossing: via Bovicampeggi, via Zanardi	BO7
Crossing: via Papiniano, via Modestino	MI8	Porta San Mamolo	BO8
Venezia square	MI9	Crossing: via Sabotino, viale Giovanni Vicini	BO9
Porta Ticinese	MI10	Crossing: via Stalingrado, via del Lavoro	BO10
Milan (woodland)		**Bologna (woodland)**	
Name of the sampling station	**Code**	**Name of the sampling station**	**Code**
Ticino parking lot	NAT 1	Talon park meadow	NAT4
Ticino woodland	NAT 2	Talon park woodland	NAT5
Ticino river	NAT 3	San Martino 1	NAT6
Ticino wall	NAT 10	San Martino 2	NAT7
Ticino field	NAT 11	Mt. Capra	NAT9
mt. Prinzera (ultramafic)		Pontecchio field	NAT12
Name of the sampling station	**Code**	Pontecchio woodland	NAT13
Case Prinzera 1	NAT 8		
Case Prinzera 2	NAT14		
Mt. Prinzera peack 1	NAT15		
Mt. Prinzera peack 2	NAT16		
Villaggio Prinzera road	NAT17		

serpentine outcrop located close to the city of Parma. The main characteristics of ultramafic stations were high Cr and Ni concentrations (derived from the weathering of locals rocks), and low levels of other HMs. The average metal content of every station is shown in Table 5.

Table 5. Average metal contents in soils of each sampled station. Mean values for each areas (urban, woodland, ultramafic) are provided in bold.

Sampling station	Zn	Pb	Cu	Ni	Cr	Cd
NAT1	0.059 ± 0.003	0.030 ± 0.008	0.011 ± 0.001	0.018 ± 0.003	0.017 ± 0.001	0.045 ± 0.013
NAT2	0.055 ± 0.014	0.010 ± 0.004	0.006 ± 0..2	0.058 ± 0.021	0.064 ± 0.012	0.041 ± 0.007
NAT3	0.064 ± 0.019	0.012 ± 0.003	0.008 ± 0.002	0.019 ± 0.002	0.009 ± 0.002	0.044 ± 0.008
NAT4	0.099 ± 0.003	0.020 ± 0.003	0.023 ± 0.005	0.061 ± 0.005	0.045 ± 0.003	0.074 ± 0.006
NAT5	0.107 ± 0.007	0.035 ± 0.016	0.024 ± 0.003	0.053 ± 0.006	0.034 ± 0.005	0.010 ± 0.001
NAT6	0.184 ± 0.011	0.023 ± 0.007	0.047 ± 0.004	0.049 ± 0.004	0.019 ± 0.004	0.291 ± 0.011
NAT7	0.130 ± 0.013	0.019 ± 0.003	0.031 ± 0.004	0.050 ± 0.011	0.017 ± 0.004	0.384 ± 0.022
NAT9	0.064 ± 0.020	0.008 ± 0.002	0.010 ± 0.002	0.095 ± 0.025	0.051 ± 0.014	0.347 ± 0.028
NAT10	0.100 ± 0.028	0.023 ± 0.001	0.016 ± 0.002	0.088 ± 0.025	0.107 ± 0.024	0.080 ± 0.005
NAT11	0.071 ± 0.019	0.019 ± 0.005	0.015 ± 0.001	0.175 ± 0.032	0.197 ± 0.055	0.076 ± 0.004
NAT12	0.081 ± 0.030	0.005 ± 0.002	0.015 ± 0.002	0.211 ± 0.041	0.163 ± 0.037	0.278 ± 0.002
NAT13	0.588 ± 0.303	0.002 ± 0.000	0.019 ± 0.001	0.148 ± 0.034	0.152 ± 0.027	0.384 ± 0.022
Mean woodland soil	**0.134 ± 0.043**	**0.017 ± 0.003**	**0.019 ± 0.003**	**0.085 ± 0.018**	**0.073 ± 0.019**	**0.171 ± 0.043**
NAT8	0.118 ± 0.014	0.007 ± 0.001	0.022 ± 0.002	1.516 ± 0.229	0.539 ± 0.043	0.389 ± 0.013
Mean ultramafic soil	**0.118 ± 0.014**	**0.007 ± 0.001**	**0.022 ± 0.002**	**1.516 ± 0.229**	**0.539 ± 0.043**	**0.389 ± 0.013**
MI1	0.934 ± 0.184	0.050 ± 0.014	0.294 ± 0.061	0.041 ± 0.007	0.061 ± 0.011	0.361 ± 0.118
MI2	0.579 ± 0.144	0.251 ± 0.033	0.492 ± 0.041	0.056 ± 0.017	0.072 ± 0.010	0.245 ± 0.072
MI3	1.226 ± 0.286	0.530 ± 0.055	0.386 ± 0.032	0.175 ± 0.005	0.228 ± 0.012	0.483 ± 0.009
MI4	1.259 ± 0.223	0.135 ± 0.005	0.540 ± 0.041	0.071 ± 0.012	0.075 ± 0.001	0.388 ± 0.013
MI5	0.511 ± 0.033	0.104 ± 0.002	0.568 ± 0.014	0.050 ± 0.001	0.071 ± 0.001	0.557 ± 0.203
MI6	0.691 ± 0.039	0.232 ± 0.029	0.271 ± 0.003	0.041 ± 0.001	0.037 ± 0.002	1.059 ± 0.188
MI7	0.359 ± 0.039	0.052 ± 0.006	0.125 ± 0.012	0.067 ± 0.019	0.032 ± 0.005	0.267 ± 0.016
MI8	0.829 ± 0.072	0.192 ± 0.004	0.407 ± 0.019	0.101 ± 0.005	0.067 ± 0.004	0.808 ± 0.070
MI9	0.268 ± 0.089	0.027 ± 0.003	0.070 ± 0.026	0.012 ± 0.002	0.028 ± 0.001	0.140 ± 0.013
MI10	0.460 ± 0.140	0.274 ± 0.085	0.244 ± 0.000	0.018 ± 0.001	0.051 ± 0.001	0.326 ± 0.019
BO1	0.983 ± 0.052	0.250 ± 0.005	0.204 ± 0.032	0.034 ± 0.002	0.039 ± 0.001	0.481 ± 0.037
BO2	0.677 ± 0.237	0.092 ± 0.022	0.179 ± 0.057	0.032 ± 0.000	0.017 ± 0.000	0.361 ± 0.036
BO3	0.505 ± 0.043	0.150 ± 0.011	0.129 ± 0.005	0.037 ± 0.004	0.039 ± 0.007	0.408 ± 0.063
BO4	0.797 ± 0.030	0.173 ± 0.015	0.243 ± 0.034	0.035 ± 0.002	0.034 ± 0.001	0.490 ± 0.035
BO5	0.562 ± 0.056	0.177 ± 0.011	0.313 ± 0.011	0.027 ± 0.002	0.098 ± 0.035	0.499 ± 0.043
BO6	0.453 ± 0.001	0.100 ± 0.006	0.391 ± 0.096	0.056 ± 0.001	0.117 ± 0.017	0.394 ± 0.006
BO7	0.408 ± 0.068	0.105 ± 0.022	0.133 ± 0.008	0.039 ± 0.004	0.039 ± 0.010	0.289 ± 0.015
BO8	0.479 ± 0.017	0.142 ± 0.009	0.114 ± 0.008	0.033 ± 0.002	0.023 ± 0.001	0.547 ± 0.127
BO9	0.317 ± 0.048	0.187 ± 0.061	0.390 ± 0.166	0.022 ± 0.001	0.030 ± 0.001	0.322 ± 0.010
BO10	0.492 ± 0.030	0.134 ± 0.023	0.175 ± 0.025	0.030 ± 0.001	0.029 ± 0.001	0.348 ± 0.009
Mean urban soils	**0.639 ± 0.082**	**0.168 ± 0.032**	**0.283 ± 0.043**	**0.049 ± 0.010**	**0.059 ± 0.014**	**0.439 ± 0.059**

1.5.8. Pot cultivation of urban plants

Urban environments are highly disturbed habitats where anthropic activities often result in a damaging or total removal of spontaneous vegetation. This situation has to be taken into consideration when the focus of the research is the collection of seeds from spontaneous plants in urban stations. Even fast growing species usually take 2 to 3 month from germination to seed production, but during this time lapse, damaging or removal interventions (from city cleaning services) could with high probability occur to the selected plants. To prevent this problem, during the present research, urban plants intended for seed production were collected in the different sampling locations and then cultivated *ex-situ* until fruiting season. The cultivation took place at the botanical garden of the University of Bologna. Plants were collected in each urban station (10 in Milan and 10 in Bologna) together with a suitable amount of soil, in order to allow their potting with the original substrate. Plants were then potted with the orginal urban soil in 10x15x5 cm non-drilled plastic trays to prevent soil leaching during irrigation (Fig. 21). In each tray (corresponding to one urban station) only one species was cultivated with a variable number of individuals. Plants were grown for 4 months (December to April 2016-17) in a cold greenhouse with natural light and irrigated with deionized water every 3-5 days.

Figure 21. Cultivation in trays of *Poa annua* (top) and *Senecio vulgaris* (bottom), coming from the 10 sampling stations of Bologna urban area.

1.5.9. Seed collection and conservation

Urban seed accessions were collected by shaking the plants (twice a week) during the four months of cultivation. In this way the collected seeds were already clean with no plant or soil residues.

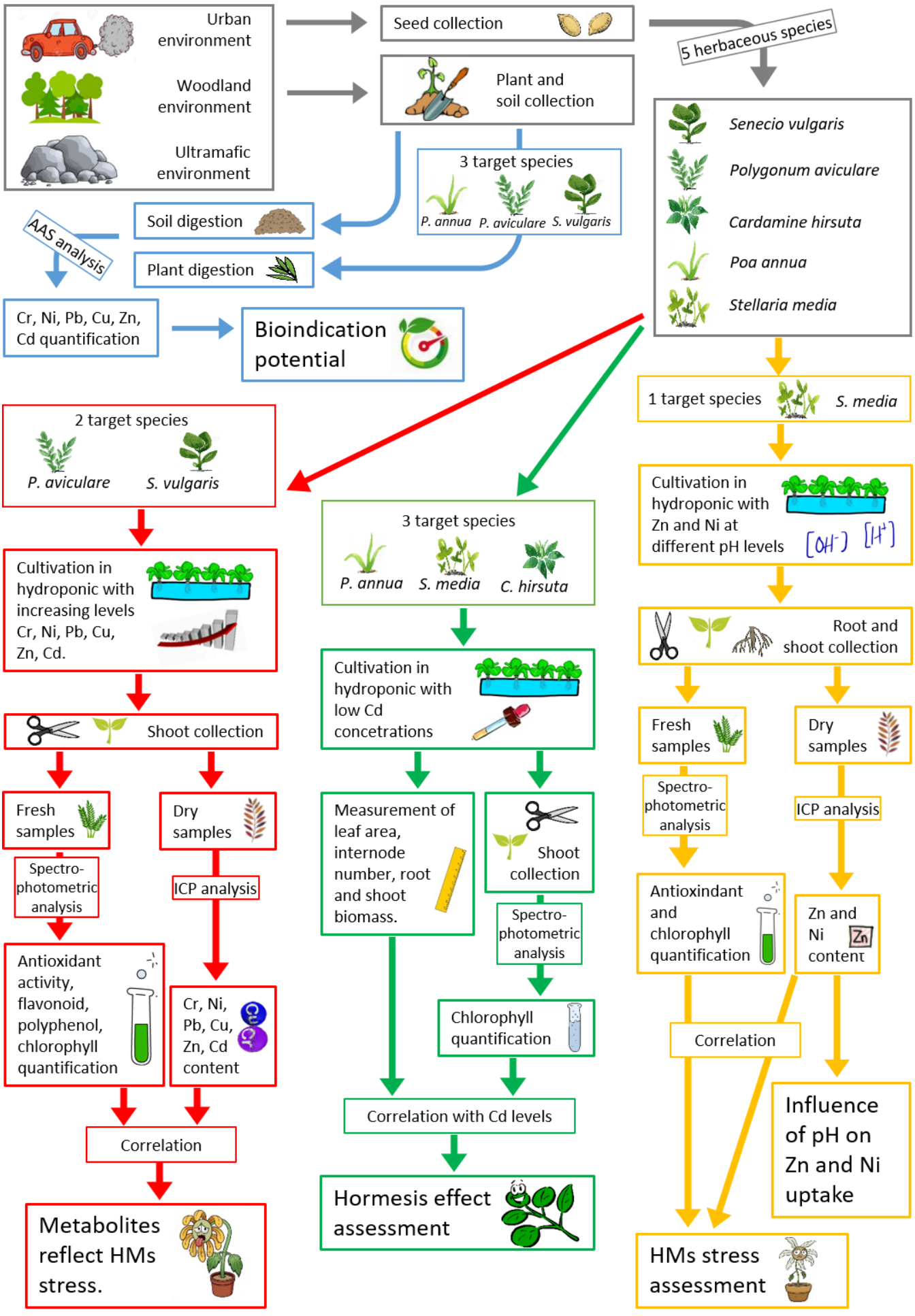
Conversely, woodland and ultramafic accessions were collected directly in the field, since there were no risks related to the artificial removal of plants in these areas. Plants were cut, air-dried for 2-3 days then shaken in a plastic bag to allow seeds removal. Seed cleaning and separation from plant residuals was carried out with fine meshes (0.5-1mm) according to seed specific dimensions (Fig. 22).

Figure 22. Sieving of *Polygonum aviculare* L. seeds. *Photo: Mirko Salinitro.*

Seeds from different stations of the same area, were pulled together in order to obtain five seed accessions for each species: Bologna urban (B), Milan urban (M), Bologna woodland (N), Milan woodland (T), Prinzera ultramafic (P). Seeds were placed in paper envelopes, labelled and stored in a refrigerator at 4 ° C until use.

1.5.10. Conceptual map of applied methods

Sample processing, lab analyses and data analyses, carried out in each single experiment are explained in detail in the Material and Methods section of every chapter. Nevertheless, an overview of the four main experiments, with the respective analytical techniques, is shown in the following conceptual map.


Urban environment
Woodland environment
Ultramafic environment
Seed collection
Plant and soil collection
5 herbaceous species
3 target species
P. annua
P. aviculare
S. vulgaris
Soil digestion
Plant digestion
AAS analysis
Cr, Ni, Pb, Cu, Zn, Cd quantification
Bioindication potential
Senecio vulgaris
Polygonum aviculare
Cardamine hirsuta
Poa annua
Stellaria media
1 target species
S. media
Cultivation in hydroponic with Zn and Ni at different pH levels
[OH⁻] [H⁺]
Root and shoot collection
Fresh samples
Dry samples
Spectro-photometric analysis
ICP analysis
Antioxidant and chlorophyll quantification
Zn and Ni content
Zn
Correlation
Influence of pH on Zn and Ni uptake
HMs stress assessment
2 target species
P. aviculare
S. vulgaris
Cultivation in hydroponic with increasing levels Cr, Ni, Pb, Cu, Zn, Cd.
Shoot collection
Fresh samples
Dry samples
Spectro-photometric analysis
ICP analysis
Antioxidant activity, flavonoid, polyphenol, chlorophyll quantification
Cr, Ni, Pb, Cu, Zn, Cd content
Cu
Cr
Correlation
Metabolites reflect HMs stress.
3 target species
P. annua
S. media
C. hirsuta
Cultivation in hydroponic with low Cd concetrations
Measurement of leaf area, internode number, root and shoot biomass.
Shoot collection
Spectro-photometric analysis
Chlorophyll quantification
Correlation with Cd levels
Hormesis effect assessment

1.6 References

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RESEARCH CHAPTERS

2. Heavy metals bioindication potential of the common weeds *Senecio vulgaris* L., *Polygonum aviculare* L. and *Poa annua* L.

Article status

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Mirko Salinitro collected the samples, analysed the data, wrote the manuscript and coordinated the study. Dora Melucci analysed the data and contributed to write the manuscript. Sonia Casolari and Francesco de Laurentiis performed the AAS analyses. Annalisa Tassoni coordinated the study and revised the manuscript. Alessandro Zappi analysed the data.

List of abbreviations

AAS = Atomic Absorption Spectroscopy

BAF = Bio-accumulation factor

BAF_BM = Bio-accumulation Factor calculated on bioavailable metal in soil

BAF_TM = Bio-accumulation Factor calculated on total metal in soil

BM = Bioavailable soil metal concentration

HMs = Heavy metals

IM = Inorganic matter

LOO-CV = leave-one-out cross-validation

LoD = Limit of detection

OM = Organic matter

PM = Plant metal concentration

TM = Total soil metal concentration

2.1. Introduction

The fast urbanization and industrialization in the last decades have resulted in increasing contents of trace metals in the environment (Wang et al., 2014; Ahmad et al., 2016; Charlerworth et al., 2011). These elements are not degradable, therefore they could accumulate in soil and become potentially hazardous to terrestrial and aquatic ecosystems, and thus to human and animal life (Melucci et al., 2018; Tchounwou et al., 2012). HMs are naturally present in the environment as a result of either natural processes or human activities (He et al., 2005; Li et al., 2009). In natural ecosystems, HMs come from ultramafic rocks and ore minerals and, during weathering that leads to soil formation, could be released in the environment (Szyczewski et al., 2009). On the other hand, anthropogenic sources (i.e. vehicular traffic, mining activities and refining processes) are nowadays the main responsible for HMs pollution (Kabata-Pendias and Mukherjee, 2007; Norgate et al., 2007). Urban areas are recognized to be the major sources for contaminants (Markert et al., 2011; Wiseman et al., 2013), and traffic is the primary source of HMs that accumulate in roadside soils (Zereini et al., 2007) and street dust (Lough et al., 2005). HMs are produced by vehicles tailpipe emissions, as well as from the wear and tear of mechanical components such as brakes, tires and catalytic converters (Zereini et al., 2007; Zereini et al., 2012).

In recent years, it has been shown that HMs levels in soil and vegetation have increased considerably due to traffic pollution, and the problem rises as daily traffic increases (Onder and Dursun, 2006). This diffuse source of pollution in areas where people live and food is produced, poses a serious threat to human health. In fact, these pollutants can enter plants directly via foliar absorption or taken up from the soil through the root system (Jozic et al., 2009) and undergo processes of bio-magnification (Locatelli and Melucci, 2012; Locatelli and Melucci, 2013). Despite the high toxicity of HMs for plants, when these elements are present in soil at low concentrations plants continue to grow healthy even while accumulating these metals. The ability of plants to accumulate HMs into their tissues may therefore be used to monitor soil pollution (Malizia et al., 2012).

Biomonitoring techniques using indicator plants (bioindication) are becoming common methods to detect toxic levels of HMs in the environments. Mosses and lichens, for example, are known to be the most sensitive indicators of atmospheric pollution (Jenkis, 1987; Jiang et al., 2018), thus they are broadly used in urban environment. Unfortunately, because of the absence of roots and their restricted presence on hard substrate, they are not suitable for soil monitoring. Many authors agree that herbaceous plants could be effective biomonitoring tools, and some common species like the dandelion (*Taraxacum officinale* Weber), nettle (*Urtica dioica* L.) and broadleaf plantain (*Plantago*

major L.) have already been successfully used (Malizia et al., 2012; Galal and Shehata, 2015; Rule, 1994; Fröhlichová et al., 2018). Not all plants are suitable as indicators; some basic characteristic of a good bioindicator were listed by Wittig, (1993). An indicator plant should: i) accumulate one or several selected elements; ii) have low sensitivity to the accumulated elements; iii) have wide distribution in various environments; iv) show a correlation between metal accumulation and input into the ecosystem.

Even when using the right plant, bioindication properties could be affected by other factors like soil properties complexation of HMs, oxidation state (Szyczewski et al., 2009; Lin and Zhang, 1990) and phenologic phase of the plants (Keane et al., 2001). Seasonality plays an important role in determining HMs concentration in plant tissues. Despite some species grow all year long, it has been demonstrated that Cu, Fe, Mn, Pb and Zn contents in dandelion leaves collected in autumn, were higher compared to those collected at the same sites in spring (Keane et al., 2001). Similar results were reported for alfalfa (*Medicago sativa* L.) with regard to Mo content (Karlsson, 1961). Soil properties, like cationic exchange capacity, clay content, pH, organic matter content, are likely to change HMs availability to plants (Kashem and Singh, 2001; Antoniadis et al., 2008). For example, Dai et al. (2004) estimated that extractable Cd, Pb and Zn in contaminated soils were positively correlated with organic matter contents due to the consequent pH decrease. Moreover, low pH is optimal for metal availability since solubility has been shown to increase with decreasing pH (Ghosh and Singh, 2005; Nanda and Abraham, 2013). Given the high variability of soils, bioindication could not be considered a technique able to precisely measure trace metal in soil, but rather a way to estimate them and their interaction with plants in some specific conditions. Therefore, it is of vital importance to assess indication properties of a species on several soils that differ for their HMs content and physical properties.

The aim of our study was to test the ruderal species *Senecio vulgaris* L., *Polygonum aviculare* L. and *Poa annua* L., as possible candidates for the biomonitoring Cu, Zn, Cd, Cr, Ni and Pb in multiple environments. Furthermore, we aimed at assessing how different type of soils can affect the predictive potential of these species.

2.2. Materials and Methods

2.2.1. Samples collection

For this study, three common weed species that had the basic characteristic to be *good biondicators* according to Wittig (1993) were selected. All the three species are ruderal plants, which makes them common in all anthropic habitats, and in particular:

1) *Senecio vulgaris* L. (groundsel) is an annual plant of the Asteraceae family. Originally with an Eurasiatic distribution, today it has become sub-cosmopolitan worldwide. The species is common in all habitats among which disturbed areas like road margins, arable fields and gardens. It preferably grows on clay soil rich in nitrogen and organic matter.

2) *Poa annua* L. (annual bluegrass) is an annual plant of the Poaceae family. Originally with an Eurasiatic distribution, today widely naturalized in the temperate areas of the globe. It's a pioneer species that grows in trampled areas, gardens and roads margins, on nitrogen-rich soils.

3) *Polygonum aviculare* L. (common knotgrass) is an annual plant of the Polygonaceae family. The species is cosmopolitan and, because of its high variability, it is adaptable to several habitats growing on all soil types and being resistant to trampling. It is widespread in urban areas, arable fields, but also woodland margins.

To maximize the metal content in the tissues, plants were harvested close to the end of their life cycle, therefore during the fruiting season (April-May 2017 for *P. annua* and *S. vulgaris*, October 2017 for *P. aviculare*). Only the aerial parts of the plants were sampled. After collection, plants where thoroughly washed with deionized water, then oven dried at 50° C until constant weight. Dried samples were powdered with an Ultraturrax IKA® A11 basic (Staufen, Germany), then stored at room temperature until analysis. In order to have high heterogeneity of soil conditions, the three herbaceous species were harvested in 8 stations belonging to three different habitats (Fig. 1). Five stations were from the urban environments of Bologna and Milan city centers (BO7, BO8, MI3, MI4, MI9), two stations were from woodland environment (Ticino Park Loc. Besate, (MI) Talon Park, Casalecchio (BO), respectively NAT1, NAT5) and one was an ultramafic station from Mount Prinzera (Parma, Italy) (NAT8)

Figure 1. Sampling locations of soils and plants used in the study. In each station one soil sample and three plant species have been collected.

The sampling stations were selected among the most polluted sites in urban environments, random choice among woodland station (since they were all similar), plus one single ultramafic station.

In every station, the three species were all present simultaneously, growing in the same bulk soil. The soil was sampled at a depth of 0-10 cm exactly below the plants, and at least 5 soil sub-samples (replicates) were collected in each location. The sub-samples were then mixed together to form one bulk sample (of around 2 kg). The bulk soil samples were homogenised and sieved at 0.5 cm to exclude stones and other coarse particles, then oven dried at 50° C until constant weight. Dried soil samples were furtherly sieved at 0.1 cm, then stored at room temperature until analysis.

2.2.2. Soil digestion

All chemicals used were ultrapure and they were purchased from Sigma-Aldrich (St. Louis, Missouri, US): 69% (w/w) HNO_3, HCl 37% (w/w), 35% (w/w) H_2O_2, 96% (w/w) sulfuric acid, ammonium citrate, iron (II) ammonium sulfate hexahydrate, potassium dichromate. Filter syringes, porosity 0.45 µm, diameter 22 mm, were employed to filter solutions after metals extraction.

To perform soil digestion a modified a version of the US EPA 3050b (1996) method was used. The dried and sieved soil (0.1 cm) was finely grinded in a mortar; then, approximately 1 g of soil and 5 mL of 69% (w/w) HNO_3 were put in a Pyrex 100-mL calibrated test tube. The tube was connected to a Vigreux column, then it was placed in a special housing on a heating plate at 150°C. The system

was left in reflux mode for 30 min. Subsequently, the tube was cooled in an ice-bath, then 5 mL of 35% (w/w) H_2O_2 were added, and the addition of H_2O_2 drops continued until the solution in the tube stopped boiling. Then, 10 mL of 37% (w/w) HCl were introduced, and another reflux step was applied for 15 min. Once digestion was completed, the solution was cooled down and filtered. Finally, the liquid phase was transferred into a 50 mL flask and brought to the final volume with 0.5 M HNO_3. The total concentration of metals in soils (TM) was measured in µg/g (briefly indicated as ppm). Blank digestions (without soil) were carried out using the same reagents as described above.

2.2.3. Plant digestions

Acid digestion on plant shoots was carried using a modified protocol adapted from Huang and Schulte (1985). An aliquot of plant powdered shoots, between 0.05 and 0.1 g, was placed in a 10-mL glass tube and 2 mL of 69% (w/w) HNO_3 were added. A pre-digestion phase was obtained by leaving the tubes at room temperature for 24 hours; then the tubes were first placed on a hot plate at 75°C for 1 h and subsequently the temperature was risen to 125°C for another 1 h. During the digestion the tubes were left open without any reflux system.
In the 2-hours digestion the volume of acid reduced to about 1 mL; then it was transferred in a 10-mL flask and brought to the final volume with MilliQ water to obtain a digestate with about 6-7% (w/w) HNO_3. Generally, even though no plant residues were visible, they were filtered by 0.45 µm filters. The concentration of metals in plants (PM) was measured in µg/g (briefly indicated as ppm). Blank digestions (without plants) were carried out using the same reagents as described above.

2.2.4. Extraction of bioavailable metal fraction from soil

The dried and sieved soil (0.1 cm) was finely grinded in a mortar, then sieved with a 0.5 mm mesh. 5 g of sieved soil was then transferred to an extraction bottle in which 5 mL of 2% (w/v) ammonium citrate solution were added. The obtained mixture was shaken on an end-over-end tube roller mixer at 30 rpm for 1 h at 20°C. The extracts were immediately separated by decantation for few minutes, followed by centrifugation for 10 min at about 3000 x *g*. The supernatant was recovered and the liquid was stored in a polyethylene container at 4°C until analysis. The concentration of bioavailable metals in soil (MeCitr) was measured in µg/g (ppm). Blank extractions (without soil) was carried out using the same reagents as described above (Quevauviller, 2002).

2.2.5. Determination of metal concentration in soils and plants

The concentration of metal in soils and plants were quantified through Atomic Absorption Spectroscopy (AAS). AAS measurements were performed using a Perkin-Elmer Mod. Analyst 400 Atomic Absorption Spectrometer (Waltham, Massachusetts, US), equipped with a deuterium background corrector, autosampler AS-72 and with HGA 800 graphite furnace. Single-element Lumina (Perkin-Elmer) hollow-cathode lamps were used. All measurements were carried out using default program for ashing and atomization curves for each element, at the detailed instrumental conditions are reported in Table 1.

Table 1. Instrument settings for Atomic absorption spectroscopy (AAS) determination.

Element	Wavelength (nm)	Slit (nm)	Drying Temperature (°C)	Pyrolisis Temperature (°C)	Atomization Temperature (°C)
Cu(II)	324.8	0.8	110	1000	2300
Pb(II)	283.3	1.05	110	950	1800
Cd(II)	228.8	1.35	110	850	1650
Cr(VI)	357.87	0.8	110	1650	2500
Ni(II)	232	1.35	110	1400	2500

All the elements, except zinc, were determined by electro-thermal AAS (ET-AAS), employing argon at flowrate 250 mL/min in all steps except during atomization (0 mL/min). Zinc was analyzed by flame AAS (FAAS) employing acetylene (4.10 L/min) and air (10 L/min).

For each of the six analyzed metals (Cu, Pb, Cd, Cr, Ni, Zn) a calibration line was created. Standards for calibration lines were purchased by Merck (Darmstadt, Germany). Three standard solutions were prepared for each metal and "outer" standard, concentrations were selected in order to stay in the linear range of each analyte, as tabulated in the software WinLab 32 (Conquer Scientific, San Diego, CA, US). Peak-area was used as analytical signal, after verifying that peak height never overcame 0.6 AU in order to stay in the absorbance linear range. Before each analysis, a blank sample was analyzed and the peak-area of the sample was subtracted to the previous blank one. For each calibration line, the limit of detection (LoD) was computed, and it was verified that it never overcame the lowest standard concentration. When analyses had to be carried out in several days, every day three standards were analyzed and projected on the calibration line, to verify its validity. Three replicates were analyzed for each standard and sample. The injected volume was 20 µL for each analysis. Samples were properly diluted in order to obtain a signal in the calibration range, and the dilution factor was kept into account to calculate the metal concentration in the sample.

In order to analyze Cd, Cr, Ni and Pb by AAS, some matrix modifiers were necessary. In particular $Mg(NO_3)_2$ (Perkin Elmer, Waltham, Massachusetts, US) for Cd and Cr, $PdCl_2$ (Fluka, Honeywell, Morris Planes, NJ, USA) for Cd, and $NH_4H_2PO_4$ (Sigma Aldrich, St. Louis, Missouri, US) for Pb. 20 µL/mL of a solution containing all of the modifiers were added to each sample, final concentrations: 200 mg/L for $Mg(NO_3)_2$, 2.3 mg/L for $PdCl_2$, 4 mg/L for $NH_4H_2PO_4$. It was also verified that the presence of an unnecessary modifiers did not influence the measurements of other metals (as Cu and Zn, which did not require any modifier).

2.2.6. Determination of organic matter and granulometry of soil

The percentage of organic matter in soils (OM) was determined by two experimental methods: titration and Loss on Ignition. The percentage of inorganic matter (IM) was calculated as 100% - OM %.

To measure OM, the titration was carried out following the method of Walkley and Black (1934). 0.5 g of soil, 10 mL of potassium dichromate 0.167 M and 20 mL of 96% (w/w) sulfuric acid were placed in a 500 mL conical flask, slowly percolating along the internal walls of the flask, not to overheat the mixture. The flask was covered with watch glass and left to rest for 30 min. Then the reaction was interrupted by adding 200 mL of distilled water, previously cooled in the refrigerator. A few drops of ferroin (redox indicator) were added, and titration was carried out with a solution of iron (II) ammonium sulfate hexahydrate 0.5 M until the color changed. At the same time, a blank test was performed with 10 mL of dichromate, 20 mL of sulfuric acid and 200 mL of distilled water. The following expression was used for the calculation of organic carbon (C) expressed in g/kg.

$$C = 3.9 \cdot \frac{(B-A)}{MSoil} \cdot MFe$$

where: *B* = volume of the solution of iron (II) ammonium sulfate hexahydrate used in the titration of the blank test, expressed in mL; *A* = volume of the solution of iron (II) ammonium sulfate hexahydrate used in the titration of the sample solution, expressed in mL; MFe = effective molarity of the solution of iron (II) ammonium sulfate hexahydrate; MSoil = mass of the soil sample, expressed in grams. To transform g/kg of organic carbon into the corresponding organic substance content, a conversion factor is applied: % OM_titr = % OM_titr x 1.724.

To validate OM content found by titration, we compared the results with the one found by Loss on Ignition method (official method for the determination of OM) as explained in Storer (1984). The two methods were comparable and gave similar results, therefore the titration method results were validated and used for statistical elaborations.

Granulometry of the samples was assessed by sieving samples with gradually smaller mashes and weighting the fraction held into each mesh. Four classes of granulometry were defined: particles > 0.5 mm (*coarse*), particles between 0.5 and 0.25 mm (*medium*), particles between 0.25 mm and 63 µm (*fine*) particles < 63 µm (*ultra fine*).

2.2.7. Data analysis

The matrix containing all collected data was composed by 86 observation (objects) and 43 variables. We used chemometrics to extract useful information from our dataset and in particular to create and validate models. Chemometrics was applied both in univariate mode (analysis of correlation, creation of linear regression) and in multivariate mode (Principal Components Analysis, PCA) (Miller and Miller, 2010; Brereton, 2007). To create linear models, the Multiple Linear Regression tool (MLR) was applied. In order to validate MLR models, besides considering the model p-values (ANOVA test), which should be close to the null value, the same data used to create the model were projected onto it, both in calibration mode (blue dots in response plots in results section) and by leave-one-out cross-validation (LOO-CV) (red dots in response plots in results section). Projection in calibration mode means that, once created the model, data are projected into it as they are. LOO-CV, on the other side, creates as many models as the number of samples, leaving each time one sample out from the model creation and projecting it onto such model. In this way, each sample is treated as if it was an external data used to validate the regression performance of the overall model. Both in calibration and in LOO-CV, response values of each sample are recalculated by projection. Then, two further response lines are computed (blue for calibration mode, red for LOO-CV), in which the independent variables are the known (experimental) response and the dependent variables are the recalculated values that appear in the response plot. The predictive model performances are evaluated by the parameters of these lines. In a perfect case, the recalculated responses would be exactly equal to the known ones, thus the response lines would be the bisector of the response plot, with slope=1, offset=0 and R^2=1. Model performances are considered acceptable if the response line parameters are close to these ideal values. A further parameter of the response lines is root-mean-squared-error (RMSE), which is a sort of sum of distances between the known responses and the recalculated ones, therefore, it should be close to zero. The potential suitability of a species as bio-indicator of one or more metals was validated in two steps. Firstly, we tested the correlation between bioaccumulation factor calculated on TM and Bioaccumulation factor calculated on BM, if that correlation was good (between 0.7 and 1), we tested the correlation

between metal content in plants (PM) and metal content in soil (TM). In Table 2 the empirical rules adopted to evaluate correlation *goodness* between variables are shown. Only plants showing high or excellent correlation values for both validation steps were used in the creation of predictive models. All statistical analyses and graphical elaborations were performed using the software The Unscrambler 10.4 (CAMO Analytics, Oslo, Norway).

Table 2. Empirical rules adopted in the evaluation of correlations.

0.3<correlation<0.5	significant
0.5<correlation<0.7	relevant
0.7<correlation<0.9	high
0.9<correlation<1	excellent

2.2. Results

2.2.1. Heavy metals in soil

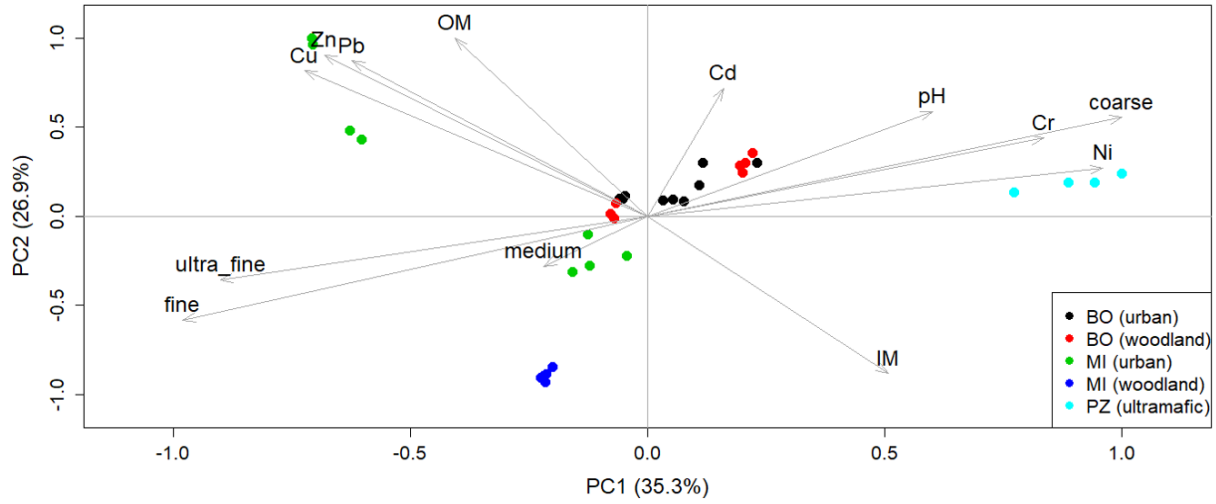


Figure 2. Soil clustering after PCA. The input data were the soil variables: granulometry, organic matter, inorganic matter and total heavy metals concentration.

The 8 soils analyzed appeared to be strongly different from each other and characterized by various metal and OM contents, texture and pH. Soil variability was analysed trough a PCA (Fig. 2) that showed a clustering of soils according to the area of collection. HMs concentrations, were closely linked to the levels of anthropogenic activity for urban and woodland soils, while were mainly from geogenic origin in ultramafic soils. The analyzed metals were present in the decreasing order: Zn>Cu>Cr>Ni>Cd for urban areas, Zn>Cu>Ni>Cr>Cd for woodland areas and Ni>Cr>Zn>Cu>Pb>Cd for ultramafic areas. All urban soils were characterized by medium to fine granulometry, high OM content (especially for Milan samples) and presence of anthropogenic metals like Pb, Zn, Cd, Cu. Woodland soils from Bologna were quite similar to those of Milan which had a slightly coarser texture and lower pH levels. Finally, mount Prinzera soils, because of their ultramafic origin, had high Ni and Cr content and were characterized by coarser granulometry respect all the others (Fig. 3).

Metal concentrations (both total and bioavailable) in each soil are summarized in Table 3. Those with higher concentrations were MI3 and MI4, while those with lowest amounts of HMs were NAT1 and NAT5, with the exception of Ni and Cr for which NAT8 showed the highest values.

Table 3. Total and bioavailable concentrations of six heavy metals in the analysed soils. OM = Organic metter, Total = total soli metal concentration, Bioavail = Bioavailable soil metal concentration

Soil	pH	OM (%)	Zn (ppm)		Cu (ppm)		Pb (ppm)		Cr (ppm)		Cd (ppm)		Ni (ppm)	
			Total	Bioavail.	Total	Bioavail.	Total	Bioavail.	Total	Bioavail.	Total	Bioavail.	Total	Bioavail.
MI3	7.73	13.05	1200 ± 300	N.A.	390 ± 30	36 ± 3	530 ± 50	28 ± 2	229 ± 10	0.48 ± 0.03	0.48 ± 0.01	0.03 ± 0.01	175 ± 5	3.80 ± 0.02
MI4	7.75	9.4	1200 ± 200	N.A.	540 ± 40	113 ± 7	135 ± 2	5.5 ± 0.3	75 ± 1	0.61 ± 0.06	0.39 ± 0.01	0.08 ± 0.01	70 ± 10	3.5 ± 0.1
MI9	8.81	9.48	270 ± 40	N.A.	60 ± 10	N.A.	22 ± 3	N.A.	100 ± 70	N.A.	0.40 ± 0.2	N.A.	60 ± 30	N.A.
BO7	9.04	7.9	410 ± 70	N.A.	133 ± 7	11 ± 1	110 ± 20	4.3 ± 0.4	40 ± 10	0.15 ± 0.01	0.29 ± 0.01	0.08 ± 0.01	39 ± 4	1.5 ± 0.1
BO8	8.91	6.59	510 ± 40	N.A.	110 ± 10	2.6 ± 0.2	120 ± 20	4.6 ± 0.4	150 ± 60	0.19 ± 0.01	0.6 ± 0.1	0.06 ± 0.01	130 ± 90	1.4 ± 0.1
NAT1	7.42	11.84	56 ± 3	N.A.	12 ± 2	2.6 ± 0.2	12 ± 4	2.8 ± 0.1	19 ± 1	0.83 ± 0.03	0.10 ± 0.05	0.19 ± 0.02	11 ± 3	0.9 ± 0.1
NAT5	8.79	4.57	184 ± 6	N.A.	50 ± 5	3.9 ± 0.4	19 ± 5	0.53 ± 0.01	22 ± 5	0.07 ± 0.01	0.33 ± 0.04	0.04 ± 0.01	49 ± 4	4.3 ± 0.3
NAT8	8.79	2.7	110 ± 10	N.A.	19 ± 2	N.A.	8 ± 4	N.A.	570 ± 50	N.A.	0.38 ± 0.03	N.A.	1900 ± 300	N.A.

It can be noted that Zn content is one order of magnitude higher in urban soils from Milan (>1000 ppm) if compared with other soils, while the lowest values can be found in woodland soils (~100 ppm). A similar trend was obtained for Cu, with urban soils from Milan having the highest values (>500 ppm) and woodland soils the lowest (~10-50 ppm), and Pb with a variable concentration from ~ 100-500 ppm for Milan urban soils down to to ~ 10-20 ppm for woodland soils. These first three metals were therefore connected to anthropogenic activities.

Substantially different is the situation of Cd, which had similar levels (around 0.35 ppm) in all the analyzed soils. Finally, Cr and Ni showed a wide range of concentrations independently of the origin of the soil, with the exception of ultramafic soils from mount Prinzera which, as expected, showed the highest concentrations of these metals (Ni ~1800 ppm, Cr ~ 500 ppm).

2.2.2. Selection of species and metals candidates for bioindication

A preliminary exploration tested the correlation between the Bioaccumulation factor calculated on TM (BAF_TM) and BAF calculated on BM (BAF_BM). This step was useful to evaluate if the response of our species was consistent both considering the total metal in soil or the bioavailable fraction. Only plants and metals that had high correlation values were kept into account as candidates for bioindication. Species with high correlation values for certain metals were likely to give consistent

Table 4. Correlation table between Bio-accumulation Factor calculated on bioavailable metal in soil (BAF_BM) and Bio-accumulation Factor calculated on total metal in soil (BAF_TM). Colours indicate the "goodness" of correlation: yellow = significant, orange = relevant, green = high, blue = excellent. N.A., not available.

Correlation BAF_TM / BAF_BM	Zn	Cu	Pb	Cr	Cd	Ni
P. annua	N.A.	0.33	0.79	-0.03	0.10	0.87
P. aviculare	N.A.	0.00	0.78	0.94	0.83	0.81
S. vulgaris	N.A.	0.97	1.00	-0.22	0.98	0.97

information on total and bioavailable metals in soil simultaneously. High correlation values (Table 4) between BAF_TM and BAF_BM were found, for all species, in at least two metals each. From this preliminary screening, *S. vulgaris* appeared to be a possible candidate for bioindication of Cu, Pb, Cd and Ni. *P. aviculare* was found to be a potential bioindicator of Pb, Cr, Cd and Ni. Finally, *P. annua* could be a possible bioindicator for Pb and Ni.

Metal concentrations for all plants are reported in Table 5.

Table 5. Metal concentrations and bioaccumulation factors for the three studied species. Plant = total metal concentration in plant, BAF = bioaccumulation factor.

Species	Soil	Zn (ppm)		Cu (ppm)		Pb (ppm)		Cr (ppm)		Cd (ppm)		Ni (ppm)	
		Plant	BAF	Plant	BAF	Plant	BAF	Plant	BAF	Plant	BAF	Plant	BAF
S. vulgaris	Mi3	17.9 ± 0.7	0.01	0.78 ± 0.05	0.02	<LoD	N.D.	<LoD	N.D.	0.05 ± 0.01	0.02	0.39 ± 0.03	0.02
S. vulgaris	Mi4	471 ± 40	0.32	9.8 ± 0.9	0.39	<LoD	N.D.	0.16 ± 0.01	0.02	0.05 ± 0.01	0.02	0.67 ± 0.03	0.03
S. vulgaris	Mi9	70 ± 1	0.39	8.6 ± 0.5	0.28	0.36 ± 0.02	0.09	0.51 ± 0.01	0.16	0.21 ± 0.02	0.01	0.64 ± 0.03	0.02
S. vulgaris	Bo7	99 ± 4	0.17	7.3 ± 0.5	0.07	0.77 ± 0.02	0.01	0.49 ± 0.02	0.02	0.21 ± 0.01	2.49	1.25 ± 0.01	0.04
S. vulgaris	Bo8	5.4 ± 0.5	0.01	0.08 ± 0.01	0.01	<LoD	N.D.	0.02 ± 0.01	0.02	0.06 ± 0.01	0.09	0.25 ± 0.02	0.02
S. vulgaris	Nat8	17 ± 1	0.16	2.9 ± 0.2	0.16	0.48 ± 0.03	0.03	1.14 ± 0.07	0.03	0.73 ± 0.02	1.86	5.1 ± 0.3	0.02
S. vulgaris	Nat1	54 ± 4	1.02	8.26 ± 0.03	0.69	0.42 ± 0.03	0.02	0.7 ± 0.01	0.05	0.76 ± 0.03	0.01	3.07 ± 0.06	0.06
S. vulgaris	Nat5	22.7 ± 0.6	0.13	5.7 ± 0.2	0.14	4.96 ± 0.03	0.31	0.16 ± 0.01	0.01	14.6 ± 0.4	0.02	2.4 ± 0.1	0.06
P. aviculare	Mi3	47 ± 3	0.03	14 ± 1	0.04	0.82 ± 0.03	0.02	1.7 ± 0.1	0.01	0.17 ± 0.01	0.36	1.93 ± 0.08	0.01
P. aviculare	Mi4	56 ± 2	0.04	13.7 ± 0.7	0.03	1.03 ± 0.08	0.01	1.58 ± 0.08	0.02	0.17 ± 0.01	0.44	0.62 ± 0.01	0.01
P. aviculare	Mi9	30 ± 3	0.16	21 ± 1	0.22	0.63 ± 0.05	0.02	3.52 ± 0.07	0.13	0.24 ± 0.01	1.86	1.8 ± 0.2	0.15
P. aviculare	Bo7	57 ± 3	0.13	19.8 ± 0.2	0.15	0.81 ± 0.01	0.01	2.5 ± 0.2	0.12	0.47 ± 0.04	5.48	0.65 ± 0.03	0.02
P. aviculare	Bo8	46 ± 5	0.20	7.8 ± 0.7	0.14	1.02 ± 0.08	0.01	1.3 ± 0.1	0.08	4.04 ± 0.07	1.72	1.23 ± 0.05	0.02
P. aviculare	Nat8	32 ± 2	0.31	39 ± 1	2.17	0.12 ± 0.01	0.01	0.22 ± 0.03	0.02	0.16 ± 0.01	0.42	2.2 ± 0.2	0.02
P. aviculare	Nat1	40 ± 2	0.75	3.6 ± 0.1	0.30	0.11 ± 0.02	N.D.	0.17 ± 0.01	0.01	0.35 ± 0.01	5.01	1.2 ± 0.1	0.06
P. aviculare	Nat5	27.4 ± 0.6	0.29	3.15 ± 0.09	0.14	0.13 ± 0.01	N.D.	0.12 ± 0.01	0.01	0.33 ± 0.02	1.43	1.6 ± 0.2	0.03
P. annua	Mi3	220 ± 10	0.15	1.8 ± 0.01	0.01	<LoD	N.D.	<LoD	N.D.	0.35 ± 0.01	0.71	4.1 ± 0.3	0.03
P. annua	Mi4	108 ± 8	0.20	14.0 ± 0.6	0.07	0.08 ± 0.01	0.01	2.33 ± 0.01	0.07	0.46 ± 0.04	1.92	5.8 ± 0.4	0.07
P. annua	Mi9	84.0 ± 0.4	0.47	14.2 ± 0.1	0.15	0.54 ± 0.02	0.02	4.14 ± 0.05	0.14	0.42 ± 0.02	3.31	6.8 ± 0.6	0.20
P. annua	Bo7	29 ± 2	0.10	11.0 ± 0.8	0.08	0.17 ± 0.03	0.01	0.45 ± 0.02	0.01	0.13 ± 0.01	0.48	0.82 ± 0.01	0.06
P. annua	Bo8	119 ± 9	0.26	20.2 ± 0.3	0.19	0.25 ± 0.02	0.01	1.52 ± 0.06	0.07	0.23 ± 0.02	0.48	2.00 ± 0.07	0.14
P. annua	Nat8	4.0 ± 0.4	0.04	4.1 ± 0.4	0.23	0.54 ± 0.02	0.03	0.38 ± 0.01	0.01	1.53 ± 0.01	3.89	17.2 ± 0.2	0.02
P. annua	Nat1	98 ± 5	1.85	5.2 ± 0.3	0.43	1.7 ± 0.1	0.08	3.1 ± 0.1	0.21	0.61 ± 0.06	8.57	3.5 ± 0.3	0.19
P. annua	Nat5	34 ± 1	0.37	7.7 ± 0.7	0.33	0.41 ± 0.03	0.01	1.6 ± 0.1	0.06	0.23 ± 0.01	1.01	6.27 ± 0.03	0.17

Table 6. Correlation table between metal in plant (PM) and total metal in soil (TM. Colours indicate the "goodness" of correlation: yellow = significant, orange = relevant, green = high, blue = excellent.

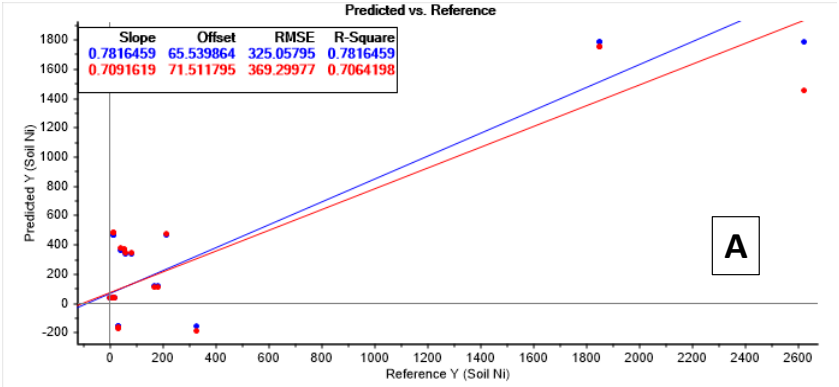
Correlation TM / PM	Zn	Cu	Pb	Cr	Cd	Ni
P. annua	0.64	0.15	-0.47	-0.47	-0.05	0.87
P. aviculare	0.59	-0.05	0.50	-0.27	0.61	0.62
S. vulgaris	0.56	0.04	-0.28	0.54	-0.02	0.73

These data was used to carry out another explorative analysis focused on the correlation between plant metal content (PM) and the soil metal content (TM). High correlations were found for two species, but only in the case of Ni (Table 6), therefore the potential of *P. annua* and *S. vulgaris* as bioindicators for Ni was furtherly explored and modelled. Some other relevant correlations were found in *P. aviculare* for Cd and Pb and for *P. annua* for Cr, but these species did not show any linear relation with the metal when furtherly tested.

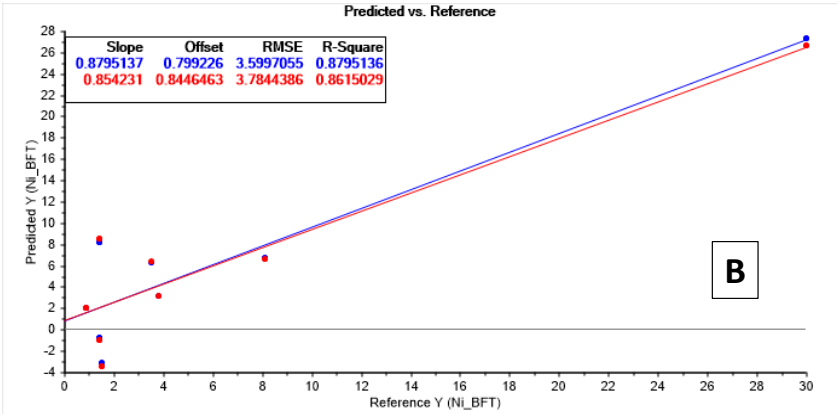
2.2.3. Bioindication of Ni using P. annua

The strong linear relation (*R= 0.841*) between total Ni in soil and Ni concentration in *P. annua* shoots is shown in the table enclosed in Fig. 3A.

This linear relation made it possible the creation of a Multiple linear regression (MLR) model with



Intercept	-415	**Intercept st.dev.**	100	**R^2**	0.841
Slope	129	**Slope st.dev.**	13.9		



Intercept	-4.56	**Intercept st.dev.**	1.08	**R^2**	0.928
Slope	1.88	**Slope st.dev.**	0.139		

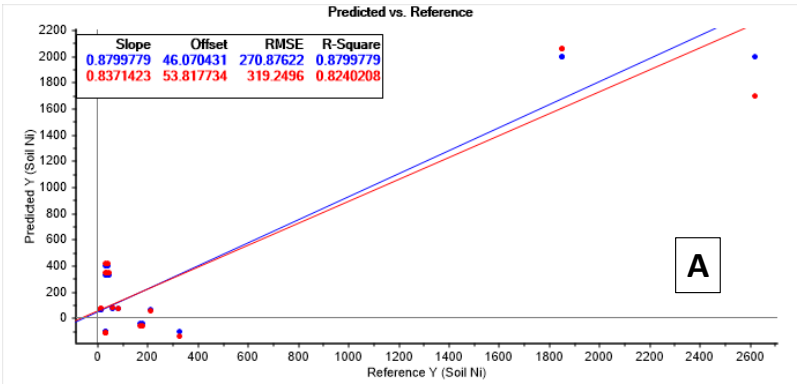
Figure 3. A) TABLE: linear regression between total Ni concentration in soil and Ni in *P. annua* shoots; PLOT: recalculated total soil Ni by the model, input data derived from the linear relation between total Ni in soil and Ni in plant. Blue dots: forecasted soil Ni concentrations in calibration mode (all soil data were used as input). Red dots: forecasted soil Ni concentrations in cross-validation mode, excluding one soil data at a time (leave-one-out mode).
B) TABLE: linear regression between bioavailable Ni concentration in soil and Ni in *P. annua* shoots; PLOT: recalculated total soil Ni by the model, input data are derived from the linear relation between total Ni in soil and Ni in plant. Blue dots: forecasted soil Ni concentrations in calibration mode (all soil data were used as input). Red dots: forecasted soil Ni concentrations in cross-validation mode, excluding one soil data at a time (leave-one-out mode).

predictive potential (response plot in Fig. 3A). The performance of the models was considered relevant to bioindication purposes since this model appeared reliable when tested by ANOVA (*p*-value related to the *F* parameter <0.05). Both in calibration (blue dots) and in cross-validation (red

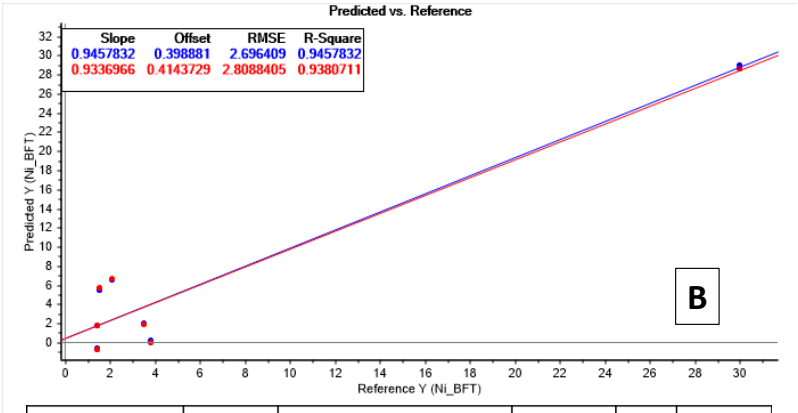
dots) modes, the two lines and the values almost overlapped. Except for high Ni values, the model appeared very accurate in the prevision of total Ni in soil using as input data Ni concentration measured in *P. annua* plants. An even better relation (*R= 0.928*) was obtained when considering the bioavailable fraction of Ni compared to Ni content in *P. annua* shoots (table enclosed in Fig 3B). The connected MLR model showed an even higher predictive potential, with high accuracy for the whole range of Ni values (response plot in Fig. 3B). The high *R* values and the solidity of both models confirmed that *P. annua* can be used as reliable Ni bioindicator.

2.2.4. Bioindication of Ni using S. vulgaris

The results regarding *S. vulgaris* showed a similar trend respect to those of *P. annua.* The data showed a clear linear relation (*R=0.908*) between Ni in soil and Ni in plant (table enclosed in Fig. 4A).



Intercept	-213	**Intercept st.dev.**	79.8	**R^2**	0.908
Slope	433	**Slope st.dev.**	37.7		



Intercept	-2.13	**Intercept st.dev.**	0.786	**R^2**	0.969
Slope	6.10	**Slope st.dev.**	0.335		

Figure 4. A) TABLE: linear regression between total Ni concentration in soil and Ni in *S. vulgaris* shoots; PLOT: recalculated total soil Ni by the model, input data derived from the linear relation between total Ni in soil and Ni in plant. Blue dots: forecasted soil Ni concentrations in calibration mode (all soil data were used as input). Red dots: forecasted soil Ni concentrations in cross-validation mode, excluding one soil data at a time (leave-one-out mode).
B) TABLE: linear regression between bioavailable Ni concentration in soil and Ni in *S. vulgaris* shoots; PLOT: recalculated total soil Ni by the model, input data are derived from the linear relation between total Ni in soil and Ni in plant. Blue dots: forecasted soil Ni concentrations in calibration mode (all soil data were used as input). Red dots: forecasted soil Ni concentrations in cross-validation mode, excluding one soil data at a time (leave-one-out mode).

From this strong relation the creation of a predictive MLR model was also possible (response plot in Fig. 4A). For low Ni values the performance of the models was high both in calibration and cross-

validation mode. While for high Ni values, results obtained in cross-validation were slightly different from the calibrations ones. The model appeared reliable when tested by ANOVA (*p*-value related to the *F* parameter <0.05) therefore significant for bioindication purposes. An even better linear relation (*R= 0.969*) was obtained when considering the bioavailable Ni pool in soil compared to *P. annua* Ni content (table enclosed in Fig 4B). The connected MLR model showed therefore an even higher predictive potential (response plot in Fig. 4B) respect to the one calculated on total Ni content. In this case, for all Ni concentrations the performance of the model was similar, with the data forecasted in cross-validation (red dots) almost overlapped to the ones calculated in calibration (blu dots). This high similarity of the two sets gave strength to the model that can be considered as highly reliable for available Ni prevision in soil.

2.3. Discussion

Polluted urban soils from Milan and Bologna, despite some peculiarities connected to parent materials that contributed to their pedogenesis, were characterized by similar amounts and types of HMs. All urban surfaces, in fact, receive deposits that mainly come from anthropic activities, like vehicle emissions, industrial discharges, domestic heating and material weathering (Gibson and Farmer, 1986; Kelly et al., 1996).

Street dust and top roadside soils in urban areas are typical sinks of HMs from atmospheric deposition and water runoff. Key HMs in these zones are: Pb from gasoline additives, Cu, Zn and Cd from car components, tire abrasion, lubricants and industrial emissions (Markus and McBratney, 1996; Wilcke et al., 1998). MI3 and MI4 (Table 4) resulted the most polluted soils as they were collected in very busy street crossing. This relation underlined the important role of vehicular traffic in contributing to soil pollution.

Woodland soils were collected in natural areas not influenced by anthropic activities and trace elements detected in such soils were those originally present in parent materials. However, a small contribution form diffuse sources of HMs pollution, like vehicles emission, cannot be excluded for these samples as well. It has been reported, in fact, that fine particulate such as that coming from tires and brakes abrasion may fall far away from source location (Hulskotte et al., 2007). Finally, in ultramafic soil, collected far from anthropic source of pollution, the level of anthropic HMs (Zn, Cu, Pb, Cd) was low, demonstrating the naturalness of this environment, but, given the ultramafic rock origin on which pedogeneseis took place, the soil was highly enriched in Ni and Cr (Kierczak et al., 2007).

The marked heterogeneity of sampled soils was the main obstacle to the calculation of plant-soil linear relations in the absorption of metals. The investigated soils showed a wide range of properties (like different texture, pH, OM content, etc.) that deeply affected the bioavailability of metal to plants (Kabata-Pendias, 2011; Yang and Ye, 2009). Morover, it is widely known that trace elements are not taken up by plants in a way directly proportional to their concentrations in soil (Curlik et al., 2016). The uptake of these elements by plants is selective: essential nutrients (like Zn, Cu and Mn) are actively uptaken and show a more linear relation to soil concentration if compared to non-essential nutrients (Kabata-Pendias, 2011; Smillie, 2015). This active absorption of micronutrients eventually results in a greater translocation and concentration in plant shoots, but also in a higher toxicity if compared to non-essential nutrients (Ralph and Burchett, 1998).

In agreement with these uptake mechanisms, the species investigated in the present research showed higher correlations between plant metal content and soil metal content for Zn and Ni (micronutrients) than for non-essential ones such as Cd (Table 6). Despite correlation coefficient for Zn where around 0.6 for all species (Table 6), no linear relations were found for this metal in all selected plant species. On the other hand, *P. annua* (*R*= 0.87) and *S. vulgaris* (*R*= 0.73) showed a linear relation between the amount of uptaken Ni and present in soil as similarly found also for *Taraxacum officinale* (Fröhlichová et al., 2018).

Another possible reason for the lack of linear relations between metal content in soils and plants is probably due to the poor translocation capacity of these elements from root to shoot. Other studies in fact demonstrated that when non-essential metals are present at high concentration in soil, most herbaceous plants tend to use exclusion strategies to prevent the uptake of these toxic elements (Weis et al., 2004; Weis and Weis, 2004). This phenomenon was observed for Cd in *Halophyla ovalis* (Ralph and Burchett, 1998) and for Pb (Sharma and Dubey, 2005). Species unable to prevent root absorption, instead limit the translocation to shoots keeping the majority of toxic elements stored in roots (Stoltz and Greger, 2002).

In the present study roots were not collected, so data about metal concentration in below ground organs were not available, but a previously published extensive literature demonstrated that soil HMs concentration better correlate to root metal concentration respect to shoot levels (Llagostera et al., 2011; Phillips et al., 2015). However, shoots are the most used parts for bioindication purposes, due to their visibility and easiness of collection. For this reason, the use of plants with limited translocation capacity of HMs in above ground parts, has low practical use (Llagostera et al., 2011).

The bioaccumulation factor (BAF), a parameter that quantifies the element transfer from soil to plant aerial parts, was lower than 1 for almost all samples and all metals. Due to this low BAF, the studied species can be considered non-accumulators, in fact, according to van der Ent et al. (2012), metal accumulator plants must have BAF parameter always higher than 1. Interestingly, several BAF values were found to be above 1 for Cd (Table 5), and similar results were also reported for *P. major* (Galal and Shehata, 2015), demonstrating that this metal at low concentrations can be easily uptaken and transferred to aerial parts.

Only for Ni and in all soils, *P. annua* and *S. vulgaris* (Fig. 5) demonstrated to have similar BAF values (on average 0.05), making them suitable for bioindication purposes. This is in line with the guidelines from EPA (2007), that state that good indicator plants should keep this parameter constant in several soil conditions. Moreover, Ni is very mobile inside the plant and is transported (bound by organic acids) via the xylematic flow from roots to shoots (Yusuf et al., 2011). The present work demonstrated that aerial parts of the common weeds *P. annua* and *S. vulgaris* can be used as environmental indicators of Ni pollution in soil. Similar results were achieved on *Urtica dioica, Taraxacum officinale, Plantago major* and two *Trifolium* species for Pb, Mn and Cu (Malizia et al., 2012). The use of common weeds can be a valid alternative to the use of lichens in assessing HMs levels in cities, especially because these herbaceous plants are common and easily recognizable.

Figure 5. A) *Senecio vulgaris*, growing in a busy street crossing in Bologna. B) *Poa annua* growing on the sidewalk in Milan

Interestingly, Malizia et al. (2012) demonstrated that similar results were achieved when assessing HMs in soil of the city of Rome by using lichens or using herbaceous plants for Cu, Zn and Pb. The promising results obtained with common weeds should encourage the research on these plants as valid alternative to lichens biomonitoring in urban areas.

Despite the previous literature about biomonitoring using herbaceous species most studies focused on *Taraxacum officinale* (Malizia et al., 2012) while only few took into account other species (Kleckerová and Dočekalová, 2014).

The results of the present study highlighted the possibility to find new species suitable for bioindication of metal pollution in anthropic environments. The importance of having several bioindicator species in each environment has been underlined by Phillips et al. (2015) who suggested a multi-species approach to bioindication in order to obtain more precise results. Finally, it was demonstrated the possibility to create predictive models when strong linear relations are present between the amount of Ni in the soil and in the plant. This chemometric approach was not aimed at the replacement of collection and analysis of samples but, instead, at further validating the results achieved by traditional field samplings and lab analysis.

2.4 Conclusions

The present results about the possible use of *S. vulgaris, P. aviculare* and *P. annua,* as HMs bioindicators, showed that metal concentrations in soils and plants mostly do not correlate under natural growth conditions. Despite metals found in soils are always present in plants, their concentration in above ground organs is deeply influenced by soil properties and plant translocation capacity. None of the studied species was suitable to be a good biondicator for Zn, Cu, Pb, Cr, and Cd; moreover, *P. aviculare* was found to be inefficient even as Ni indicator. On the contrary, the present work demonstrated the feasibility to use *P. annua* and *S. vulgaris* as bioindicators of Ni concentration in soil. The two species were reliable indicators both of total and bioavailable Ni fractions. In fact, the metal analytical data achieved in soil and plant aerial parts of these two species allowed us to develop good chemometric models, which were able to forecast with great accuracy Ni concentration in soil staring from the amount of Ni in plants.

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3. PH variation influences Ni and Zn uptake in different populations of *Stellaria media* L. Vill. grown in hydroponics

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Mirko Salinitro performed the experiment, carried out the analyses and wrote the manuscript, Antony van der Ent, coordinated the study, Alice Tognacchini perfomed the ICP-MS analyses, Annalisa Tassoni, revised the manuscript.

List of abbreviations:

AA: ascorbic acid,

ABTS: 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonic) acid,

CAT: catechin,

GA: gallic acid,

HMs: heavy metals

3.1. Introduction

The correlation between metal availability and soil parameters has been intensively studied over the last decades, to better understand which were the main parameters that influence metals activity in soil, hence uptake and toxicity for plants (Kukier et al., 2004; Pérez-Esteban et al., 2012;

Pérez-Esteban et al., 2014; Zia et al., 2018). A significant number of studies have shown that soil pH and dissolved organic carbon (DOC) strongly influence metal concentrations and speciation in soil solutions, although the specific effects vary between different metals (Kunhikrishnan et al., 2017; Schneider et al., 2016). Other studies have considered the influence of total organic matter content, with Fe-Mn oxides and pH as major factors for metal availability in soil solution (Pérez-Esteban *et al.*, 2012; Pérez-Esteban et al., 2014; Zeng et al., 2011). Ni and Zn are typically strongly correlated to soil solution pH and are weakly influenced by other factors (Bhogal et al., 1993; Kukier et al., 2004; Zia et al., 2018). It has been demonstrated that soluble organic carbon did not affect Zn concentrations in soil solution because of the lower affinity of Zn for organic compounds (Wong et al., 2007), while for Ni, soil characteristic (like clay content and cationic exchange capacity) (CEC)) and pH have significant effect on metal availability (Zia et al., 2018). Nonetheless, most authors agree that pH is the major factor in influencing metal availability and uptake (Kukier et al., 2004; Park et al., 2011; Walker et al., 2004). For Zn a negative correlation between pH and soluble Zn (at low pH more solubility) has been observed in many studies and conclusion on this metal appear consistent in the literature (Pérez-Esteban *et al.*, 2014; Zeng *et al.*, 2011; Zia *et al.*, 2018).

The situation is very different for Ni. A study on the effect of different soil pH on Ni concentrations of the hyperaccumulators *Alyssum murale* and *Alyssum corsicum,* concluded that an increase in soil pH was associated with an increase in shoot Ni concentration (Li et al., 2003). Those results were in contrast with other data demonstrating that the increase of soil pH caused a decrease of Ni concentrations in various non-accumulator species (Kukier and Chaney, 2004; L'Huillier and Edighoffer, 1996) and in the hyperaccumulators *Berkheya coddii* and *Alyssum bertolonii* (Robinson et al., 1999; Robinson et al., 1997). Moreover, contrasting results were found by (Kukier et al., 2004) studying *A. corsicum* and *A. murale* in different Ni-contaminated soils. Additionally, it has been recently stressed that the bioavailability of metals varies with plant species as a result of rhizosphere mechanisms such as root acidification or alkalinisation (Chaignon et al., 2002; Hinsinger and Courchesne, 2008). For instance, a study found that the availability of Zn was higher than expected in tobacco plants due to roots-induced pH decrease (Loosemore et al., 2004).

Despite many studies have been carried out on soil evaluating the synergic influence of many factors, very limited research (i.e Kumar et al., 2012) has been done in hydroponic, to investigate how a single factor (*i.e.* pH) could influence the availability and the subsequent uptake of a certain metal in plants.

If evaluating the effect of a single factor can be difficult in a complex system like the soil, it is conversely possible in hydroponic conditions in which the nutrient solution parameters can be

singularly manipulated. Moreover, roots acidification or alkalinisation is effectively counterbalanced in hydroponics, making these root processes uninfluential. Following this approach, the aim of the present study was to evaluate the effect of pH on the uptake and toxicity of Ni and Zn, on the non-accumulator plant *Stellaria media* grown in hydroponics. Moreover, the plant content of chlorophylls, polyphenols, flavonoids and the antioxidant activity were analysed in order to correlate them with Ni and Zn accumulation and metal stress effects in *S. media*.

3.2. Material and Methods

3.2.1. Selected species

Stellaria media (L.) Vill. (Caryophyllaceae) is an annual herbaceous plant native to Europe and widely naturalized in all continents. It commonly grows in disturbed habitats, such as road margins, crop fields and bare soils deposits. Five different accessions of *Stellaria media* were collected in the year 2017 in five different locations. For each location, seeds were taken in several stations to sample the whole genetic variability of each place. Two accessions were respectively from the urban environment of Milan and Bologna (Italy), two accessions were from woodland stations located at the outskirt of Milan and Bologna, one accession was from ultramafic soils naturally enriched of Ni and Cr and located at mount Prinzera (Parma, Italy) (see detailed table at page 28). Urban stations were characterized by high and polymetallic pollution of soils, woodland station were conversely characterized by low heavy metal (HMs) levels, ultramafic station were characterized by high levels of Cr and Ni only. Seed collections was carried out at the end of the vegetative season of the plants (late April). Plants were cut and air-dried, then shook to allow the release of seed. Seeds were sieved to remove plant particles, air-dried for 1 week, than stored in plastic tubes at room temperature until sowing. For the germination, seeds were soaked overnight in tap water, then placed on a substrate composed by perlite, vermiculite and quartz sand in a ratio of 1:1:1. Seeds were watered with tap water and kept in the dark at 20°C for 2-3 days until germination. Seedlings were then transferred in a growth cabinet with 12-12 hour light-dark at 25°C degrees for 1 week, before transplanting them into the hydroponic system.

3.2.2. Hydroponic culture system

The hydroponic system was composed of 4 separate tanks (25 L each) filled with Vega Classic nutrient solution (Canna, Brisbane QLD, Australia) diluted 1:250, containing: 16.3 mM N, 1.2 mM P, 5.7 mM K, 4.4 mM Ca, 1.3 mM Mg, 1.1 mM S, 14 µM Fe-DTPA (diethylenetriamine penta acetic acid ferric complex), 26 µM B, 0.6 µM Cu, 10.2 µM Mn, 0.8 µM Mo, 4.3 µM Zn.

The nutrient solution was spiked with $Zn(NO_3)_2 \cdot 6H_20$ or $Ni(NO_3)_2 \cdot 6H_2O$ to obtain a concentration of 0.55 mM and 0.10 mM respectively (Table 1).

Table 1. Different pH treatments with Ni and Zn in hydroponics. N.d., not determined.

pH	**Zn (mM)**	**Ni (mM)**
5.0	0 (Control)	0 (Control)
5.0	0.55	0.10
5.5	n.d.	0.10
6.0	0.55	0.10
6.5	0.55	0.10
7.0	0.55	no growth

Zinc was tested at pH 5 to 8, while Ni was tested at pH 5 to 6.5 because at higher pH the combination of Ni stress and induced Fe deficiency inhibited plant growth (Table 1). Control plants were cultivated at pH 5 with no added metal in the solution. The pH was automatically maintained at the set value ± 0.1 with 0.1 M KOH solution (Table 1). A 5% (v/v) replacement of the nutrient solution was performed daily. For each accession, five plants were grown in 5 cm plastic baskets filled with a foam disk and immersed in the nutrient solution. Before the transplant in the hydroponic systems, plants have been thoroughly washed to remove any soil residues. Plants were grown for 20 days with 12-12 hours light-dark, under LED light (intensity ~20000 lumen, temperature 7500 K), at 20° C

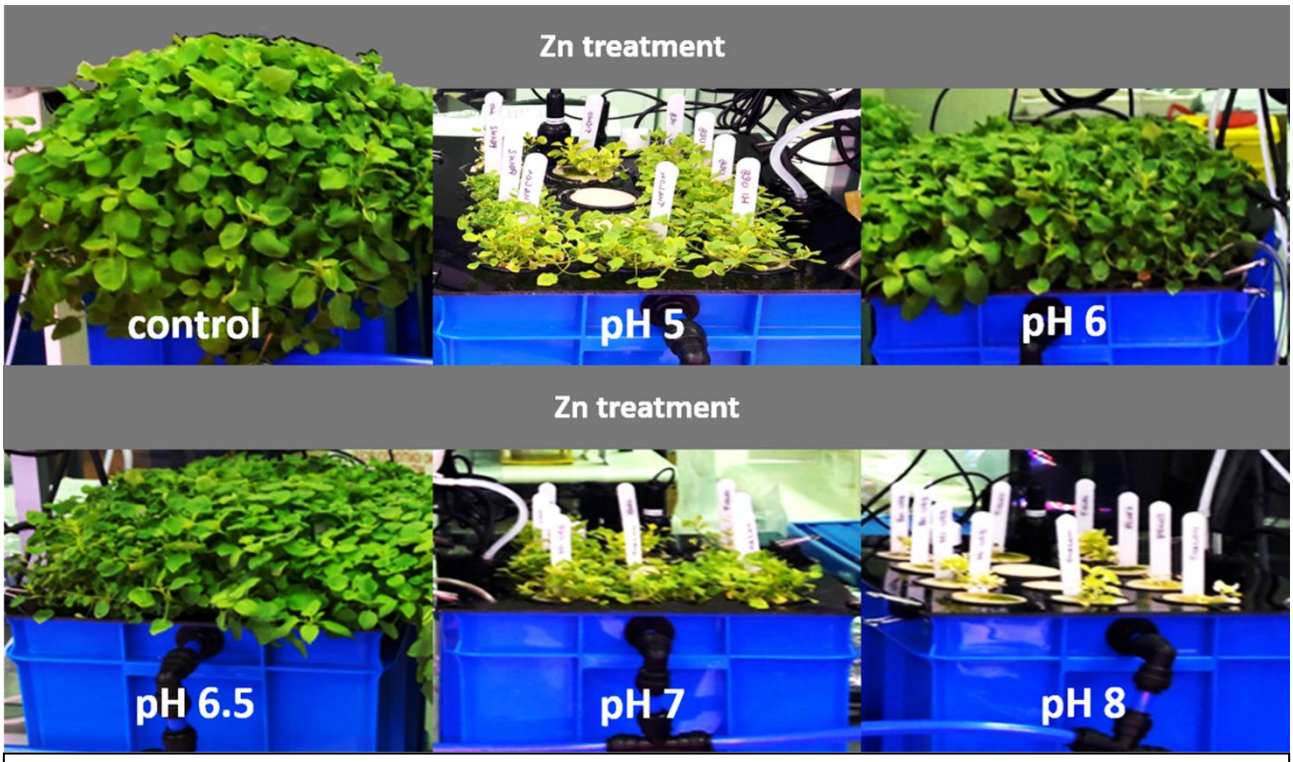

Figure 1. *Stellaria media* plants after 20 days of growth in hydroponics with the addition of 0.55 mM of Zn. Each tank corresponded to a pH treatment (pH 5 to 8). The control tank had no Zn and was kept at pH 5.

(Fig. 1).

At the end of the cultivation period, plants were harvested and divided into shoots and roots. Plants were rinsed with de-ionised water then grinded in liquid nitrogen. The bulk samples were divided into two aliquots: fresh aliquots (stored at -80° C) used for spectrophotometric analyses and dry aliquots for metal content analyses. To obtain dry samples, the aliquots were at 60°C for 24 hours. Dried shoot weight was on average 13.5 % of the fresh one, dried root weight was on average 7.3 % of the fresh one.

3.2.3. Spectrophotometric quantifications

For spectrophotometric analyses, 0.1 g of freshly grinded plant samples were extracted with 1 mL of 95% (v/v) methanol and shaken overnight at room temperature. The supernatant was then recovered after centrifugation at 15,300 x *g* for 5 minutes at room temperature and used in the total polyphenols, total flavonoids and antioxidant activity quantification reactions.

Total polyphenols colorimetric quantification was performed through Folin-Ciocalteu assay (Ferri et al., 2013; Singleton et al., 1999). The results were expressed as mg of gallic acid equivalents per g of fresh weight (mg GA eq/gFW) by means of a dose-response calibration curve (between 0 and 15 µg of gallic acid).

Total flavonoids colorimetric quantification assay was performed as in Zhishen et al. (1999). The results were expressed as mg of catechin equivalents per g of fresh weight (mg CAT eq/gFW) by means of a dose-response calibration curve (between 2 and 14 µg of catechin).

The antioxidant activity quantification was performed through ABTS (2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonic) acid) reagent decolorimetric assay (Ferri et al., 2013; Re et al., 1999) and the results were expressed as grams of ascorbic acid (AA) equivalents per g of fresh weight (mg AA eq/gFW) by means of a dose-response calibration curve (between 0 and 2 µg of AA).

For the determination of photosynthetic pigments, a modified method from Radwan et al. (2007) and Metzner et al. (1965) as used. 0.1 g of freshly grinded plants, were extracted with 1.5 mL of 85% (v/v) acetone and mixed 2 times for 30 seconds, the samples were then centrifuged at 4°C, 665 x *g* for 5 minutes and the supernatant recovered. Plant powder resulted completely bleached at the end of the extraction.

The supernatant was analysed at three different wavelengths (663, 644 and 452.5 nm) and the obtained absorbance values were processed to give the pigment concentrations in mg/gFW with the following equations:

chlorophyll *a* = 10.3 x Abs_{663} – 0.98 x Abs_{644},

chlorophyll *b* = 19.7 x Abs_{644} – 3.87 x Abs_{663},

carotenoids = 4.2 x $Abs_{452.5}$ – [(0.0264 x chl-*a*) + (0.426 x chl-*b*)]

All the spectrophotometric analyses were performed with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

3.2.4. Nickel and zinc quantification

For the quantification of metals, grinded roots and shoots were oven dried at 80°C until constant weight. Samples were pre-digested at room temperature with 2 mL 70% (v/v) HNO_3 for 1 day, then

digested on a heat block 1 h at 70°C and 1 h at 125°C following a modified method from Huang et al. (1985). After the digestion, samples were brought up to 10 mL with de-ionised water. Five replicates of reference material (Apple leaves NIST® SRM® 1515) and blanks were included in the digestions. The analyses were performed with an iCAP 7400 series ICP-OES simultaneous spectrometer (Thermo Fisher Scientific, Cambridge, UK). Data were expressed as ppm related to sample dry weight.

3.2.5. Data analysis

All the statistical analyses were performed using R software version 1.3.5 (R Core Team, Vienna, Austria). The differences in metal uptake and metabolites production were evaluated among the 5 different plant populations as well as the different pH treatments. Data were tested for normality using Shapiro-Wilk normality test and for homogeneity using the Levene's Test for Homogeneity of Variance with default parameters from the package "car" (https://CRAN.R-project.org/package=car). The non-parametric Kruskal-Wallis test, followed by Dunn's multiple pairwise comparison post-hoc test from dunn.test package (https://CRAN.R-project.org/package=dunn.test), were used to evaluate the differences among compared groups (*p-values* are reported in brackets in the section Results) . Spearman correlation coefficients were calculated to determine the relationship between Zn and Ni uptake and metabolites production. Linear regression model was used to describe the relations between metal uptake and flavonoids, polyphenols and antioxidants. Non-linear regression was used to describe the relation between Ni uptake-fresh biomass and Zn uptake-chlorophylls production (R^2 and *p-values* are reported in brackets in the section Results). Graphical elaborations where performed using the R package ggpubr (https://CRAN.R-project.org/package=ggpubr). To calculate free ionic activity of Ni and Zn at every pH tested a simulation using the software GEOCHEM-EZ 1.0 (free software available at http://www.PlantMineralNutrition.net) was performed. The salts contained in the nutrient solution and solution pH were used as input data, and the parameter *precipitation allowed* was set as default.

3.3. Results

3.3.1. Population variability

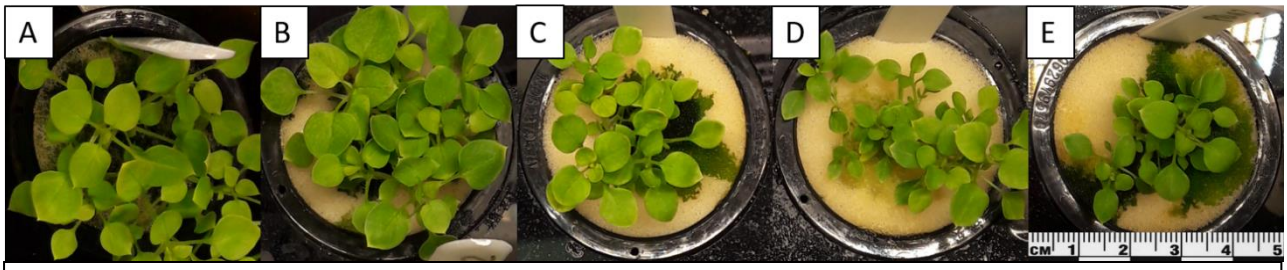

Figure 2. Different plant populations of *Stellaria media* L. Vill. after 1 week of hydroponic growth in control conditions (no metal and pH 5.0). A) Bologna woodland; B) Milan woodland; C) Milan urban; D) Bologna urban; E) ultramafic.

Population variability was mainly observed in control plants and in treatments were Zn and Ni

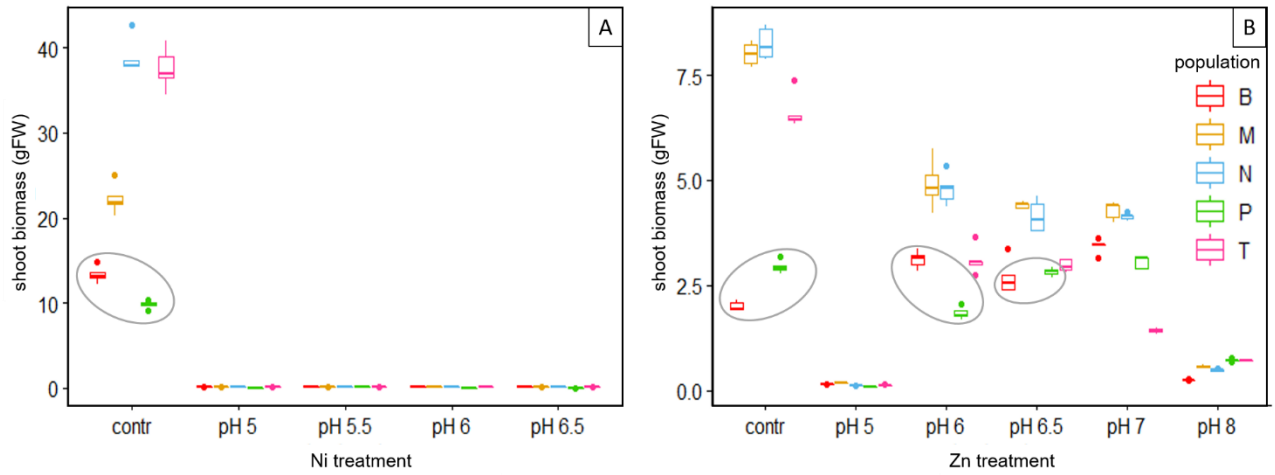

Figure 3. Shoot biomass variability in *Stellaria media* plants from five different populations, after hydroponic culture at different pH. Plants treated with Ni (A) and with Zn (B) at different pH. B, Bologna urban; M, Milan urban; N, Bologna woodland; P, ultramafic; T, Milan woodland. Highlighted with grey circles the ultramafic and Bologna urban accessions, which always showed the smallest size plants in low stress conditions.

toxicity resulted low (Fig. 2). The smallest plants were those coming from ultramafic and Bologna urban accessions with an average shoot weight of 7.7 gFW and 6.4 gFW, respectively.

The Milan urban accession showed an intermediate shoot weight with an average of 15.1 gFW, while Milan and Bologna woodland accessions displayed a higher average shoot weight of 22.8 gFW. These differences disappeared when Zn and Ni concentrations in plants tissue were higher and toxic effects buffered population variability (Figs. 3A, B).

3.3.2. PH influence on Ni and Zn uptake

An overall different biomass production (at all tested pH) was noticed between plants grown with Ni and plants grown with Zn (Fig. 3). For the first, the average weight of treated plants was 0.11 gFW

and 0.04 gFW, for shoots and roots respectively. For the second, the average weight of treated plants was 2.18 gFW and 0.52 gFW, for shoots and roots respectively.

Table 2. Ni and Zn ionic activity at different pH treatments calculated with the software GEOCHEM-EZ. The ionic activity of a metal expresses the ease with which it undergoes chemical reactions and interactions with biological membranes (like root hairs) and other elements. To run the simulation the salts contained in the nutrient solution and solution pH were used as input data and the parameter precipitation allowed was set as default.

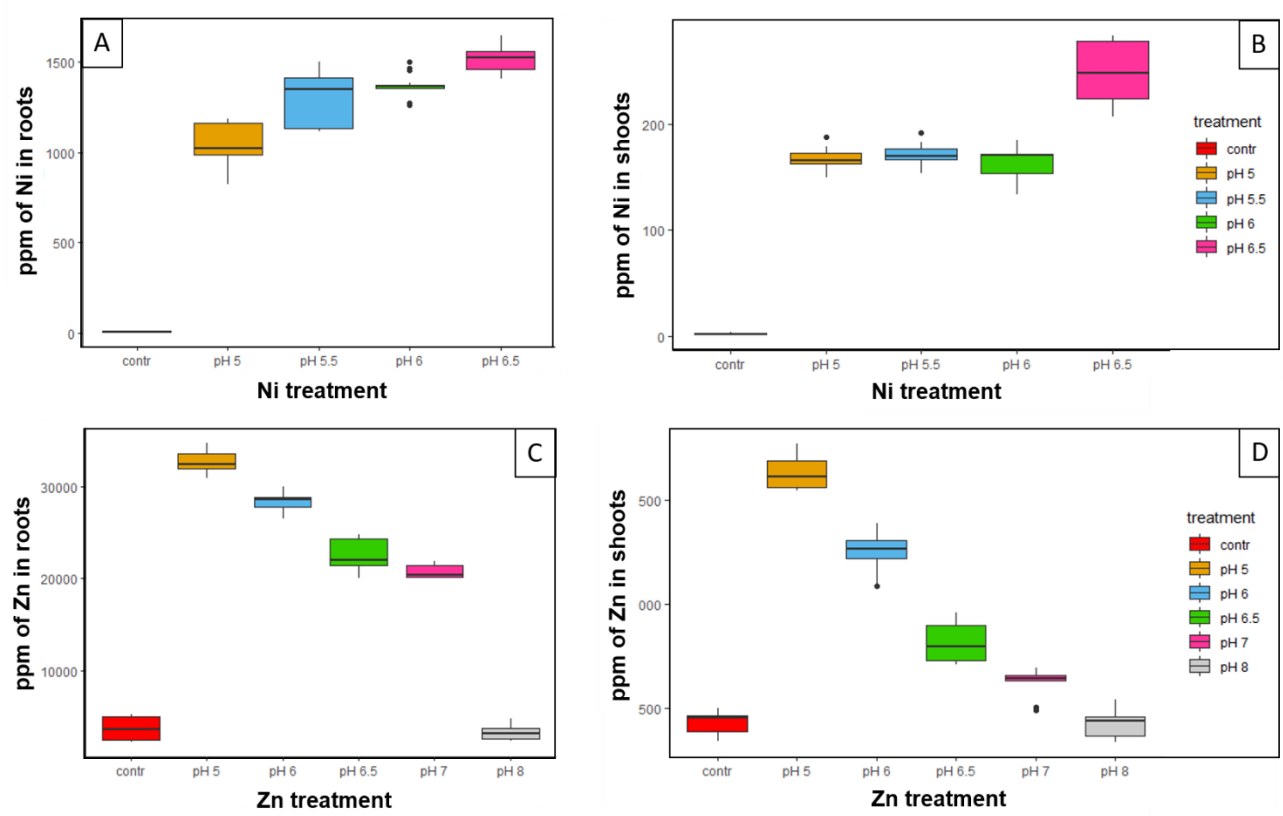

Figure 4. Ni and Zn uptaken concentrations in *Stellaria media* roots and shoots after hydroponic culture at different pH. Ni uptake in roots (A) and shoots (B). Zn uptake in roots (C) and shoots (D). All treatments resulted significantly different (*p<0.05)* after Kruskal-Wallis test and Dunn's post-hoc test, except pH 5, 5.5, 6 in Ni treatment (*p>0.05*).

Data showed that different pH treatments clearly affected the uptake of Ni and Zn (*p= < 0.01* both for shoots and roots) but in an opposite way (Fig. 4). Ni accumulation increased with the increase of solution pH, both in roots and shoots (Figs. 4A, B) with roots accumulating on average six-times more Ni than shoots. Ni concentration in plant roots varied from an average 1036 ppm at pH 5 to 1522 ppm at pH 6.5 (Fig. 4A). A similar trend was observed in shoots that ranged between an average of 167 ppm, at pH 5, to 250 ppm at pH 6.5. Unlike Ni, Zn accumulation decreased with the increase of pH (Figs. 4C, D). The concentration in roots was from five to 20-fold higher than in shoots respectively in pH 8 and pH 5 treatments. Zn concentration in roots, varied between an average of 32700 ppm, in plants grown at pH 5, and 3390 ppm in plants at pH 8 (Fig. 4C). In shoot Zn uptake ranged on average between 164 ppm, in plants grown at pH 5, and 435 ppm at pH 8 (Fig. 4D).

Ion activity of Ni did not show significant changes among pH treatments, but only a slight decrease at the increase of pH, conversely Zn activity decreased at higher pH treatment (Table 2).

The translocation of both Ni and Zn from roots to shoots was affected by their availability. The

	Ionic activity in Zn treatment			**Precipitates µg/L**		
pH	Zn	Fe	PO_4^-	$ZnPO_4$	$FeOH^-$	$FePO_4$
8	3.904×10^{-7}	3.198×10^{-21}	9.213×10^{-9}	4.672	0.192	-
7	2.221×10^{-6}	3.190×10^{-18}	6.752×10^{-9}	1.104	0.187	-
6.5	7.006 v 10^{-6}	3.311×10^{-17}	1.202×10^{-10}	1.079	0.183	-
6	2.642 v 10^{-5}	2.395×10^{-16}	1.653×10^{-11}	0.990	0.185	-
5	2.584×10^{-4}	1.533×10^{-14}	2.590×10^{-13}	-	-	0.090
	Ionic activity in Ni treatment			**Precipitates µg/L**		
pH	Ni	Fe	PO_4^-	$ZnPO_4$	$FeOH^-$	$FePO_4$
6.5	3.337×10^{-5}	2.339×10^{-17}	1.668×10^{-10}	-	-	0.093
6	3.517×10^{-5}	1.788×10^{-16}	2.252×10^{-11}	-	-	0.093
5.5	3.590×10^{-5}	1.595×10^{-15}	2.520×10^{-12}	-	-	0.093
5	3.654 v 10^{-5}	1.535×10^{-14}	2.616×10^{-13}	-	-	0.093

translocation of Ni from roots to shoots was around 25 % in control plants, where Ni levels were very low, whereas in all the other treatments the translocation was around 12% to increase up to 16% at pH 6.5 ($R^2 = 0.604$, *p<0.01*) (Fig. 5A). When Zn levels were low (*i.e*. in control treatment) or its availability was low (*i.e.* in the pH 8 treatment) plant transferred up to the 15% of Zn from roots to shoots. Conversely, with the increase of Zn availability, the translocation decreased around 3% and went slightly up at 5% at pH 5 ($R^2 = 0.913$, *p<0.01*) (Fig. 5B).

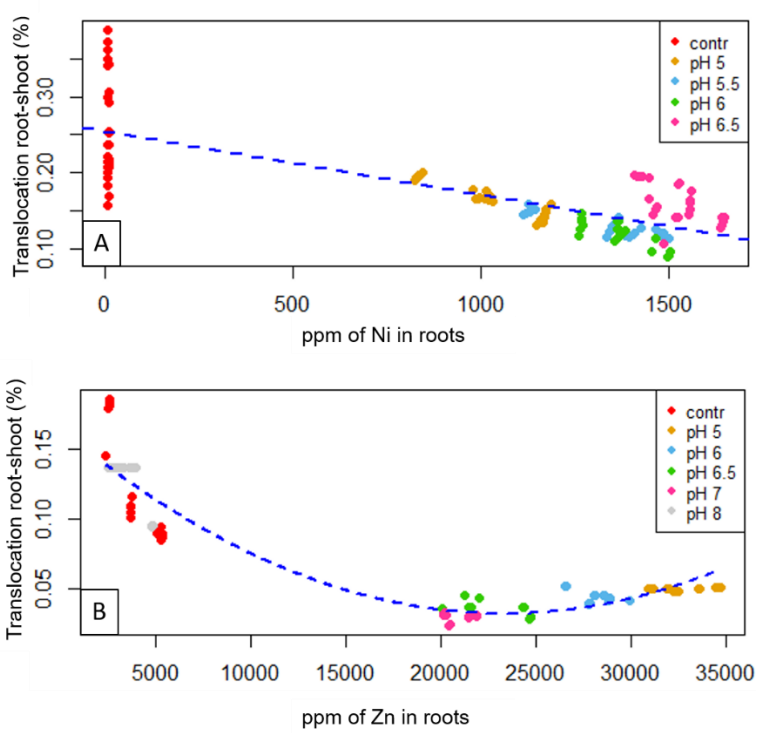

Figure 5. *Stellaria media* root-shoot Ni and Zn translocation gradients in relation to metal concentrations in roots after hydroponic culture at different pH. Ni (A) and Zn (B) translocation. A) Linear model (R^2 = 0.604, *p<0.01*); B) Non-linear model (R^2 = 0.913, *p<0.01*).

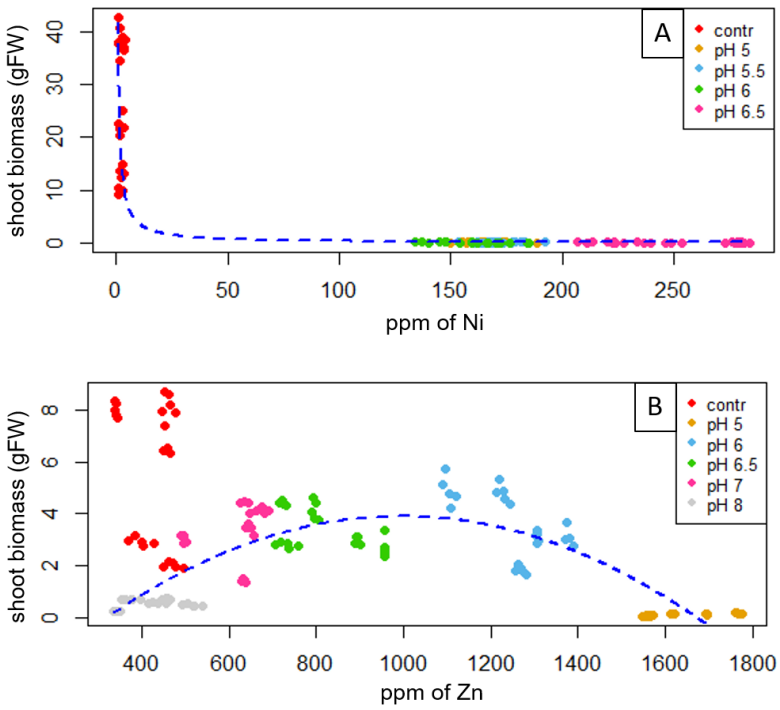

Figure 6. *Stellaria media* shoot biomass variation in relation to Ni or Zn uptaken concentration after hydroponic culture at different pH. Biomass variation in Ni (A) and Zn (B) treatments. A) Non-linear model (R^2 = 0.890, *p<0.01*); B) Non-linear model (R^2 = 0.652, *p<0.01*).

3.3.3. Assessment of Ni and Zn toxicity

One of the parameters which better reflected the plant stress connected to the uptake of Ni and Zn was the reduction of shoot biomass. Ni toxicity was similar among all plants grown at different pH with a biomass reduction of around 95% compared to the control for all treatments; a strong negative correlation between the two variables was assessed (R^2 = 0.890 *p<0.01*) (Fig. 6A). The complete Zn uptake curve, from toxicity to deficiency caused by pH,

was highlighted in this study and the correlation between Zn uptake and shoot biomass reduction was high (R^2 = 0.652, *p<0.01,* control was not included in the regression) (Fig. 6B). Plants showed strong Zn toxicity at pH 5, with a reduction in shoot biomass of 97% compared with the control. At pH 6, 6.5 and 7 plants had similar biomass values as the control, while at pH 8 plants suffered a marked biomass reduction (90% compared to control) due to Zn and iron deficiency caused by alkaline pH (Table 2).

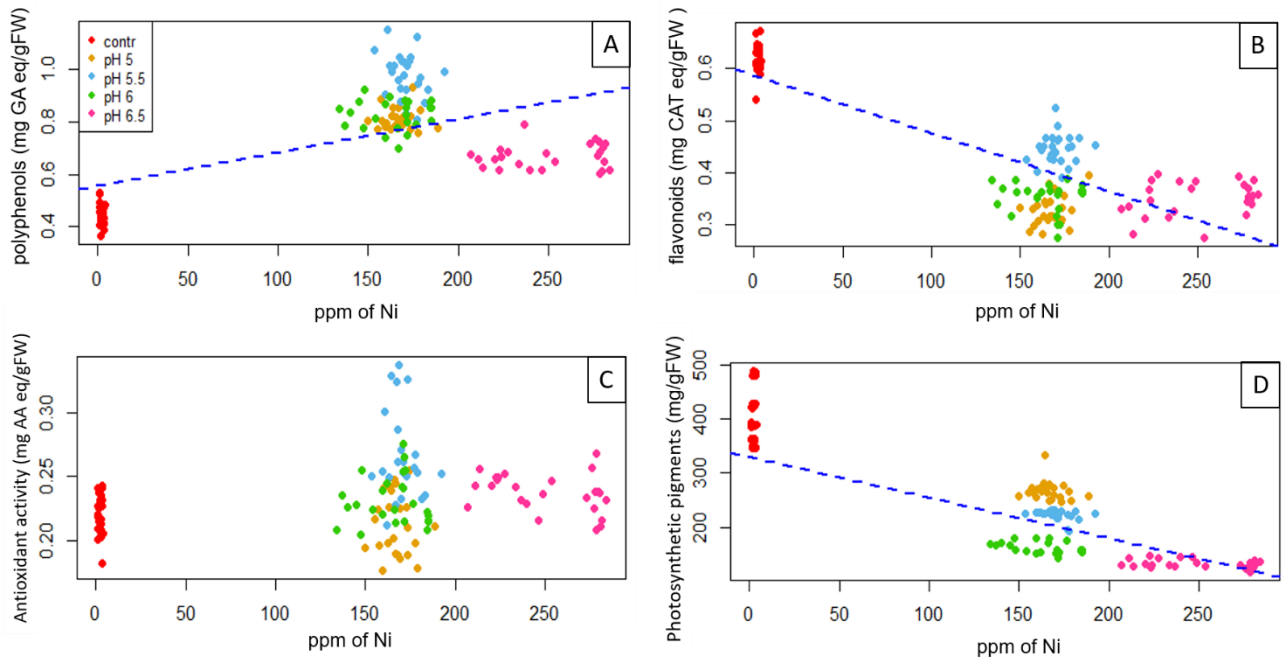

Figure 7. Polyphenol, flavonoid, photosynthetic pigment and antioxidant activity levels in *Stellaria media* shoots in relation to Ni uptaken concentration after hydroponic culture at different pH. A) Total polyphenols, expressed as mg of gallic acid (GA) equivalents/gFW; B) total flavonoids, expressed as mg of catechin (CAT) equivalents/gFW; C) antioxidant capacity, expressed as mg of ascorbic acid (AA) equivalents/gFW; D) total content of chlorophyll-*a*, chlorophyll-*b* and carotenoids, expressed in mg/gFW. A) Linear model (R^2 = 0.309 *p<0.05*); B) Linear model (R^2 = 0.793 *p<0.01*); C) Linear model (R^2 = 0.037 *p*=0.17); D) Linear model (R^2 = 0.680 *p<0.01*).

In addition to shoot biomass reduction, Ni increasing concentrations caused a reduction in total flavonoid amount and total content of chlorophyll-*a*, chlorophyll-*b* and carotenoids in plant aerial parts (R^2 = 0.793 *p<0.01* and R^2 = 0.680 *p<0.01,* respectively) (Figs. 7B, D). Conversely, total polyphenols were weakly positively correlated with Ni concentration (R^2 = 0.309 *p<0.05*) (Fig. 7A). Finally, antioxidant activity, to which both polyphenols and their subclass flavonoids contribute, seemed not to be correlated with Ni uptake (R^2 = 0.037 *p*=0.17) (Fig. 7C).

General trends were substantially clearer as regards Zn uptake. The values for pH 8 treatment were reported in the graphs (in grey) but not included in regression models because, as previously explained, the stress symptoms derived from metal deficiency (Zn and Fe) instead of Zn and Ni toxicity. The main differences were found in flavonoid content and antioxidant activity. For the first, the trend was reversed compared to Ni, in fact in shoots flavonoids increased dependently to Zn

concentration (R^2 = 0.852 *p<0.01*) (Fig. 8B). Moreover, as similarly reported for Ni, total polyphenols concentration was positively correlated with the increase of Zn (R^2 = 0.728 *p<0.01*) (Fig. 8A). Because both polyphenols and flavonoids may contribute to antioxidant activity, this parameter also showed a clear positive relation with Zn concentration (R^2 = 0.751 *p<0.01*) (Fig. 8C). Photosynthetic pigments content slightly increased in treatments at pH 6 and 6.5 compared to the control, then sharply decreased at pH 5 (R^2 = 0.476 *p=<0.05*).

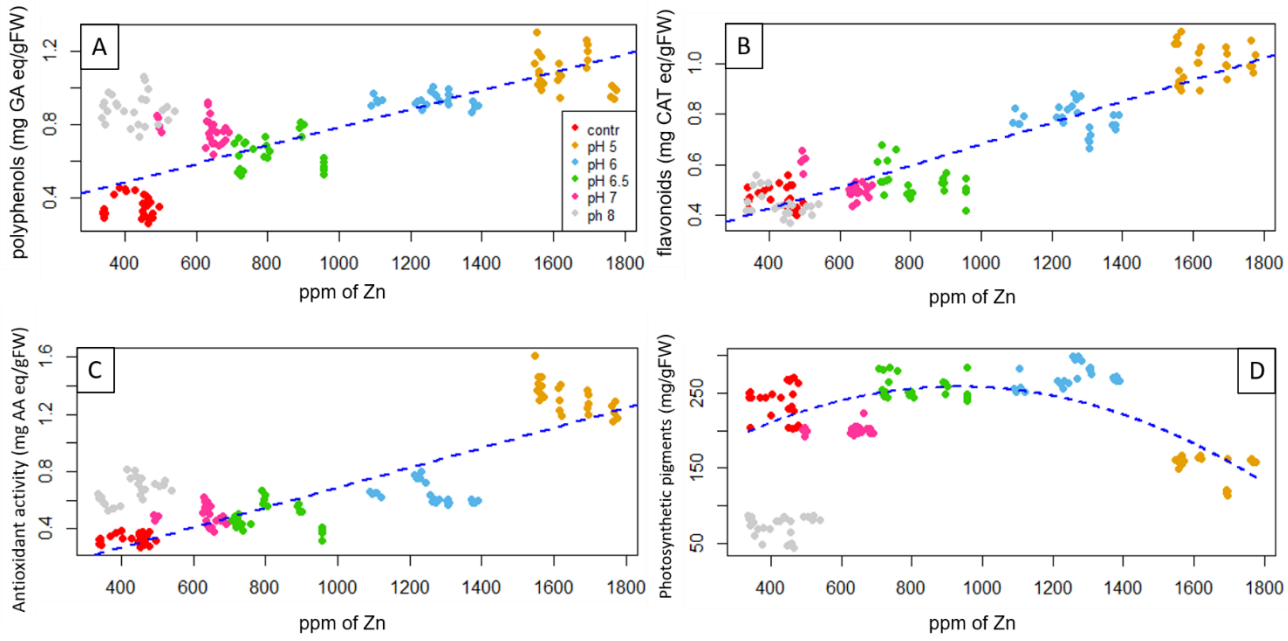

Figure 8. Polyphenol, flavonoid, photosynthetic pigment and antioxidant activity levels in *Stellaria media* shoots in relation to Zn uptaken concentration after hydroponic culture at different pH. A) Total polyphenols expressed as mg of gallic acid (GA) equivalents/gFW; B) total flavonoids expressed as mg of catechin (CAT) equivalents/gFW; C) antioxidant capacity expressed as mg of ascorbic acid (AA) equivalents/gFW; D) total content of chlorophyll-*a*, chlorophyll-*b* and carotenoids expressed in mg/gFW. A) Linear model (R^2 = 0.728 *p<0.01*); B) Linear model (R^2 = 0.852 *p<0.01*); C) Linear model (R^2 = 0.751 *p<0.01*); D) Non-linear model (R^2 = 0.476 *p=<0.05*).

3.4. Discussion

The aim of our research was to study the interactions between Zn and Ni uptake and the variable pH on a non-accumulator plant (*Stellaria media*) in hydroponic culture. This approach allowed us to observe changes in Zn and Ni uptake, with no interference due to soil characteristic like cationic exchange capacity, organic carbon content or root acidification processes. The lack of *buffer factors* in the experiment made clearer the effects caused by pH, but, on the other side, made plants more vulnerable to Ni and Zn toxicity. All plants in the Ni treatments suffered chlorosis due to Ni toxicity, while in the Zn treatment at pH 8 the plants suffered Zn and Fe deficiency (Table 2). In general, the deleterious effects of Ni were more marked than Zn ones, partly because the intrinsic higher toxicity

of Ni (Kabata-Pendias, 2010) and partly because higher translocation occurred from roots to shoots (Fig. 5A, B).

The present experimental design moreover, aimed at the assessment of possible variations among the tested five different *S. media* plant populations. The hypothesis that different populations coming from different habitats, could have developed different abilities in metal tolerance, has been already confirmed by several studies (Bickham et al., 2000; Collier et al., 2010; Keane et al., 2005), that took into consideration populations growing on standard and mine soils. Our data did not confirm such hypothesis and all populations appeared homogeneous for Zn and Ni uptake (*p=0.92* for Ni, and *p=0.91* for Zn) and for flavonoid, polyphenol and photosynthetic pigment productions. Therefore, it appears that differences in Ni and Zn concentrations and availability between urban, woodland and ultramafic soils were not enough marked to allow diversification among populations. Despite that, population indeed differed for their size, in fact plants coming from contaminated environments, such as urban and ultramafic accessions, were smaller then woodland accessions (Figs. 2, 3A, 3B). It was observed that, at the increase of pH there was a correspondent increase in Ni uptake both in roots and shoots of *S. media* (Fig. 4). The conceptual model of such response is known as *biotic ligand model* (Di Toro et al., 2001). When metal ionic activity is kept constant, an increase in pH may cause higher binding of metal cations by biotic ligands (biological membrane and transporter proteins) because of the deprotonation of transporters (Lòpez et al., 2000). Our results showed that Ni activity can be considered almost constant at all pH tested (Table 2), and this ideal situation could happen only in total absence of other factors able to bind or release Ni ions from the solution. Similar results were never achieved in pot experiments. In fact the only few studies carried out on non-accumulator plants like crop plants (Kukier and Chaney, 2004; L'Huillier and Edighoffer, 1996; Sanders et al., 1986) reported opposite trends.

The uptake of Ni may vary according to the type media (i.e. soil, perlite, hydroponic, etc.) in which the plant is grown. In fact, studies conducted on oats (Weng et al., 2003) found that Ni concentration in shoots increased with the increase of pH, analogously to our results (Fig. 4A).

As stated in several previous studies (Pérez-Esteban *et al.*, 2014; Zeng *et al.*, 2011; Zia *et al.*, 2018), soil pH is the main variable affecting bioavailability and uptake of Zn. This study is consistent with the literature since a strong negative correlation in Zn uptake at the increase of pH was observed (Fig. 4). This behaviour, can be explained by the chemical forms that Zn assumes at different pH (Kukier *et al.*, 2004). Solution pH influences the speciation of Zn: at pH values below 7.7, Zn^{2+} predominates and plants can easily absorb it, but above pH 7.7, $ZnOH^{+}$ is the main species and is not bioavailable anymore (Kiekens, 1995; Reddy et al., 1995). Moreover, as confirmed by the

GEOCHEM-EZ simulation (Table 2), free Zn activity decreased with increasing pH values, coupled with Zn precipitation at the high pH treatments.

Unlike Ni, the behaviour of Zn in hydroponics closely simulates the one in soil, as soluble Zn is mainly present as inorganic species, mostly as the free cation (94–98%) in soil solution. Moreover, few interactions with soil and organic matter may occur (Meers *et al.*, 2006; Pérez-Esteban *et al.*, 2014; Weng *et al.*, 2002).

Zn and Ni toxicity, induced by differential uptake among pH treatments, influenced several plant parameters, first of all the amount of biomass. As expected Zn and Ni toxicity caused a reduction of biomass and chlorosis, as widely stated in the literature (Jayakumar *et al.*, 2007; Weng *et al.*, 2003) (Fig. 6, 7D, 8D). Beside these effects, the production of secondary metabolites (like flavonoids and polyphenols) connected with the decrease of oxidative stress, was investigated. Interestingly the correlation between the production of these compounds and metal uptake was strong both for Ni (R^2 = 0.309, R^2 = 0.793, for flavonoids and polyphenols respectively) and Zn (R^2 = 0.852, R^2 = 0.728, for flavonoids and polyphenols respectively) (Fig. 7A, 7B, 8A, 8B). In Ni treatments data were grouped in two main clusters: control and pH treatments. All treated plants exhibited acute Ni toxicity, therefore the production of polyphenols and flavonoids and photosynthetic pigments was quite similar for all pH treatments and strongly different from control (Fig. 7). Flavonoids (a subfamily of polyphenols) were negatively correlated with Ni uptake, despite most of studies (Chalker-Scott, 1999; Winkel-Shirley, 2002) reported opposite trends, but total polyphenols were positively correlated. As a result, no correlation was detected for antioxidant capacity (Fig. 7C). This negative trend was probably caused by the high toxicity of Ni which suppressed the production of flavonoids as found by Jayakumar et al. (2007), Similarly, a decrease in polyphenols in the treatment at pH 6.5 was detected, despite the overall growing trend, a similar situation was reported in maize plants by (Kisa et al., 2016). The lower toxicity of Zn instead, allowed us to observe a gradual production of antioxidant which steadily increased from control treatment to high Zn stress (pH 5 treatment), with no signs of inhibition by the excessive toxicity. Polyphenols, flavonoids and total antioxidant capacity were all increasing at the increase of metal concentration acting clearly against the subsequent oxidative stress (Chalker-Scott, 1999; Jayakumar et al., 2007; Kisa et al., 2016; Kumar et al., 2012; Winkel-Shirley, 2002). Conversely, photosynthetic pigments content was negatively affected by Zn uptake which caused chlorosis (Fig. 8D).

3.5. Conclusions

This study showed that the variable pH is positively correlated with Ni uptake in hydroponic culture, while an opposite trend was detected in similar studies carried out on soil. Conversely, Zn uptake negatively correlated with pH and results found in soil and hydroponic are consistent. As consequence data pointed out that in soils, many other factors (such as organic matter and clay content) play an important role in controlling Ni solubility and uptake, while Zn is chiefly controlled by pH. *Stellaria media* plants had high Ni and Zn uptake and showed several toxicity symptoms and this study demonstrated that production of antioxidant substances (like polyphenols) is proportional with Zn and Ni concentration in shoots, while on the contrary biomass production and chlorophyll contents were supressed. Finally, our results demonstrated the suitability of using hydroponic systems to study single variables affecting metal uptake. These simplified systems can effectively avoid interference with many other substrate variables that cannot be excluded when experiments are performed using soil. Taking into consideration the relations between pH and the uptake of different metals, can be a starting key to maximize the success of biomonitoring campaigns and phytoremediation interventions in metal polluted sites.

3.6. References

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4. Antioxidant compounds (polyphenols and flavonoids) chlorophylls and biomass as tools for the evaluations of heavy metal stress

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Mirko Salinitro cultivated the plants, analysed the samples, elaborated the data, wrote the manuscript and coordinated the study. Sonia Casolari and Alice togancchini performed the AAS and ICP-MS analyses. Dora Melucci and Alessandro Zappi contributed to analyse the data Annalisa Tassoni coordinated the study and revised the manuscript.

List of abbreviations:

AA: ascorbic acid,

ABTS: 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonic) acid,

CAT: catechin,

GA: gallic acid,

HMs: heavy metals

ROS: Reactive oxygen species

4.1. Introduction

Metal contamination is one of the most important contemporary environmental problems. Metals are naturally present in soil, but, due to industrial and agricultural activities (such as smelting activities, vehicular traffic, mining wastes, use of sewage sludges, etc.), their concentrations have increased up to toxic levels in several areas (Vassilev et al., 2004, Rout and Das, 2009).

Plants growing on heavy metals' (HMs) contaminated soils cannot prevent their uptake, due to the absorption of these contaminants together with other essential nutrients, but they are able to differently accumulate them inside their tissues (Baker, 1981).

According to their behaviour in dealing with HMs, plants show different survival strategies which could be divided into four different categories. I) *Accumulators*, are highly tolerant plant capable of extracting heavy metals from soils and concentrate them into their above-ground tissues. II) *Indicators*, are quite tolerant plants in which the internal metal concentration of their above-ground tissues is directly proportional to the external concentration present in the soil. III) *Excluders*, are tolerant plants capable to limit the internal levels of heavy metals. IV) *Non-accumulators*, are sensitive plants that do not possess specific mechanisms of uptake, translocation or exclusion of HMs, but mainly rely on passive absorption (Barker, 1981; Mganga et al., 2011).

Accumulators and excluders are naturally adapted to high metal concentrations in soil, but the majority of plant species can be classified as non-accumulators or indicators and have to cope with heavy metals when growing on polluted soil (Viehweger, 2014).

Nevertheless, a basal heavy metal tolerance can be found in all plant species, as they all coordinate a complex system of uptake/exclusion, transport/sequestration and detoxification of such elements in order to protect sensitive organs from metal stress (Viehweger, 2014). This system is composed by different strategies for metal tolerance and detoxification (Emamverdian et al., 2015). As a first step, plants adopt the so called *avoidance strategy*, which aims at the restriction or exclusion of metal uptake from the soil, thus preventing metal entry into the roots (Viehweger, 2014). If this action fails and the metal enters the plant, tolerance mechanisms for detoxification are activated, such as metal sequestration and compartmentalization in different intracellular compartments (Patra et al., 2004). These strategies include metal transportation into the vacuole, binding to the cell wall, biosynthesis and accumulation of compounds aimed at metal complexation, such as prolines and metallothioneins (Dalvi and Bhalerao, 2013; John et al., 2009). When all these measures result unsuccessful and the plant begins to suffer the effects of metal toxicity, the activation of the antioxidant defence mechanisms is then pursued (Manara, 2012).

The presence of HMs in plant cells can disrupt physiological and biochemical functions causing the formation of reactive oxygen species (ROS) (Ovecka and Takac, 2014; Kisa et al., 2016; Hall, 2002; Sharma and Dietz, 2009). ROS are produced because of metals interfere with electron transport, especially in the chloroplast membranes. ROS molecules consist of radical and non-radical oxygen species formed by the partial reduction of oxygen, such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) (Yadav, 2010). The increase in ROS exposes plant cells to oxidative stress and may lead to lipid peroxidation, alteration of different molecules and membranes and rupture of DNA strands (González et al., 2017). Reactive oxygen species are also generated in plant cells during normal metabolic processes (for example; superoxide anion is produced by the photosynthetic electron transport system), however, under normal conditions the toxicity is tightly controlled by the ability of plant´s antioxidant molecules to eliminate or reduce the damage caused by ROS (Bhaduri and Fulekar, 2012).

Plants have managed to overcome this oxidative stress by a wide range of antioxidant molecules and enzymes protecting cells against the oxidative injuries (Kisa, 2018). Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) can be identified as the most important antioxidant enzymes while glutathione, carotenoids and ascorbate (vitamin C) represent the non-enzymatic components (Sies, 1997).

Among the antioxidant secondary metabolites, polyphenols, such as flavonoids and anthocyanins, have been indicated as important components of plant detoxification systems, by acting as metal chelators or by directly scavenging some active oxygen species (Michalak, 2006). The antioxidant activity of phenols is mainly due to their redox properties, which allow them to act as reducing agents. The accumulation of phenolic compounds in plants can be induced by various biotic and abiotic stresses, such as UV radiation, low temperature, wounding and low nutrients (Dixon and Paiva, 1995).

Among polyphenols, an important sub-class of antioxidant molecules are flavonoids. Their power against ROS is well known, however, they also perform a vast set of biological functions, including stress protection against diverse environmental perturbations and pathogen attacks (Winkel-Shirley et al., 2001).

Numerous studies have already reported the effectiveness of antioxidants molecules in reducing plant HMs stress. For instance, Tian et al. (2014) reported that the high presence of complexing antioxidants in shoots of *Brassica napus*, resulted in a diminished damage of plants grown under high Pb concentrations, if compared to plants with low antioxidants levels. Recent results showed evidence that flavonoids can facilitate heavy metal tolerance in *A. thaliana* (Keilig and Ludwig-Müller

2009). Quercetin for example, besides being able to chelate iron, undergoes also a complexation with copper (El Hajji et al., 2006; Pękal et al., 2011) and uranium ions (Geipel et al., 2010) and additionally exhibits reductive activity towards redox-active metals. This system of complex formation and reduction of non-essential HMs has a double function: on one hand, it can be used as a defense strategy producing less soluble metal complexes by excreting these metabolites in the soil, on the other hand, it can stabilize unstable metal redox states reducing cell oxidative stress (Viehweger et al., 2014).

The presence of metals and metalloids in plant organs does not only cause oxidative stress, but also exerts a wide array of consequences which include: plant growth decrease, low chlorophyll synthesis, change in the ratio of chlorophyll *a* / chlorophyll *b* (Mysliwa-Kurdziel et al., 2004; Viehweger and Geipel, 2010), poor photosynthetic activity (Küpper et al., 2007) and transpiration rate impairment (Vernay et al., 2007; Chandra and Kang, 2016). However, one of the most visible effect of metal toxicity is a diffuse leaf chlorosis (Fig. 1).

Figure 1. Chlorotic leaves of *P. aviculare* under Ni treatment.

Paunov et al. (2018) demonstrated that Cd and Zn exposure strongly diminished chlorophyll and carotenoid concentrations in wheat, the first being much more effective than the second. It was in fact demonstrated that Cd can competitively bind to the Ca-binding sites in PSII during the photoactivation of the water-splitting system (Faller et al., 2005) bringing to a consequent partial inactivation PSII, which is converted in a so-called energy sink, thus transforming the excitation energy of the antenna chlorophylls into heat (Paunov et al., 2018). Moreover, indirect effects of Cd on chlorophyll content via the induction of micronutrient deficiencies have also been reported. These effects strongly resemble those of Fe deficiency and are characterized by the inhibition and disorganization of chlorophyll-protein complexes by the formation of a Cd-chlorophyll complex (Küpper et al., 2000).

Similar effects have been in general demonstrated for most metals. In a study carried out on *Brassica juncea*, it was found that after 90 days of cultivation on Pb, Cu, Cd polluted soil, chlorophylls *a* and *b* contents in leaves were markedly lower compared to control plants (Karak et al., 2013). Moreover, according to the results obtained on poplar hybrids, a proportional decrease in chlorophyll and carotenoid contents was demonstrated in plants cultivated with increasing concentrations of Cd, Cu, Cr and Zn (Chandra and Kang, 2016).

Despite the high correlation between heavy metal stress, antioxidant response and photosynthetic pigments content, some authors argued that the overall effects of most abiotic stress were often dependent on the plant species, genotype and other plant characteristics (Le Gall et al., 2015). Nonetheless, photosynthetic pigments reduction caused by HMs was frequently used to determine plant stress level (Aggarwal et al., 2012). In order to widen the knowledge on the various parameter used to evaluate HM stress, the aim of the present study was investigate the differences in physiological responses to increasing concentrations of six HMs (Cd, Cr, Cu, Ni, Pb, Zn), with particular attention to antioxidant activity, polyphenol and flavonoid production, photosynthetic pigments content and biomass production. The plants used were *P. aviculare* and *S. vulgaris,* each of them represented by in five different populations, grown in hydroponic culture. The relations between HMs uptake, antioxidant metabolites production and photosynthetic pigments content, was then tested as reliable HM stress biomarkers.

4.3. Materials and Methods

4.3.1. Species selection

In this study, two annual weeds have been selected: *Polygonum aviculare* (Fig. 2) and *Senecio vulgaris* (Fig. 3), because of their widespread presence in numerous environments, from disturbed one to almost pristine. These two species are tolerant to HM pollution, fast growing and produce abundant seeds with high viability, for the previous reasons were perfect candidates for our research.

Figure 2. *Polygonum aviculare* (L.). Photo: Mirko Salinitro

P. aviculare is an annual plant belonging to the *Polygonaceae* family, is a cosmopolitan species, growing in several disturbed habitats and in particular in frequently trampled areas. It germinates in spring and it continuously flowers during the hot season to finally produce seeds in autumn. The stems are creeping and grow up to 45-50 cm long, it has dark-green oval leaves and small whitish flowers growing at the axil of each, followed by small triangular nuts.

S. vulgaris (Fig. 3), is an annual plant belonging the *Asteraceae* family, is a cosmopolitan species that grows on disturbed soils, like arable fields and wasteland. It germinates in autumn and/or spring, depending on the latitude and climate and flowers all year long. The plant is erected, 35-40 cm tall and well branched, it produces several yellow flower heads, quickly followed by hairy seeds adapted to wind dispersion.

Figure 3. *Senecio vulgaris* (L.). Photo: Mirko Salinitro.

For both the selected plant species, five different populations were collected in five different locations: Bologna urban area, Bologna woodland area, Milano urban area, Milano woodland area and Mt. Prinzera serpentine area (see Table 4 at page 28 and Fig. 1 at page 46 for further details on sampling locations). The five different populations were adapted to different HM levels in soil, since they were growing on totally different substrate. The urban population was adapted to highest levels of HMs in soil, the serpentine population tolerated high levels of Ni and Cr, finally the woodland population was adapted to soils with low HMs content.

4.3.2. Plant cultivation

Figure 4. *P. aviculare* after transplant in pots.

Before sowing, seeds were sterilized in 70% (v/v) ethanol for 30 seconds and rinsed 3 times with sterile water to remove possible pathogens. Seeds were then placed on humid coarse quartz sand in transparent plastic boxes. *Polygonum aviculare* seeds were placed on the surface because germination required light and warm temperature and kept at 20°C with 16/8 hours light/dark until germination. Conversely *S. vulgaris* seeds were covered with a thin layer of sand, as they better geminate in the dark at low temperature, and therefore kept at 10°C in the dark for 3 days then transferred at 20°C with 16/8 hours light/dark until complete germination.

Plants took around 10 days from sowing, to develop the first leaf, after that seedlings were transferred from the plastic boxes to their final containers in a number of 3-5 per pot (Fig. 4). The final pots consisted of Ø 5 cm plastic container, immersed in trays filled with 200 ml of half strength Hoagland's nutrient solution (Waters et al., 2012), which was enough to cover 1/3 of the pot (Fig. 5). The nutrient solution contained: 2 mM KNO_3, 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mM NH_4NO_3, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.25 mM KH_2PO_4, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnCl_2 \cdot 4H_2O$, 2 µM $ZnSO_4 \cdot 7H_2O$, 0.5 µM $CuSO_4 \cdot 5H_2O$, 0.075 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.15 µM $CoCl_2 \cdot 6H_2O$, 0.05 µM $NiCl_2 \cdot 6H_2O$, 40 µM Fe-EDTA. The solution pH was adjusted to 5.8 ± 0.2 with 1 M NaOH, then at each treatment was added with the proper metal concentration, starting from a concentrated stock solutions of CdCl2 · 2,5 H_2O 0.1 M, CrCl · $6H_2O$ 1M, $CuSO_4 \cdot 5H_2O$ 1M , $NiCl_2 \cdot 6H_2O$ 1M, Pb-EDTA 1M, $ZnSO_4 \cdot 7H_2O$ 1M, according to the experimental plan shown in Table 1.

Figure 5. Modified hydroponic system.

Treatment concentration *A* was calculated as the average of the four more concentrated values for each metal in urban soils (total metal). This values was multiplied by different factors (2, 5, 25 100 etc.), according to the toxicity of each element until concentration *D*, which was the maximum that allowed plant growth and development.

Trays and pots were placed in closed plastic containers in order to limit surface evaporation, hence concentration of nutrient and metals in the solution. Plants were cultivated in this modified hydroponic system for four weeks: the first week after the transplant in half strength Hoagland's nutrient solution without any metal, and three weeks in half-strength Hoagland's nutrient solution spiked with different metal concentrations. The solution was totally replaced every week in order to avoid nutrient depletion. Plants were cultivated in a growth chamber at a constant temperature of 21.5 ± 0.5°C, with 16/8 hours light/dark.

Table 1. Final concentrations of each tested metal. 0= control, A= urban metal concentration, B= medium metal concentration, C= high metal concentration, D= max metal concentration tolerated by the plant.

Treatment	**Zn**	**Pb**	**Cu**	**Ni**	**Cr**	**Cd**
Control	0 mM	0 mM	0 mM	0 mM	0 mM	0 µM
Conc. A	0.28 mM	0.025 mM	0.13 mM	0.025 mM	0.036 mM	0.09 µM

Conc. B	0.56 mM	0.25 mM	0.26 mM	0.12 mM	0.18 mM	9.46 µM
Conc. C	1.11mM	1.25 mM	0.66 mM	0.25 mM	0.36 mM	47.29 µM
Conc. D	2.78 mM	2.50 mM	1.32 mM	0.62 mM	0.89 mM	94.59µM

4.3.3. Sample collection and preparation

At the end of the fourth week of culture, only plant shoots were harvested. They were rinsed with deionized water, then dried up with paper towels before weighting (fresh weight). Once weighted, fresh samples were grinded in liquid nitrogen to obtain a homogeneous powder. The bulk powders were then stored at -80°C for further analysis.

4.3.4. Metal quantifications

For the quantification of metals, 0.5 gFW of grinded shoots were oven dried at 80°C 24 hours until constant weight. About 0.1-0.2 gDW of samples were pre-digested at room temperature with 2 mL 70% (v/v) HNO_3 for 1 day, then digested on a heat block 1 h at 70°C and 1 h at 125°C following a modified method from Huang and Schulte, (1985). After the digestion, samples were taken up to 10 mL with deionised water. Five replicates of reference material (Apple leaves NIST® SRM® 1515) and blanks (only nitric acid) were included in the digestions. Metal concentration analysis were performed with ICP-MS Elan 9000 DRCe, (Perkin Elmer, Waltham, Massachusetts, USA). Data were expressed as ppm related to sample dry weight.

4.3.5. Spectrophotometrical analysis

Total flavonoids quantification assay was performed as explained in Zhishen et al. (1999) and Ferri et al. (2013). This spectrophotometric analysis is based on the progressive colouration (orange) of $AlCr_3$ and $NaNO_2$ in presence of increasing concentration of flavonoids in alkaline environment. The same methanolic extracts obtained for antioxidant activity were used for total flavonoid quantification. The supernatant was then recovered after centrifugation at 12000 rmp for 5 minutes. The calibration curve was made with growing concentration of (+)catechin hydrate (CAT) from 2 to 14 µg/mL) and as blank control deionised water was used. An appropriate volume of sample was put in 1.5 mL tubes, 400 µL of deionized water were added then 30 µL of $NaNO_2$ 5% (w/v). The solution were mixed and after 5 minutes 30 µL of $AlCl_3$ 10% (w/v) were added. The

solutions were mixed and after 6 minutes 200 µL of NaOH 1 M were added then each sample was brought up to a 1 mL with deionised water. Samples absorbance was detected at 510 nm for flavonoids quantification. Data were expressed as mg CAT equivalent/gFW

Total polyphenols quantification assay was performed by Folin-Ciocalteu assay as explained in Singleton et al. (1999) and Ferri et al. (2013). This spectrophotometric analysis is based on the progressive colouration (blue) of the Folin-Ciocalteu reagent (a solution of phosphomolybdic acid and phosphotungstic acid) in presence of increasing concentration of polyphenols in alkaline environment. The same methanolic extracts previously obtained for antioxidant activity were used for total polyphenol quantification. The calibration curve was made with growing concentration of gallic acid (GA) from 0 to 150 µg/mL) and as blank control the 0 of the curve was used. An appropriate volume of sample was put in 2 mL tubes, each sample was brought up to the 1.6 mL with deionised water. 100 µL of Folin-Ciocalteu regent were added and the solution was mixed and incubate at room temperature for 5 minutes. 300 µL of 20% (w/v) Na_2CO_3were added, the samples were mixed and incubated at 40°C for 30 minutes. Samples absorbance was detected at 765nm for polyphenols quantification. Data were expressed as mg GA equivalent/gFW

Total antioxidant quantification was performed by the ABTS assay (Re et al., 1999, Ferri et al., 2013). This spectrophotometric analysis is based on the progressive decolouration (from blue to transparent) of the ABTS reagent (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) in presence of increasing concentration of antioxidant substances. 0.1 gFW of frozen fresh grinded samples were extracted with 1 mL of 95% (v/v) methanol and shaken overnight at room temperature. The supernatant was then recovered after centrifugation at 12000 rmp for 5 minutes. The calibration curve was made with growing concentration of ascorbic acid (AA) (from 0 to 80 µg/mL) and as blank control de-ionised water was used. An appropriate volume of sample was put in 1.5 mL tubes, then 1 mL of ABTS working solution (ABS_{734} = 0.7±0.05) was added and samples were incubated for 30 minutes at 30°C. After incubation samples absorbance was detected at 734 nm in a VersaMax™ Microplate Reader (Molecular Devices, San Jose, California) spectrophotometer, for total antioxidant quantification. Data were expressed as mg AA equivalent/gFW.

For the determination of photosynthetic pigments a modified method from Radwan et al. (2007) and Metzner et al. (1965) was used. 0.1 gFW of frozen grinded sample were extracted with 85% (v/v) acetone and mixed 2 times for 30 seconds, the samples were then centrifuged at 2500 rpm for 5 minutes and the supernatant recovered.

During all the operations, samples were kept at a temperature below 4°C to void the degradation of the pigments. The quantification of chlorophyll *a*, *b* and carotenoids was carried out by a plate-reader spectrophotometer (Fig. 6) at three different wavelengths: 663 nm (absorbance peak for chlorophyll *a),* 644 nm (absorbance peak for chlorophyll *b*) and 452.5 nm (absorbance peak for carotenoids), and pure acetone 85% was used as blank control. The obtained absorbance values were processed to give the pigment concentrations (µg/mL) with the following equations:

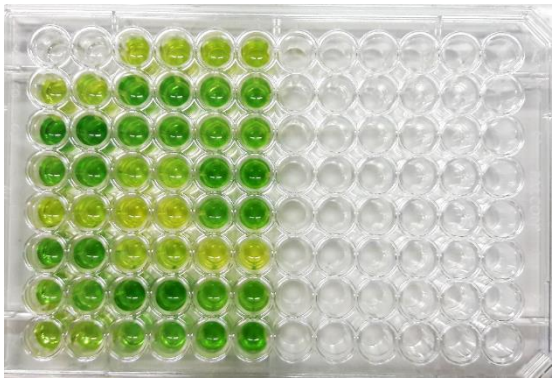

Figure 6. Acetone extracts ready for photosynthetic pigments quantification.

Chlorophyll a = $10.3 \cdot Abs_{663} - 0.98 \cdot Abs_{644}$

Chlorophyll b = $19.7 \cdot Abs_{644} - 3.87 \cdot Abs_{663}$

Carotenoids = $4.2 \cdot Abs_{452.5} - [(0.0264 \cdot \text{chl-}a) + (0.426 \cdot \text{chl-}b)]$

4.3.6. Data analysis

All the statistical analyses were performed using R software version 1.3.5 (R Core Team, Vienna, Austria). The differences in metal uptake and metabolites production were evaluated among the 5 different plant populations, as well as the different metal treatments and dosage. Data were tested for normality using Shapiro-Wilk normality test, and for homogeneity using the Levene's Test for Homogeneity of Variance with default parameters from the package "car" (https://CRAN.R-project.org/package=car). Since data resulted non-parametric, the Kruskal-Wallis test, followed by Dunn's multiple pairwise comparison post-hoc test from dunn.test package (https://CRAN.R-project.org/package=dunn.test), were used to evaluate the differences among compared groups (*p-values* are reported in brackets in the section Results) . Spearman correlation coefficients were calculated to determine significant relationship between metal dosage (for each metal) and total antioxidants, polyphenols, flavonoids, photosynthetic pigment, shoot biomass. Linear regression models were used to describe the relations between metal uptake (or metal dosage) and all the previous parameter (R^2 and *p-values* are reported in brackets in the section Results). Principal Component analisys (PCA) was performed with the function prcomp, using default values. Graphical

elaborations where performed using the R package ggpubr (https://CRAN.R-project.org/package=ggpubr).

4.3. Results

4.3.1. Metal accumulation

Both *Polygonum aviculare* and *Senecio vulgaris* grew well in hydroponic condition, showing toxicity at high metal concentrations (B, C, D)

In both plant species, metal concentration in plant shoots was directly proportional to the amount of metal contained in each treatment with no exceptions among the six tested metals. On average, *P. aviculare* shoots had on average lower concentrations if compared to those of *S. vulgaris*. Considering the average levels among A to D treatments, the concentrations, respectively for *P. aviculare* and *S. vulgaris*, of Cd were 111 and 407 ppm, of Cr 19 and 276 ppm, of Cu 196 and 176 ppm, of Ni 90 and 240 ppm, of Pb 364 and 1805 ppm, of Zn 937 and 1548 ppm (Figs. 7a, 7b).

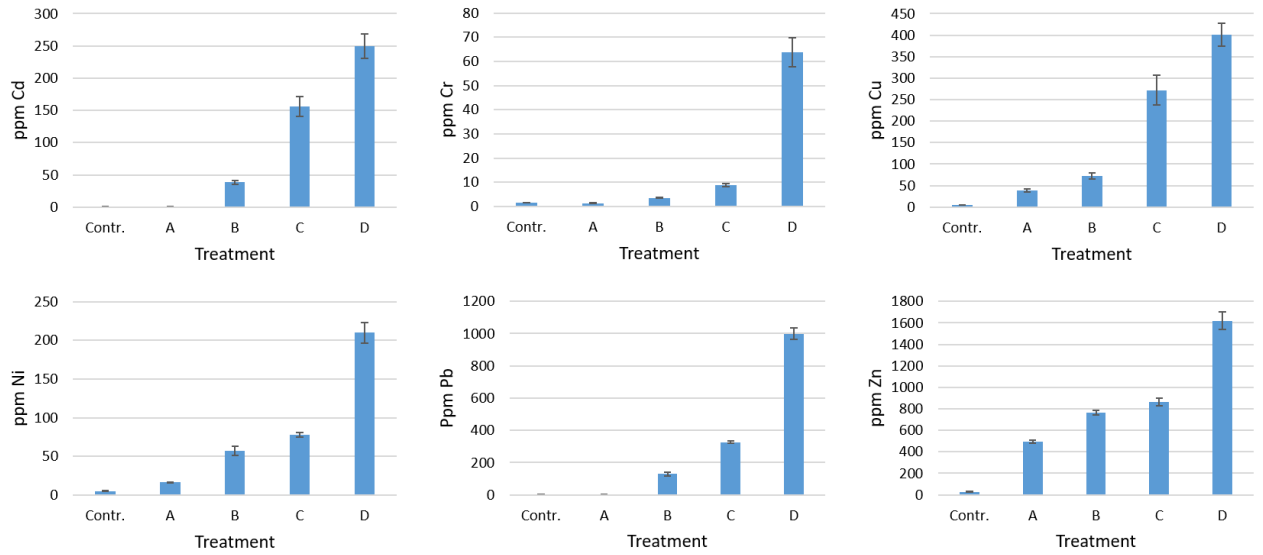

Figure 7a. Metal contents in *Polyogonum aviculare* shoots. From the top-left: Cd, Cr, Cu, Ni, Pb, Zn. Metal treatment increases from sample A to D, where treatment A corresponds to the urban soil concentration of each tested metal, and D is the maximum concentration allowing plant growth.

Overall, the greatest differences in metal uptake between the two plant species were detected for Cr and Pb treatments. For Cr treatment, A, B, C and D samples were respectively 6, 13, 22, and 13-times higher in *S. vulgaris* compared to *P. aviculare.* For Pb treatment, A, C and D samples were

respectively 6, 7 and 5-times higher in *S. vulgaris* compared to *P. aviculare*. The only metal which was similarly absorbed by both species was Cu, whose concentrations were slightly lower in all samples in *S. vulgaris* shoots compared to *P. aviculare* ones (Figs. 7a, 7b). *S. vulgaris* resulted to be above the hyperaccumulation threshold set for Pb (>1000 ppm; Van der Ent et al., 2013), in the treatments C and D with respectively 2401 and 4580 ppm of metal accumulated in plant shoots. Also for Cd the set hyperaccumulation threshold (> 100 ppm; Van der Ent et al., 2013) was overtaken in treatments B, C and D (226, 630 and 765 ppm respectively) for *S. vulgaris* and C, D for *P. aviculare* (156 and 250 ppm).

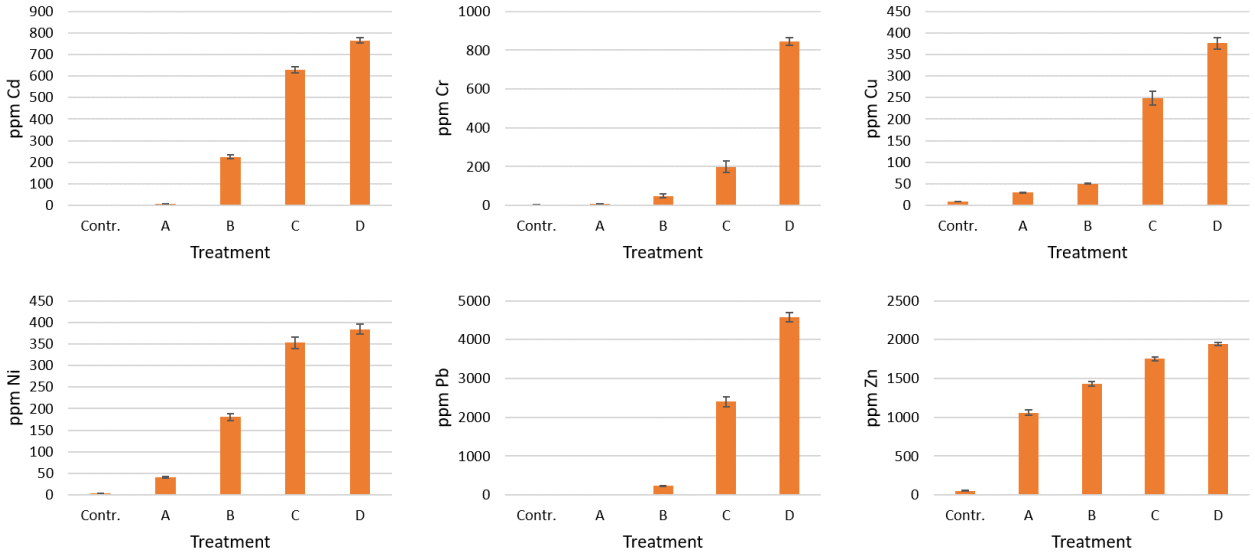

Figure 7b. Metal contents in *Senecio vulgaris* shoots. From the top-left: Cd, Cr, Cu, Ni, Pb, Zn. Metal treatment increases from A to D samples, where treatment A corresponds to the urban soil concentration of each tested metal, and D is the maximum concentration allowing plant growth.

The different metals were differentially transferred from soils to shoots according to the type of species and metal. This parameter is called bioaccumulation factor (BAF) and is calculated as the ratio between metal concentration in soil (in this case in the hydroponic solution) and the metal concentration in plant shoots. BAFs for *P. aviculare* and for *S. vulgaris* were reported in Tables 2a and 2b. Overall, BAFs for all metals were higher for *S. vulgaris*, in accordance with the general higher metal content detected, if compared to *P. aviculare*. The only exception was Cu, where the uptake and transfer efficiencies in *S. vulgaris* were slightly lower than in *P. aviculare*. With the exception of A, B, C Cr treatments and A treatment for Pb in *P. aviculare*, all BAFs were > 1, showing that the two studied species were able to concentrate metals in their aerial parts when cultivated in hydroponic. The most easily transferred metals were Cd, followed by Zn and Ni with maximum BAFs respectively of 416, 57, 34 in *S. vulgaris* and of 222, 27, 13 in *P. aviculare* (Tables 2a, 2b). From the present data, it can be clearly noticed that for Cd, Zn and Ni, at the increase of the metal concentration in the

Treatment	Cd	Cr	Cu	Ni	Pb	Zn
A	222.8	0.7	4.5	13.7	0.3	26.9
B	37.4	0.4	4.4	8.7	2.4	21.1
C	29.5	0.5	6.5	5.3	1.3	11.9
D	23.4	1.4	4.8	5.8	1.9	8.9

Table 2a. Bioaccumulation factor for *Polygonum aviculare* shoots. Metal treatment increases from A to D, where treatment A corresponds to the urban soil concentration of each tested metal, and D is the maximum concentration allowing plant growth.

Treatment	Cd	Cr	Cu	Ni	Pb	Zn
A	416.3	4.0	3.6	34.5	1.6	57.0
B	205.4	5.2	3.1	25.5	4.4	39.1
C	119.2	10.7	5.9	24.1	9.3	24.1
D	71.7	18.3	4.5	10.6	8.8	10.7

Table 2b. Bioaccumulation factor for *Senecio vulgaris* shoots. Metal treatment increases from A to D, where treatment A corresponds to the urban soil concentration of each tested metal, and D is the maximum concentration allowing plant growth.

nutrient solution, metal transfer decreased. An opposite trend was conversely observed for Cr and Pb, where BAFs generally increased at the increase of metal concentration in the nutrient solution. For Cu, the BAF seemed not to be dose-dependent remaining quite constant in all treatments for both plant species.

At the increase of metal accumulation in shoots an analogous increase of dry weight/fresh weight (DW/FW) ratio was detected. In both species and for all metals DW/FW ratio ranged between 0.18-0.20 in treatments A (lowest metal concentration) while increased up to 0.28-0.32 in treatments D (highest metal concentration).

P. aviculare	Cu	Pb	Cr	Ni	Cd	Zn
B	0.978	0.928	0.992	0.980	0.980	0.928
M	0.964	0.966	0.882	0.983	0.962	0.757
N	0.980	0.895	0.912	0.982	0.980	0.896
T	0.975	0.977	0.980	0.686	0.977	0.981
P	0.922	0.882	0.882	0.985	0.975	0.980
S. vulgaris	**Cu**	**Pb**	**Cr**	**Ni**	**Cd**	**Zn**
B	0.983	0.980	0.855	0.979	0.974	0.984
M	0.958	0.982	0.882	0.955	0.987	0.980
N	0.980	0.977	0.882	0.978	0.912	0.882
T	0.967	0.980	0.977	0.980	0.981	0.986
P	0.969	0.984	0.425	0.977	0.972	0.979

Table 3. Correlation coefficient (R^2) between metal concentration in the nutrient solution and metal concentration in plant shoots. All R^2 were calculated with R software using the function cor.test method Spearman. Poorly significant correlations ($R^2 < 0.5$) were highlighted in yellow. Populations: B= Bologna urban, M= Milan urban, N= Bologna woodland, T= Milan woodland, P= Serpentine.

For all metals, there was a statistically significant high correlation between metal amount in the nutrient solution and metal detected in plant shoots, with R^2 coefficients in most cases above 0.9 (Table 3). These high and positive correlations also underline the possibility of using the two species as bioindicators since at the increasing of metal content in the nutrient solution, a corresponding increased metal level was detected in plant shoots.

4.3.2. Metal effects on the production of antioxidants metabolites and photosynthetic pigments

The correlations between metal concentration in shoots and the production of some secondary metabolites and of photosynthetic pigments, were tested by multiple cor.test using R software and all R^2 are reported in Table 4.

	P. aviculare	Cu	Pb	Cr	Ni	Cd	Zn	S. vulgaris	Cu	Pb	Cr	Ni	Cd	Zn
population B	FW	-0.890	-0.859	-0.850	-0.904	-0.835	-0.901	FW	-0.976	-0.928	-0.841	-0.683	-0.920	-0.877
	Flav	0.786	0.923	0.691	0.950	0.446	0.823	Flav	0.836	0.570	0.890	0.584	0.749	0.723
	Poly	0.638	0.770	0.881	0.678	0.632	0.575	Poly	0.709	0.739	0.896	0.628	0.166	0.600
	Tot_antiox	0.281	0.752	0.615	0.836	0.235	0.825	Tot_antiox	0.768	0.868	0.298	0.143	0.710	0.683
	Pigm	-0.908	-0.852	-0.540	-0.871	-0.117	-0.830	Pigm	-0.839	-0.509	-0.600	-0.576	-0.720	-0.873
population M	FW	-0.714	-0.858	-0.740	-0.787	-0.829	-0.742	FW	-0.807	-0.904	-0.874	-0.587	-0.807	-0.711
	Flav	0.936	0.940	0.205	0.915	0.733	0.484	Flav	0.612	0.342	0.664	0.414	0.531	0.761
	Poly	0.646	0.919	0.365	0.632	0.821	0.695	Poly	0.708	0.952	0.862	0.928	0.864	0.891
	Tot_antiox	0.599	0.880	0.898	0.925	0.146	0.270	Tot_antiox	0.928	0.270	0.877	0.329	0.881	0.746
	Pigm	-0.587	-0.456	-0.289	-0.476	-0.623	-0.683	Pigm	-0.859	-0.729	-0.410	-0.906	-0.758	-0.898
population N	FW	-0.935	-0.875	-0.805	-0.967	-0.965	-0.755	FW	-0.867	-0.955	-0.786	-0.872	-0.883	-0.877
	Flav	0.608	0.114	0.801	0.564	0.501	0.853	Flav	0.667	0.663	0.655	0.478	0.442	0.863
	Poly	0.879	0.813	0.836	0.866	0.856	0.828	Poly	0.643	0.890	0.569	0.131	0.560	0.611
	Tot_antiox	0.122	0.238	0.503	0.499	0.660	0.657	Tot_antiox	0.216	0.131	0.211	0.325	0.770	0.578
	Pigm	-0.408	-0.501	-0.328	-0.733	-0.823	-0.512	Pigm	-0.807	-0.582	-0.270	-0.870	-0.518	-0.824
population T	FW	-0.910	-0.893	-0.844	-0.656	-0.895	-0.916	FW	-0.883	-0.958	-0.893	-0.926	-0.897	-0.741
	Flav	0.946	0.849	0.956	0.170	0.472	0.677	Flav	0.905	0.676	0.765	0.653	0.585	0.862
	Poly	0.585	0.662	0.493	0.162	0.116	0.856	Poly	0.838	0.671	0.959	0.514	0.848	0.956
	Tot_antiox	0.695	0.877	0.692	0.370	0.684	0.696	Tot_antiox	0.967	0.924	0.576	0.182	0.661	0.985
	Pigm	-0.902	-0.941	-0.410	-0.765	-0.931	-0.697	Pigm	-0.948	-0.903	-0.257	-0.863	-0.125	-0.929
population P	FW	-0.908	-0.853	-0.885	-0.931	-0.894	-0.846	FW	-0.945	-0.935	-0.416	-0.913	-0.905	-0.911
	Flav	0.816	0.859	0.669	0.487	0.126	0.343	Flav	0.806	0.613	0.337	0.197	0.634	0.602
	Poly	0.922	0.901	0.644	0.724	0.611	0.878	Poly	0.333	0.294	0.692	0.616	0.119	0.868
	Tot_antiox	0.929	0.891	0.836	0.822	0.650	0.960	Tot_antiox	0.614	0.223	0.566	0.940	0.256	0.975
	Pigm	-0.893	-0.824	-0.579	-0.940	-0.935	-0.959	Pigm	-0.937	-0.620	-0.707	-0.842	-0.778	-0.963

Table 4. Correlation coefficients (R^2) between metal concentration in plant shoots and plant fresh weight, levels of some secondary metabolites, photosynthetic pigments. All R^2 were calculated with R software using the function cor.test method Spearman. Poorly significant correlations ($R^2 < 0.5$) were highlighted in yellow, non-significant correlations ($R^2 < 0.3$) were highlighted in red. Populations: B= Bologna urban, M= Milan urban, N= Bologna woodland, T= Milan woodland, P= Serpentine. FW: shoot fresh weight, Flav: flavonoids, Poly: polyphenols, Tot_antiox: antioxidant activity, Pigm: photosynthetic pigments (chl a + chl b+ carotenoids).

Correlation coefficients can be divided into 4 classes: < ±0.3= no correlation, ±0.301 < ±0.5 = low correlation, ±0.501 < ±0.7 = good correlation, > 0.7 = excellent correlation.

With respect to both plant species, only the 10% and 8% of the R^2 indicated respectively no or low correlation between parameters (red and yellow cells Table 4). The 25% of the R^2 showed a good correlation and the 57% and excellent correlation. In particular, both in P. *aviculare* and *S. vulgaris*, HM shoot contents were negatively correlated with photosynthetic pigments content and fresh weight. Overall, in both species, Zn and Cu were the metals having the highest correlation with all measured variables, while Cd and Ni were those with the lowest correlation.

Also the PCA analyses (Fig. 8a, 8b), performed taking into consideration antioxidant activity, polyphenol and flavonoid content, fresh and dry weight, metal concentration in shoots, grouping together data coming from all the five different populations only according to the metal dosage (low (A) to high (D), confirmed the presence of a clear general trend. In *P. aviculare* (Fig. 8a), the major data distribution trend is guided by photosynthetic pigments, antioxidant activity, polyphenols, flavonoids and metal shoot content. In fact, photosynthetic pigments were higher in control and A

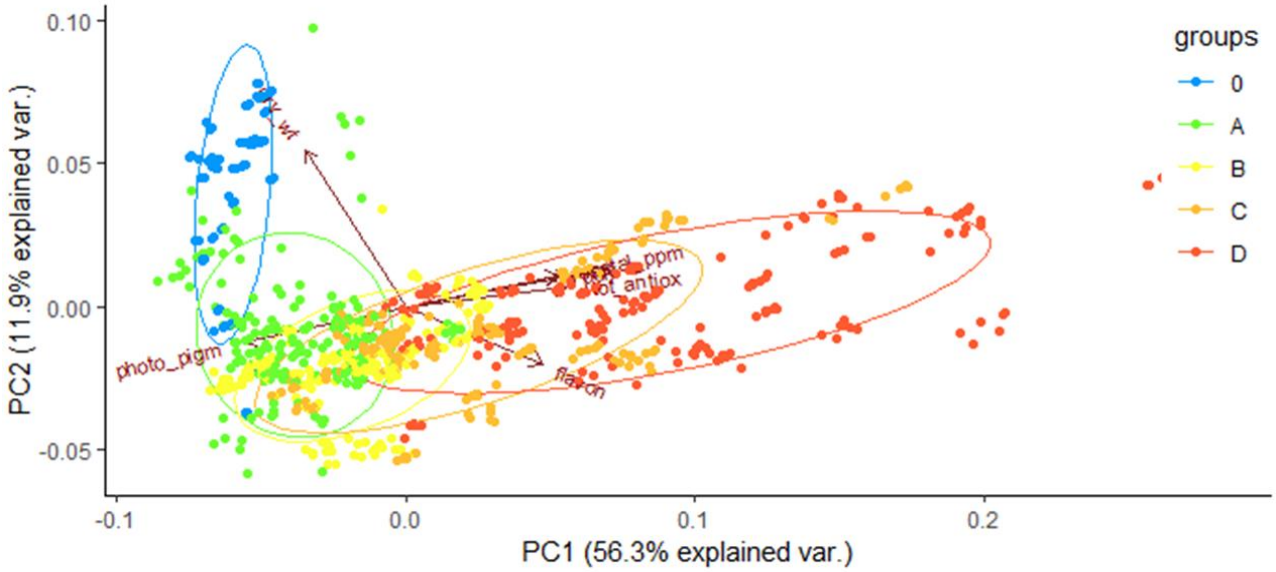

Figure 8a. PCA analysis showing the grouping of treatments *in Polygonum aviculare*. The following variables were considered: polyphenols, flavonoids and photosynthetic pigment content, antioxidant activity, fresh weight, dry weight, metal concentration in shoots (average of the six metals according to the treatment). 0= control, A= urban metal concentration, B= medium metal concentration, C= high metal concentration, D= max metal concentration allowing plant survival.

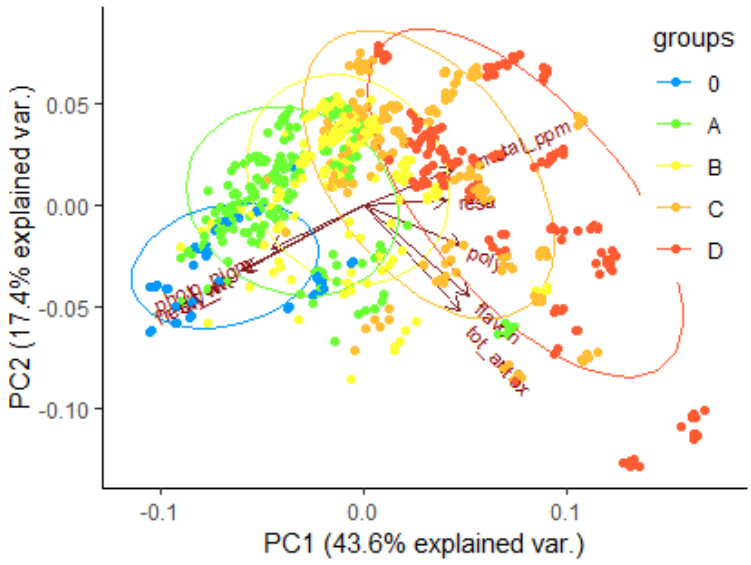

Figure 8b. PCA analysis showing the grouping of treatments in *Senecio vulgaris*. The following variables were considered: polyphenols, flavonoids and photosynthetic pigments content, antioxidant activity, fresh weight, dry weight, metal concentration in shoots (average of the six metals according to the treatment). 0= control, A= urban metal concentration, B= medium metal concentration, C= high metal concentration, D= max metal concentration allowing plant survival.

treatment to progressively decrease in B, C and D dosages (on average 224.6, 203.8, 172.4, 123.3 ug/gFW respectively). Conversely, polyphenols, flavonoids, antioxidant activity and metal contents

were higher in C and D samples (on average 9.4 mg/gFW AG eq., 3.4 mg/gFW CAT eq., 6.6 mg/gFW AA eq.) to decrease in A and control shoots (on average 5.7 mg/gFW AG eq., 1.9 mg/gFW CAT eq., 3.4 mg/gFW AA eq.). Control treatment was characterized by high fresh weight of biomass (0.98 g), which decreased in sample A and reached a lower similar amount in B, C and D (on average 0.04 g). In *S. vulgaris* (Fig. 8b), the general data distribution trend was instead mainly guided by photosynthetic pigments, metal contents and fresh weight variables, while levels of polyphenols and flavonoids only had a minor role in the grouping of samples.

Shoot fresh weight and photosynthetic pigments content steadily decreased from control to D treatment (from 138.9 to 68.6 ug/gFW), while an opposite trend was observed for metal concentration in shoots and, to some extent, for DW/FW ratio. The vertical distribution of the data given by the axis PC2 (Fig. 8b), was mainly due to polyphenols, flavonoids and antioxidant activity. All these parameters showed higher values in treatments C and D (on average 1.4 mg/gFW AG eq., 0.9 mg/gFW CAT eq., 0.7 mg/gFW AA eq.), even though in each dosage group (except for the control), the concentrations of antioxidants compounds are quite variable.

In Fig. 9 as example, the correlations between shoot Pb content and other metabolite parameters in *P. aviculare*, are reported.

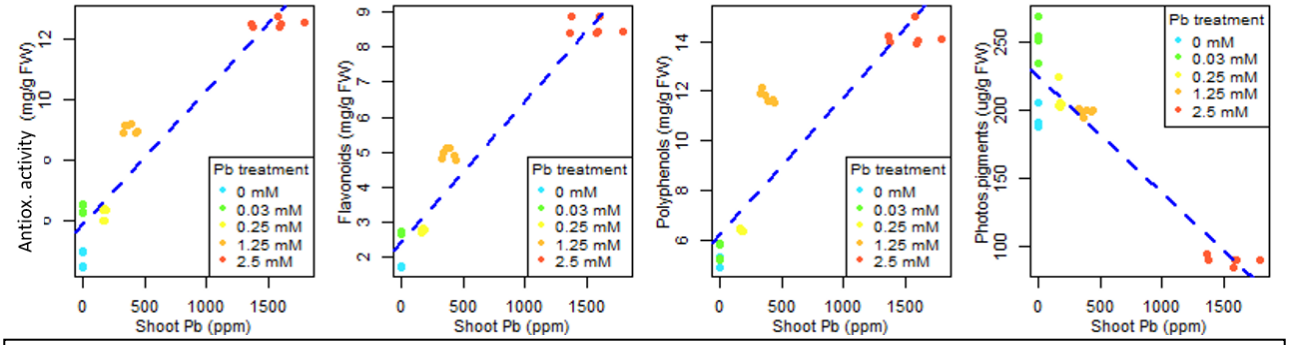

Figure 9. Examples of correlation between shoot Pb content and other metabolite parameters Milan urban population of *P. aviculare*. From the left: antioxidant activity, flavonoids, polyphenols, photosynthetic pigments content. Antioxidant activity is expressed as mg ascorbic acid equivalent (AA) per gFW, flavonoids are expressed as mg equivalents of catechin (CAT) per gFW; polyphenols are expressed as mg equivalents of gallic acid (GA) per gFW; photosynthetic pigments are expressed in μg/gFW and are the sum of chlorophyll *a*, chlorophyll b and carotenoids single amounts.

The overall consequent effects of increasing dosages of any tested metal in both species, were increase in some secondary metabolites and antioxidant activity accompanied by the simultaneous decrease of photosynthetic pigments and fresh weight (Tables 5a, 5b). Nonetheless, some exceptions to this general trend were pointed out. In *P. aviculare,* the flavonoid levels remained almost constant at all Cd treatment levels and low concentrations of Cr (A, B) and Ni (A) caused an

increase of photosynthetic pigments. In *S. vulgaris,* polyphenols only increased at the highest dosage of any metal, remaining almost constant between control, A and B treatments; photosynthetic pigments slightly increased respect to the control, at low levels (concentration A) of Cd, Cr, Ni and Pb, to decrease again at higher dosages of the same metals. Data in Tables 5a and 5b also indicate which metals were most toxic for the two plant species. Acute toxicity can be observed in a plant, when a dramatic decrease in plant biomass and photosynthetic pigments is accompanied by an opposite sharp increase in secondary metabolites. *P. aviculare* was in general less sensitive to HMs than *S. vulgaris* since it showed acute toxicity only in few cases and in particular under Cu, Pb, Zn treatments at dosage D (Table 5a, data highlighted in yellow). Instead, in *S. vulgaris* acute toxicity was detected in a higher number of treatments: Cd (sample D), Cu (D), Ni (D), Pb (C, D) and Zn (C, D) (Table 5b, data highlighted in yellow).

Table 5a. Average levels of all populations, of fresh weight (gFW), flavonoids (mg CAT eq/gFW), polyphenols (mg GA eq/gFW), antioxidant activity (mg AA eq/gFW) and photosynthetic pigments (μg/gFW) in *P. aviculare*. Highlighted in yellow the concentrations that gave acute toxic effects.

Treatment	FW	Flav	Poly	Antiox. Act	Pigm
control	1.20	1.93	4.97	4.80	235.93
Cd_A	0.38	2.63	4.90	3.62	218.76
Cd_B	0.12	2.36	5.15	3.50	208.24
Cd_C	0.05	2.40	5.66	3.04	164.76
Cd_D	0.02	2.42	7.13	4.33	139.44
control	1.20	1.79	5.09	4.76	201.27
Cr_A	0.55	2.00	5.68	2.54	234.96
Cr_B	0.08	2.02	6.23	3.01	228.11
Cr_C	0.02	2.31	7.80	3.76	221.52
Cr_D	0.02	3.59	9.61	6.70	186.23
control	1.11	1.58	5.55	4.23	200.36
Cu_A	0.02	1.94	5.51	3.37	192.90
Cu_B	0.00	2.33	6.06	3.08	202.67
Cu_C	0.00	2.76	9.24	7.36	146.66
Cu_D	0.00	4.18	10.78	8.70	77.21
control	0.96	1.41	6.51	3.27	217.48
Ni_A	0.26	2.03	5.08	4.17	238.42
Ni_B	0.04	2.76	5.82	5.21	199.18
Ni_C	0.02	3.22	7.16	5.36	184.36
Ni_D	0.01	3.75	8.82	7.75	133.82
control	0.79	1.58	6.13	2.00	236.42
Pb_A	0.31	2.66	5.89	4.29	230.77
Pb_B	0.08	2.81	6.48	4.63	217.52
Pb_C	0.01	3.61	9.23	6.93	177.98
Pb_D	0.01	5.01	11.25	9.04	122.67
control	0.65	2.53	6.54	0.56	240.42
Zn_A	0.11	1.66	6.92	4.15	236.65
Zn_B	0.11	2.11	7.29	4.43	167.21
Zn_C	0.03	2.26	9.04	5.59	139.14
Zn_D	0.01	4.65	15.57	10.39	77.80

Table 5b. Average levels of all populations, of fresh weight (gFW), flavonoids (mg CAT eq/gFW), polyphenols (mg GA eq/gFW), antioxidant activity (mg AA eq/gFW) and photosynthetic pigments (μg/gFW) in *S. vulgaris*. Highlighted in yellow the treatments that gave acute toxic effects.

Treatment	FW	Flav	Poly	Antiox. act	Pigm
control	0.84	0.33	1.06	0.38	118.76
Cd_A	0.55	0.50	0.98	0.55	154.16
Cd_B	0.23	0.44	0.95	0.57	110.92
Cd_C	0.14	0.67	1.48	0.51	112.11
Cd_D	0.09	0.91	1.35	0.54	68.15
control	0.61	1.00	1.00	0.67	136.03
Cr_A	0.39	0.51	0.98	0.50	160.42
Cr_B	0.25	0.70	1.07	0.57	148.75
Cr_C	0.08	0.85	1.16	0.68	136.07
Cr_D	0.06	1.17	1.61	0.82	119.30
control	0.84	0.67	1.07	0.56	131.46
Cu_A	0.08	0.84	1.02	0.62	127.11
Cu_B	0.05	0.79	1.03	0.68	121.84
Cu_C	0.03	0.76	1.22	0.71	96.95
Cu_D	0.01	1.36	1.41	0.96	56.75
control	0.99	0.34	1.10	0.49	133.73
Ni_A	0.70	0.64	1.02	0.52	143.57
Ni_B	0.60	0.50	1.10	0.46	94.66
Ni_C	0.42	0.59	1.19	0.52	64.43
Ni_D	0.16	0.90	1.50	0.80	50.32
control	0.97	0.42	1.01	0.56	149.65
Pb_A	0.38	0.50	1.02	0.48	156.15
Pb_B	0.07	0.54	1.08	0.47	146.16
Pb_C	0.02	0.84	1.40	0.55	70.09
Pb_D	0.01	0.97	1.63	0.66	70.35
control	1.06	0.76	0.79	0.53	164.13
Zn_A	0.50	0.43	0.99	0.44	129.98
Zn_B	0.56	0.59	1.16	0.59	99.95
Zn_C	0.23	1.03	1.53	0.84	69.08
Zn_D	0.07	1.49	1.90	1.15	53.04

4.4.3 Species and population differences

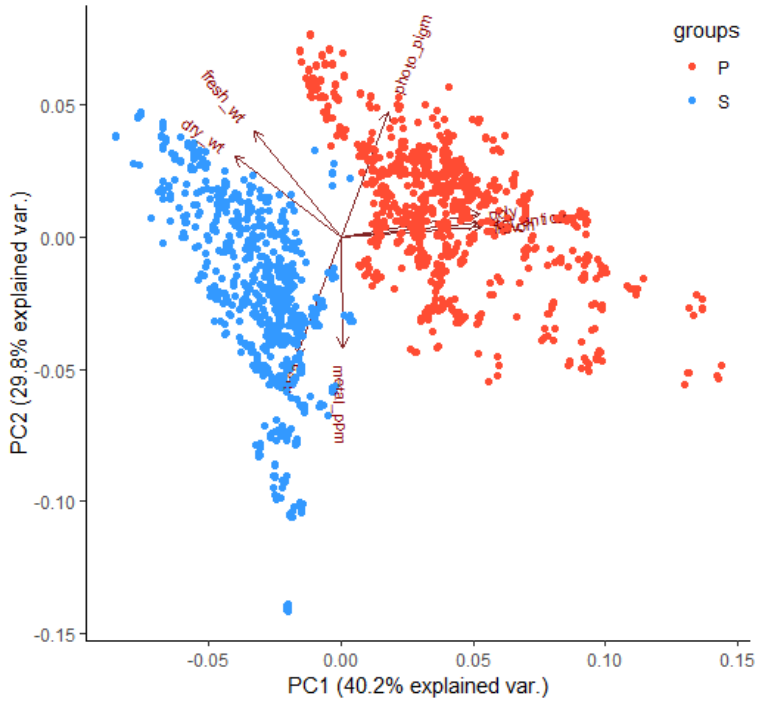

Figure 10. PCA analysis showing the different behaviour of the two species on the basis of the following variables: polyphenols, flavonoids, antioxidant activity and photosynthetic pigment content, fresh weight, dry weight, DW/FW ratio, metal concentration in shoots. P= *Polygonum aviculare*, S= *Senecio vulgaris.*

On the basis of the PCA analysis performed (Fig. 10) taking into consideration polyphenols, flavonoids, antioxidant activity and photosynthetic pigment content, fresh weight, dry weight, DW/FW ratio, metal concentration in shoots, a clear separation of the data related to *P. aviculare* and *S. vulgaris* was pointed out. In general, *P. aviculare* plants (red dots) were characterized by higher photosynthetic pigments, flavonoid and polyphenol contents, antioxidant activity and a lower DW/FW ratio than *S. vulgaris* plants (blue dots). Shoot metal content, despite being in general higher for *S. vulgaris* respect to *P. aviculare*, was however represented by dots quite scattered on a gradient that reflects the four increasing metal treatments. A similar distribution gradient was detected also for fresh and dry weights in both species.

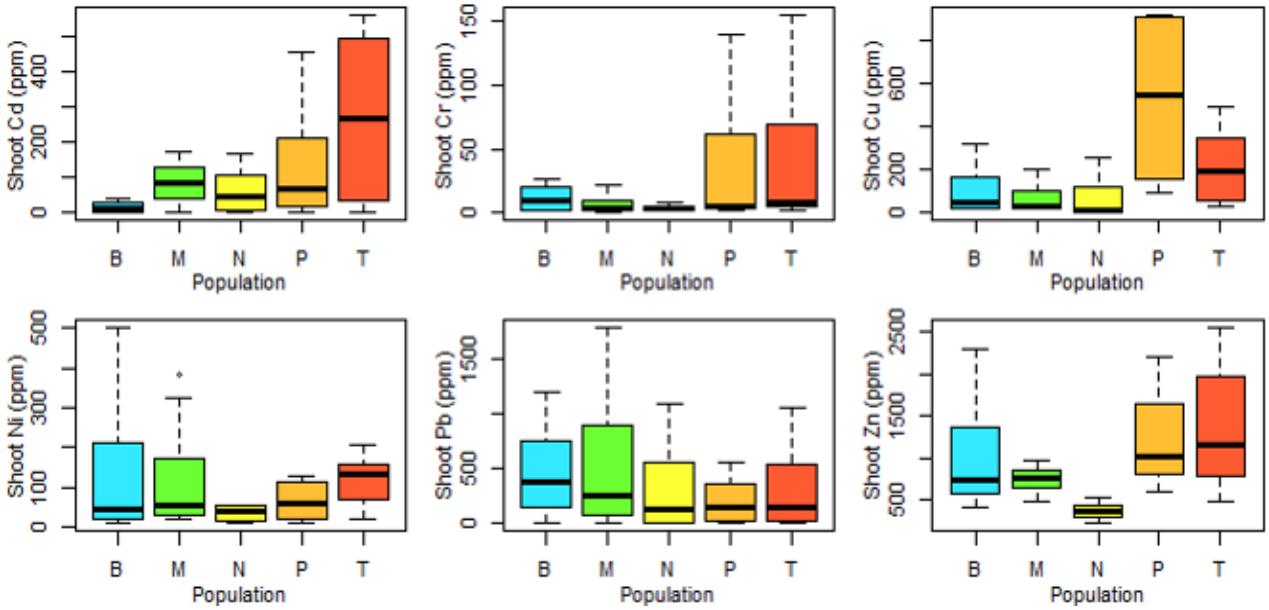

Figure 11. Metal accumulation in the shoots of different *P. aviculare* populations. From the top-left: Cd, Cr, Cu, Ni, Pb, Zn. Populations: B= Bologna urban, M= Milan urban, N= Bologna woodland, T= Milan woodland, P= Serpentine. Significant differences were calculated with Dunn.test using R software and *p* values are reported in the text.

Several differences were also evidenced within the five populations of the two plant species, which showed a different behaviour with regard to the accumulation of the six tested metals. For *P. aviculare* (Fig. 11), Cd, Cr, Cu, Ni and Zn the Milan woodland plants (T) always showed significantly higher shoot metal concentrations if compared with the other populations ($p < 0.01$). Similarly, but only for Cu and Zn, the serpentine population (P) showed a metal concentration 50-70 % above average levels of other populations ($p < 0.01$). The Bologna woodland population (N), was generally the one that accumulated the lowest concentration of all metals (e.g. for Cd – 91 % less than the others) if compared to the others, even if these differences were only significant for Cr, Ni and Zn ($p < 0.01$). Interestingly, with respect to Zn uptake, the urban populations of Milan (M) and Bologna (B) showed a similar average accumulation of 744 ppm and 997 ppm which was 57% higher and 47% lower than population N and P, T respectively, creating three different groups ($p < 0.01$).

The differences among *S. vulgaris* populations were conversely low with similar levels of accumulation of all tested metals (Fig. 12). For Ni treatments, the Bologna urban plants (B) resulted to have significant less Ni in shoot (on average 100 ppm) compared to other populations (on average

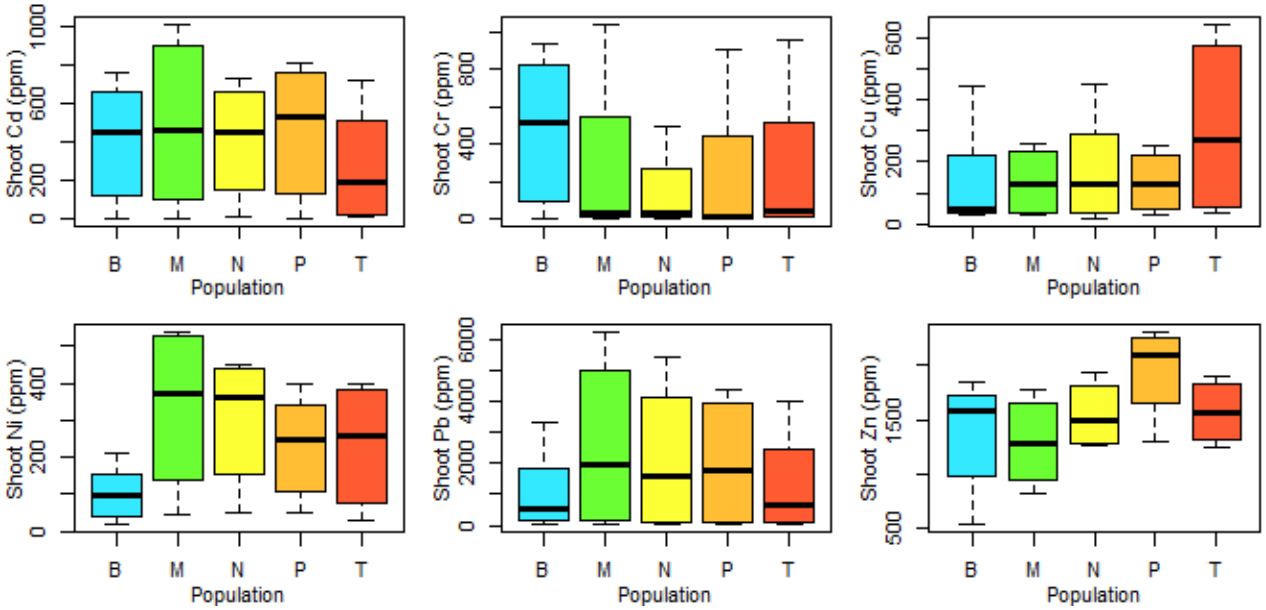

Figure 12. Metal accumulation in the shoots of different *S. vulgaris* populations. From the top-left: Cd, Cr, Cu, Ni, Pb, Zn. Populations: B= Bologna urban, M= Milan urban, N= Bologna woodland, T= Milan woodland, P= Serpentine. Significant differences were calculated with Dunn.test using R software and *p* values are reported in the text.

274 ppm) ($p < 0.01$). The serpentine population (P) showed a 26% above average Zn shoot concentration (1953 ppm) respect to the others.

Some differences among different populations were also present with regard to metabolites production. In *P. aviculare* for example, a constant similar trend of the two woodland populations (N and T) and of the two urban population (B and M), was detected. In particular N e T had higher polyphenols and lower flavonoids and antioxidant activity, compared to the other populations ($p < 0.01$). Conversely, urban populations (B and M) had opposite trends with low polyphenols and high flavonoids and antioxidant activity. Bologna urban population showed higher photosynthetic pigments content ($p < 0.01$) compared to all other populations. In *S. vulgaris,* some differences were also present among the five plant populations but no constant trends were detected. In particular, Milan urban population (M) showed higher values for polyphenols, flavonoids and antioxidant activity compared to all other populations ($p < 0.01$); N and T plants showed higher polyphenol contents compared to others ($p < 0.01$); B, N and T populations had lower flavonoid levels compared to P and M ($p < 0.01$) while in addition B and N samples also showed lower antioxidant activity ($p < 0.01$). As regards photosynthetic pigments, the serpentine population (P) showed higher photosynthetic pigments compared to the other tested plants.

No differences were reported for shoot biomass of all populations under the same treatment conditions ($p > 0.05$), that happened both *for P. aviculare* and *S. vulgaris*.

4.4. Discussion

Five populations (2 urban, 2 woodland and 1 serpentine) of two common weeds, *P. aviculare* and *S. vulgaris*, were cultivated in hydroponic, with increasing doses of 6 selected HMs, (Cd, Cr, Cu, Ni, Pb, Zn) (Table 1). All the plants grew well at low metal concetration (A), while started to show toxicity simptoms at concentration B, C and D.

The present data demonstrated that, in both *P. aviculare* and *S. vulgaris*, the concentration of all the six tested metals was proportional to the treatment dosage in hydroponic culture conditions (Figs. 7a, 7b). Because of this proportional absorption, the studied species can be considered good candidates as indicator plants, even though the metal uptake efficiency of the two species was significantly different. *P. aviculare* was more efficient, when compared to *S. vulgaris,* in preventing the absorption of metals going toward an excluder behavior especially in the presence of Cd and Pb. In fact, all metals appeared to be more concentrated in *S. vulgaris* shoots respect to *P. aviculare*, from - 60% in the case of Zn to - 93 % in the case of Cd. The only exception was Cu, which was instead absorbed in similar amounts (176 ppm in *S. vulgaris* and 195 ppm in *P. aviculare*) in both species. It was in fact previously demonstrated in *Arabidopsis thaliana,* that Cu requirement is strictly regulated by plants, so to prevent uncontrolled uptake from the substrate (Kampfenkel et al., 1995; Penarrubia et al., 2010) leading to hypothesise a similar mechanism of exclusion also for *P. aviculare* and *S. vulgaris*.

For some metals at high dosages, the hyper-accumulation thresholds fixed by Van der Ent et al. (2013) on the basis of a global database of hyperaccumulator plants, were largely exceeded. That has happened for Cd (threshold of > 100 ppm) and Pb (threshold of > 1000 ppm), with some *S. vulgaris* shoot samples being above 500 ppm for Cd and 5000 ppm for Pb and some *P. aviculare* shoot samples being above 250 ppm for Cd (Fig. 7a, 7b). Nonetheless the previous data, the two selected plant species cannot be considered hyper-accumulators as, in the present experiments they were not cultivated on soil, but in hydroponics, which is one the essential requirements to evaluate the hyper-accumulation capacity of a species (Van der Ent et al., 2013). Furthermore, the hyper-accumulation threshold was exceeded only for highest metal doses in the nutrient culture solution. These conditions may have caused, an uncontrolled breakthrough of metal due to a generalized physiological disruption of plant cells (Baker, 1981), consequently leading to high levels

of HMs in plant tissues. Hence, it was concluded that the tested species did not show a hyper-accumulation mechanisms, but the high Cd and Pb concentrations reached in some samples, were dose-dependent as a consequence of plant cells disruption phenomenon.

All metals in both species (except for Cr in *P. aviculare*) had a shoot bio-accumulation factor (BAF) > 1, demonstrating the ability of these plants to concentrate metallic ions within the aerial parts in hydroponic conditions (Tables 2a, 2b). This situation is usually not present when plants are grown on soil, where metals are usually less available due to the interactions with soil particles and pH. In fact, similar ruderal species like *Plantago major* and *Taraxacum officinale* collected on roadside soils enriched in several trace metals, showed BAFs <1 for most elements (Galal and Shehata, 2015; Kleckerová and Dočekalová, 2014). *P. aviculare* itself, when grown on natural soils, was not capable of concentrating HMs in its shoots (Chapter 2 of the present thesis and Salinitro et al., 2019) on the contrary to what detected in hydroponic conditions (Tables 2a, 2b).

In both plant species, BAFs decreased with the increase of Cd, Ni and Zn concentrations in the solution (Table 2a, 2b), demonstrating a controlled absorption mechanism for these elements, aimed at limiting their uptake at toxic concentrations. The trend was different for Cr and Pb, which levels showed a possible passive uptake through the root membrane as already shown for some non-essential elements, among which Cr, by Shanker et al. (2005) who confirmed the absence of specific Cr transporter. In fact, at Cr and Pb increasing doses in the nutrient solutions, higher BAFs were also observed in both species (Tables 2a, 2b). Cu BAFs remained almost constant at all treatment concentrations (with the exception of C samples for both species), again confirming the strict regulation of the uptake of this metal (Tables 2a, 2b).

The two species, beside being different in the HMs absorption capacity, with *P. aviculare* accumulating less metals than *S. vulgaris*, were also quite different in their reaction toward HM-caused stress as shown by results related to the levels of some plant metabolites (Fig. 10). Although *P. aviculare* plants had a more limited HM uptake, they seemed to be more equipped in the activation of the antioxidant system, producing more polyphenols, flavonoids, and having a higher antioxidant activity (with respect to shoot gFW), when compared to *S. vulgaris* (Fig. 10, Tables 5a, 5b). Secondary metabolites, such as polyphenols and flavonoids, show in fact a well documented antioxidant activity and play an important role in mitigating metal stress toxic effects (such as ROS production), along with superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) enzymes and with phytochelatins (Rice-Evans and Miller, 1996; Sies, 1997; Michalak, 2006)

The differential absorption of all tested metals in *P. aviculare* populations (Fig. 11) was not easily explainable on the basis of the metal levels to which plants were adapted to in their original habitat

which were previously reported in table 5 at page 29, and in Salinitro et al. (2019). *P. aviculare* B and M urban populations absorbed more Ni, Pb and Zn than other populations, while the serpentine (S) and Milan woodland (T) populations showed higher levels of Cr, Cd, Cu and Zn.

On one hand, the similar behavior of the two urban populations (that showed higher uptake rate of very common metals in urban environment such as Pb and Zn, could be explained by a major tolerance capacity of the plants toward these elements, probably caused by a pre-adaptation to environmental characteristics. On the other hand, it seemed difficult to explain the similar uptake behavior of the serpentine (P) (adapted to high Ni and Cr levels) and the Milan woodland (T) (not adapted to metals) population, which was in contrast with the other woodland population (N) being the one accumulating the lowest amount of all tested elements (Fig. 11). Similar results were previously reported for *Paspalum distichum* and *Cynodon dactylon*, the populations of which, when growing on mine tailings, have evolved a greater metal tolerance compared to those growing on unpolluted soils (Shu et al., 2002).

In *S. vulgaris*, the metal uptake differences among populations tended to disappear, with the exception of Ni, in which population B accumulated - 60 % compared to the other accessions and Zn, in which population P accumulated 26% more than the others. These results could not be explained with the pre-adaptation model, unlike *P. aviculare* were urban and woodland population behaved consistently (Figs. 11, 12).

When analysing the differences among the five different *P. aviculare* populations with regards to the production of some plant metabolites (such as polyphenols and flavonoids) having antioxidant activity, they also reflected a similar trend based on the environment of origin. For example, the two urban B and M populations behaved similarly having a high content of polyphenols, flavonoids and antioxidant activity levels on the opposite of the two woodland populations (T and N) which showed low levels. Similarly to what has been detected for HM uptake, *S. vulgaris* has no fixed population groups that could reflect the plant pre-adaptation to environmental metal stress levels. A possible hypothesis could be the fact that *S. vulgaris* needs more time than *P. aviculare* to differentiate ecotypes because of its wind-dispersion strategy. In facts, long distance dispersion favour genetic mixing, maintaining low differences among populations, as demonstrated for several tropical species (Hamrick et al., 1993).

The effect of increasing concentrations of metals on inducing the production of some plant metabolites (Table 4), seemed in agreement with patterns already reported in the literature for other species: a general increase in antioxidant activity and antioxidant molecules (such as flavonoids and polyphenols) and a decrease of photosynthetic pigments content and of shoot

biomass (i.e Michalak, 2006; Martins and Mourato, 2006; Viehweger, 2014). In particular, Lavid et al. (2001), demonstrated that increasing Cd accumulation in *Nymphaea alba* leaves, was accompanied by increasing polyphenols and peroxidase activity levels. Polyphenols in fact, can act as scavengers of HMs thanks to their capacity to form insoluble complexes with divalent and trivalent cations, reducing their cellular concentrations (Lavid et al., 2001). Vajpayee et al., (2000) also reported severe toxic effects of Cr on *N. alba* with regards to photosynthetic pigments. This plant in fact, when exposed to 200 uM Cr for twelve days showed a decrease in Chl a of about 81.3% and Chl b of 61.4%.

There are also evidences that flavonoids can play a role in Al toxicity resistance in *Zea mais* (Kidd et al., 2001), despite no data were specifically reported regerding flavonoids in relation with the six metal tested in the present study.

The present research demonstrated that 82% of the metal treatments showed a good or excellent correlation between the amount of polyphenols and flavonoids, antioxidant capacity and the metal concentration in plant shoots, confirming that in most cases the metal stress level and the amount of secondary metabolites showing antioxidant activity were strictly connected.

The production of different types of polyphenols, flavonoids and of other antioxidant molecules, is rather ubiquitous in plants when subjected to different abiotic or biotic stress, among which HMs, drought, UV radiation, pathogens, etc (Dixon and Paiva, 1995). Consequently, information like metal concentration or type of stressor cannot be inferred just looking at antioxidants concentrations in plant tissues. Another issue is the widespread lack of linearity of these metal-metabolite relations that are represented in many cases by logarithmic or exponential dose-response curves (Fig. 13a, 13b). In the case of a logarithmic curve, the plant immediately reacts to low concentrations of HM, producing high contents of antioxidant molecules, to soon reach a plateau after which increasing HM concentrations do not stimulate further antioxidants production (Fig. 13a). In the case of an exponential curve, the plant do not react to a wide range of HM concentrations up to a specific HM level which triggers the sharp production of antioxidant metabolites starts (Fig. 13b).

The high variability of antioxidant responses in plants, leads to the need to integrate the data regarding the amounts secondary metabolites with several other markers, in order to obtain a complete and reliable evaluation of the plant stress. These may include, among many other, shoot and root biomass, photosynthetic pigments content and antioxidant activity.

For instance, several literature papers reported that HMs strongly affect photosynthetic processes, causing both a reduction of photosynthetic pigments amount and plant growth (Küpper et al., 1998; Chandra and Kang, 2016; Bidar et al., 2007; Zornoza et al., 2002) and of antioxidant capacity (Rout

and Das, 2003). For instance, it has been demonstrated that *Plantago major* (Kosobrukhov et al., 2004) and *Brassica napus* (Tian et al., 2014; Bilal Shakoor et al., 2014) plants grown with high Pb concentrations, showed a reduced production of photosynthetic pigments and a lower biomass compared to control treatments. Similarly, the antioxidant activity of tomato plants, treated with increasing concentrations of Cd, Cu and Pb, was directly proportional to the level of metal treatment (Kisa, 2018).

In the present study, by integrating the data regarding polyphenols and flavonoids content, antioxidant activity, photosynthetic pigments content and shoot biomass (Tables 5a, 5b), it was possible to understand which metals were the most toxic for the two studied species. The drastic reduction of photosynthetic pigments and shoot biomass and the simultaneous sharp increase in the production of antioxidant compounds were considered biomarkers of HM acute stress conditions. This situation occurred more frequently in *S. vulgaris* compared to *P. aviculare,* the second being therefore considered more resistant to HM stress than the first one. The metals that induced the highest toxicity effects in *P. aviculare* were Cu, Pb and Zn, especially at their maximum tested concentrations (table 5a). In *S. vulgaris,* metal toxicity was recorded for high and medium concentrations of Cd, Ni, Cu, Pb and Zn (table 5b). It can be hypothesised that the greater sensitivity to HMs detected in *S. vulgaris* respect to *P. aviculare*, could be due to several factors, such as the higher metal uptake capacity and the lower production of detected antioxidant metabolites of *S. vulgaris* leading to a reduced protection capacity against HM-induced oxidative stress.

The acute toxicity responses observed in this study for some metal treatments like Cu and Zn, seems to be in line with other studies which reported these elements as extremely toxic, with effects comparable to those exerted by Pb and Hg (Zenk, 1996). Nonetheless, the most toxic element even at very low concentrations resulted to be Cd, as also shown by Jagodin et al. (1995) who reported a Cd toxicity in plants 2 to 20-fold higher than that of other HMs.

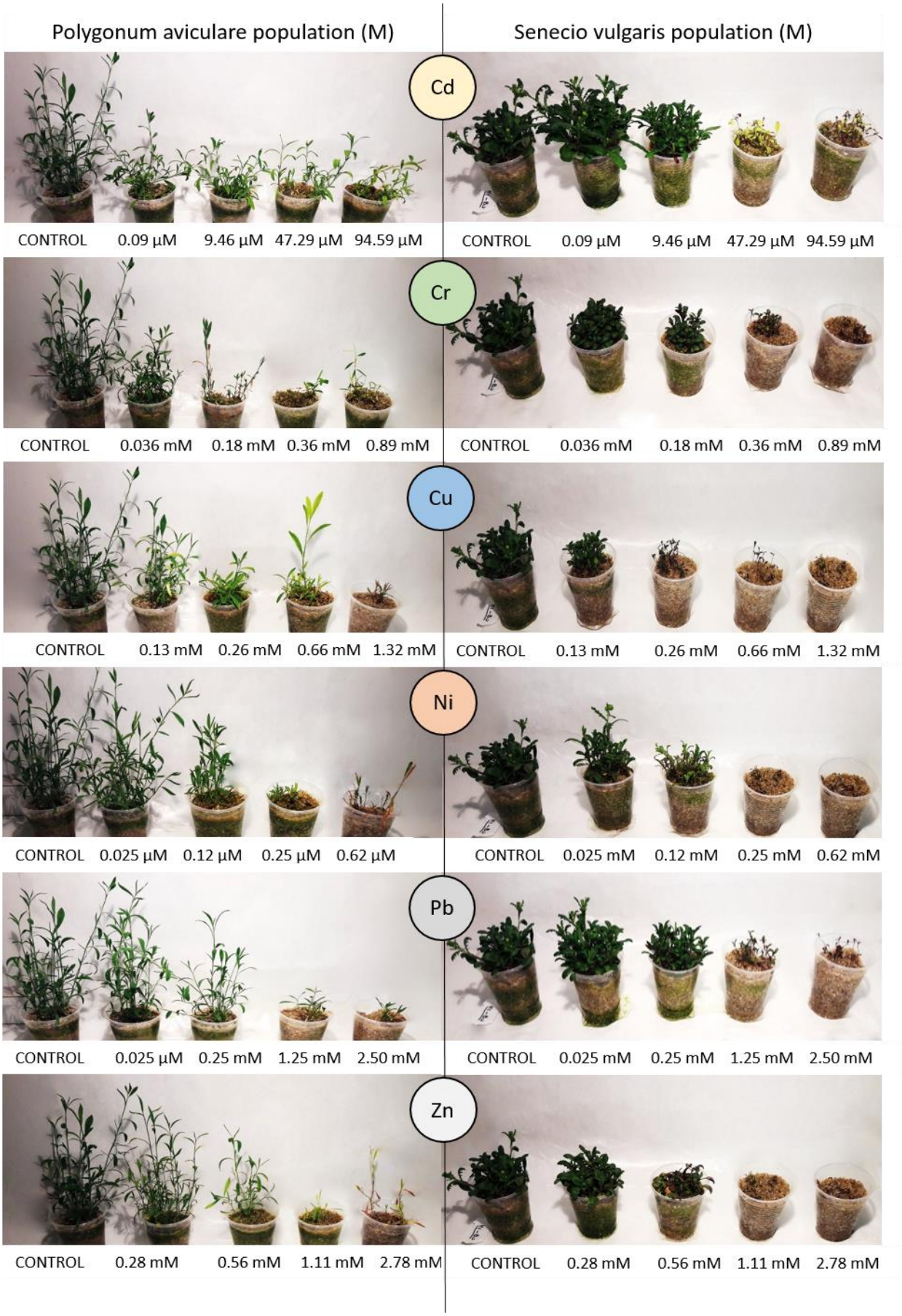

Figure 14. Decreasing shoot biomass and chlorosis showed by *P. aviculare* (left) and *S. vulgaris* (right) Milan urban population (M) plants under different metal stress conditions.

4.5. Conclusions

The present study demonstrated that *P. aviculare* and *S. vulgaris* had a linear uptake of Cd, Cr, Cu, Ni, Pb and Zn, when cultivated in hydroponic conditions in the presence of increasing metal concentrations (Fig. 14). Because of this behaviour, the two studied species can be suitable as metal indicators in the several environments since their widespread presence. For the highest Cd and Pb treatments, the commonly set hyper-accumulation threshold was largely exceeded nonetheless in these condition the two studied species showed severe toxicity symptoms, therefore highlighting their incapability to hyper-accumulate metals. *P. aviculare* demonstrated to be more tolerant than *S. vulgaris* to high HMs levels in the solutions suggesting its possible use in for phytostabilization purposes. Differences in HMs accumulation and in some metabolites production were detected among the five *P. aviculare* populations, being instead absent among *S. vulgaris* populations. These results showed that some species, that have low mobility (i.e. with seeds dispersed by gravity) can develop urban ecotypes adapted to polluted soils.

Our study demonstrated that flavonoids, polyphenols, antioxidant activity, photosynthetic pigment production and shoot biomass, were mostly correlated with metal content in plant shoots and therefore can be effectively used as a marker of HM stress in plants. Nonetheless, the amount of secondary metabolites produced by plants in response to HMs is wider, and the inclusion of antioxidant enzymes (SOD, POD, CAT), phytochelatins and metallothioneins in plant stress evaluation can furtherly improve the results. The mere quantification of antioxidant metabolites, cannot give information about the type of metal that caused the oxidative stress, neither the concentration of the stressors. In fact, high dosage of poorly toxic metal give similar effects of low doses of highly toxic metals.

Because of the limits discussed above, the integration of biological parameters can be an effective way to evaluate metal stress and toxicity in plants, only when coupled with the quantification of trace elements in plant tissues.

4.6. References

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5. Hormesis: when heavy metals become beneficial, the case of cadmium tested on three annual weeds

Article status

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Author Contributions

Mirko Salinitro, analysed the data, contributed to write the manuscript and coordinated the study. Giorgia Guardigli, cultivated the plant, collected the data and contributed to write the manuscript. Annalisa Tassoni coordinated the study and revised the manuscript.

List of abbreviations

ROS = Reactive oxygen species

HMs = Heavy metals

5.1. Introduction

The term hormesis appeared for the first time in the scientific literature in 1943, when Southam and Ehrlich (1943) tested the effect of the extracts of the red cedar tree on the growth of a large number of fungal species. Since then, there has been an increasing attention to the topic and publications about hormesis have kept growing in the last years (Calabrese, 2015b).

The term hormesis describes a biphasic dose-response relationship characterized by opposite effects caused by low and high doses of the same substance (Calabrese & Blain, 2009; Calabrese, 2015a, 2015b). These substances include organic compounds (i.e. weed-killers), biological molecules (i.e. polyphenols), physical stressors (i.e. radiations) and chemicals (i.e. heavy metals) (Kendig et al., 2010). Hormesis effect includes both positive and negative responses, because the fundamental requirement of this phenomenon is to display reversal response between low and high substance doses, regardless to its beneficial or harmful effects on the studied organism (Calabrese et al., 2009).

The typical graphs that describe this biphasic response phenomenon are the U-shaped curve and the inverted U-shaped curve (Fig. 1), depending on the measured endpoints. If the endpoints are dysfunctional (such as carcinogenesis and disease incidence) they will be high in the control treatment then decrease at low doses of a given hormetic substance, to increase again at higher doses, creating a U-shaped curve (Fig. 1a).

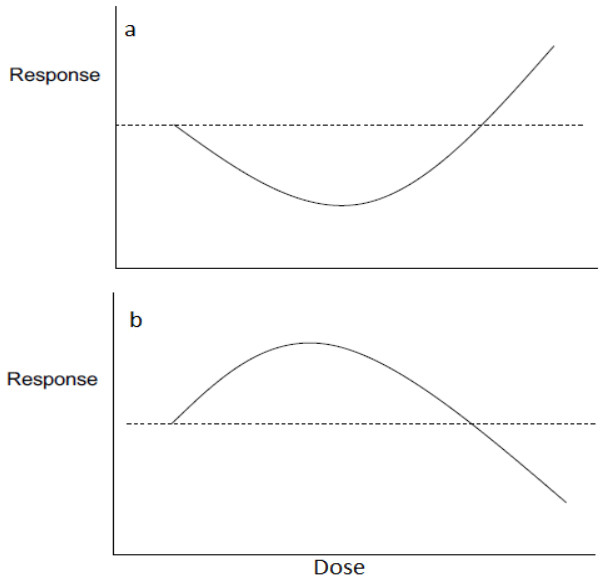

Figure 1. U-shaped and inverted-U shaped curves. a) The hormetic dose–response curve depicting low-dose reduction and high-dose enhancement of adverse effects such as disease incidence. b) The most common form of the hormetic dose–response curve depicting low-dose stimulatory and high-dose inhibitory responses of normal functions like biomass growth. Adapted from Jia et al., 2015

Conversely, the inverted U-shaped curve (Fig. 1b) occurs if the endpoints evaluated are related to normal plant functions (such as fertility and growth), showing an increase at low doses and a decrease of the effects at higher substance doses (Jia et al., 2015).

Hormesis is a widespread dose-response phenomenon that occurs in numerous organisms (bacteria, plants, animals, etc.) as a result of the application of a large array of compounds that applied in several fields. For example, some studies investigated the effects of the low-dose radiation (LDR) in different experimental models including cultured cells and laboratory animals (Liu, 2003; Luckey, 1982; Yang et al., 2016; Ji et al., 2019). It was also demonstrated a possible application of hormesis in medicine, in fact low radiation doses induced hormesis specifically in cells belonging to the immune and hematopoietic systems, being a possible treatment for cancer and diabetic complications (Ji et al., 2019).

Other studies focused on toxic organic molecules like (caffeine, aldicarb, rotenone, charybdotoxin) (Cutler, 2013), showing evidence that these chemicals, having a demonstrated toxicity at higher concentrations, can have instead beneficial effects in insects when used in low amounts. Nicotine for instance, whose chemical structure provided the template for the development of neonicotinoids (common insecticide molecules), is proven to induce an increase in sucrose sensitivity and improved retention of olfactory learning in bees (Cutler and Rix, 2015).

More abundant are the examples of hormesis reported for plants. Cedergreen et al. (2009), studied the effect of glyphosate on *Hordeum vulgare* (barley), demonstrating that this herbicide can increase plant growth of the 12-15 % if applied at dosage of 2.5–20 g/ha^{-1}, corresponding to less

than 1% of the rate suggested to kill weeds. Another study has investigated the hormesis effect induced by the herbicide metamitron on *Chenopodium album*, showing that hormetic doses of this weed-killer, stimulated seed yield of the 45% above untreated plants. This study gives important insight to understand the evolution of herbicide resistance in weeds (Belz, 2018).

Hormesis effects, have also been widely reported as a reaction to low concentration of heavy metals (HMs) (i.e. Agathokleous et al., 2019; Seth et al., 2007; Hajiboland et al., 2013). In a recent study, the dose-response effect of Lanthanum, a rare earth element that has numerous applications in modern industries, was evaluated in a large number of plant taxa. It was observed that, the maximum biological response to low La doses was at 56 µM, at which concentration La affected numerous biological processes in plants like: biomass amount, cell growth rate, chlorophyll content, peroxidase activity, flavonoids content and many other (Agathokleous et al., 2019).

In general it is assumed that hormesis is an adaptive response to stress, possibly triggered by an initial disruption of homeostasis followed by a compensatory process aimed at the re-establishment of the previous status (Calabrese, 2015a). These responses are often over-compensatory and occur thanks to several mechanisms still unclear, that differ depending on the biological system studied and the type of stress applied.

When focusing specifically on the hormesis effect induced in plants by heavy metals, three main action mechanisms could be distinguished (Poschenrieder et al., 2013):

- Substrate interactions: the ionic interactions between different chemicals present in the soil can affect nutrient absorption in a positive or negative way.
- Metal-induced activation of specific defense reactions, such as translation of metal tolerance genes.
- Metal-induce general defense reactions, triggered by the generation of reactive oxygen species (ROS) as consequence of the metal-induced oxidative stress, leading to the activation of the antioxidant response determining the final hormesis stimulation effect.

Since, the main mechanisms of hormesis are still largely unknown, it is important to figure out how widespread this phenomenon is among plants and which substances can cause it. For this reason we chose common weeds as model species, since these plants can be easily found in places (i.e. urban environments) characterized by HMs pollution, including Cd. This metal, in fact has been widely studied for its acute toxicity but also for its capacity to stimulate hormetic response in several organisms. The aim of this study is therefore to assess the presence of hormesis effect in three herbaceous species (*Poa annua, Stellaria media, Cardamine hirsuta)* caused by the exposure to micro-doses of Cd.

5.2. Materials and Methods

5.2.1. Species selection

Poa annua (L.) (annual blue grass) of the Poaceae family is a cosmopolitan species that grows on a wide variety of soils. It tolerates trampling, mowing and frozen conditions. This plant shows a rapid growth, it can go from seed to adult plant, flowering and producing seeds in about 6 weeks. The stems are 15-25 cm high and the leaves clasp the stem. Flower and seeds are grouped in a panicle.
Cardamine hirsuta (L.) (hairy bittercress) is an annual member of the Brassicaceae family common on disturbed soils, fields and meadows. This species is native of Europe and western Asia but has spread all over the word. *C. hirsuta* may complete two generations in a year during warm season, in spring and fall. The plant shows a basal rosette of compounded leaves, with 3-9 round leaflets. From the rosette a 10-30 cm high stem produces several white flowers. The flowers are grouped in a raceme and the seeds are contained in upright pointing siliquae.
Stellaria media (L.) (common chickweed) is an annual herbaceous plant of the Caryophyllaceae family. It is native of Europe but now naturalized in all continents. This species commonly grows in lawns, wastelands and in disturbed habits such as road margins and bare soil deposits. Stems are slender, branched and slightly swollen at the joints with sparse hairs all along. The plant has a creeping habit, and the stems can produce adventitious roots. The leaves are oval and opposite flowers are white and small and dehiscent capsules contain several seeds.
All seeds used in the experiment were collected in the Ticino Natural Park (Locality Besate, (MI), Italy)(see table at page 28). The area is characterized by undisturbed sandy soil, with low organic matter content and good water availability during all year. The Ticino Park has low anthropic pollution and widespread presence of native woods rarely interrupted by fields. The analysis of soils (See page 52) revealed very low HMs concentrations, therefore we did not consider this population as pre-adapted to HMs stress.

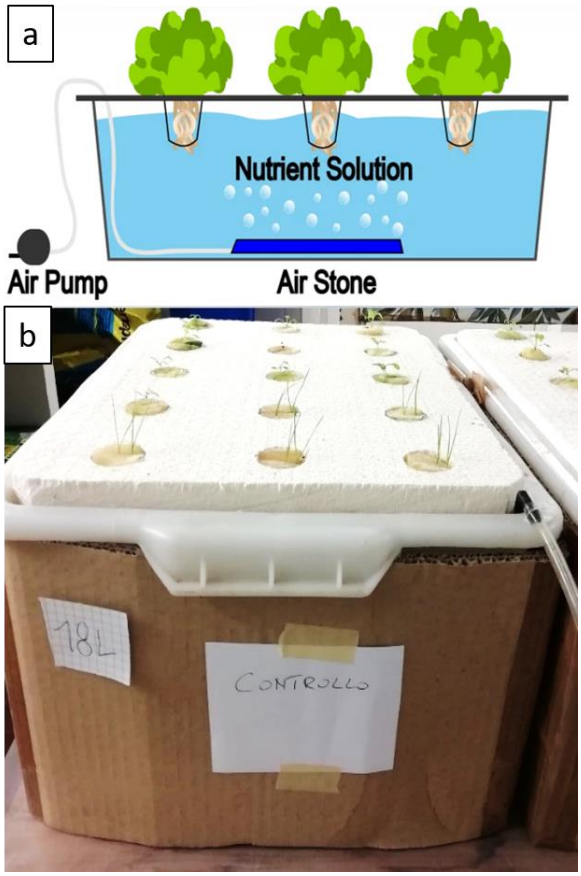

Figure 2. a) Schematic representation of the tank functioning. b) Control tank with two-day-old seedlings.

5.2 .2. Plant cultivation

A hundred seeds per species were sowed in a germination medium composed by 50% compost and 50% of coarse sand. The container with seeds were sealed with plastic film to prevent the soil from drying out. After germination, seedlings were counted to determine seed viability, which was: 98% for *P. annua*, 53% for *C. hirsuta* and 30% for *S. media.* At the cotyledon stage, plants were transferred in the hydroponic system. To remove plants from the growth medium, trays were saturated with water so that the soil would soften and easily detach from root surfaces. Plants were then carefully extracted from the ground using tweezers, taking care not to damage the roots. Roots were carefully washed with tap water in order to remove all soil particles, then plants were placed on rounded foam supports. During the two days after transplantation the foam supports were humidified with de-ionized water, to ensure plant survival until roots would have been long enough to touch the nutrient solution. To carry out this experiment a deep-water culture hydroponic system was built and used (Fig. 2a). In this system, the roots float free in the nutrient solution and, at same time, oxygen is provided through an air pump producing bubbles from the bottom.

The system was composed by six plastic tanks, coated with dark cardboard to prevent light penetration. Every tank had a capacity of 18 liters and was topped by a styrofoam cap having fifteen circular holes (Fig. 2b). One plant per hole was fixed with a soft foam cylinder. In each tank, five plants per species were cultivated. The hydroponic system was kept at 22 ± 0.5 ° C with 16/8 hours of light/dark.

The tanks were filled with modified half-strength Hoagland's solution (Waters et al., 2012), containing: 2 mM KNO_3, 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mM NH_4NO_3, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.25 mM KH_2PO_4, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnCl_2 \cdot 4H_2O$, 2 µM $ZnSO_4 \cdot 7H_2O$, 0.5 µM $CuSO_4 \cdot 5H_2O$, 0.075 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.15 µM $CoCl_2 \cdot 6H_2O$, 0.05 µM $NiCl_2 \cdot 6H_2O$, 40 µM Fe-EDTA

Five increasing micro-concentrations of cadmium ($CdCl_2 \cdot 2.5H_2O$) were tested: 0.5 µM, 0. 75 µM, 1 µM, 1.5 µM, 2 µM obtained by dilution of a 0.1 M $CdCl_2$ stock solution. The nutrient solution was adjusted by adding NaOH 1 M, until a pH value of 5.8 ± 0.1. A 10% replacement of the nutrient solution and a pH adjustment to the initial value, were performed every two days.

5.2.3. Samples collection

The plants were cultivated for four weeks, then harvested four weeks and divided into shoots and roots. The roots were rinsed with deionized water and dried with paper towels before weighting, while shoots were directly weighted. Once weighted plants were wrapped in tinfoil in order to obtain for each plant two separate samples: one for roots and one for shoots. Every sample was marked with a specific code keeping track of the treatment the plant had undergone. Fresh samples were grinded in liquid nitrogen to obtain a homogeneous powder. All samples were stored at - 80° C until further analysis.

5.2.4. Traits measurement

At the second and the fourth week of cultivation, nodes and the leaf area were measured. Plant nodes were counted following a different method according to the morphological differences of each species. For *P. annua* the number of lateral sprouts generated by the primary stem, including the primary stem, were considered as nodes (Fig. 3a). For *C. hirsuta* every node corresponded to one leaf, due to its rosette habit in the juvenile phase. For *S. media* every node corresponded to a pair of leaves, counted on the primary stem, while nodes of the secondary branches were not considered. To measure leaf area, an empirical rule was followed in order to select the best leaf to be measured. For *P. annua* the penultimate complete leaf of the main sprout was measured, both at 2 and 4 weeks. For *C. hirsuta* the third leaf after the cotyledons was

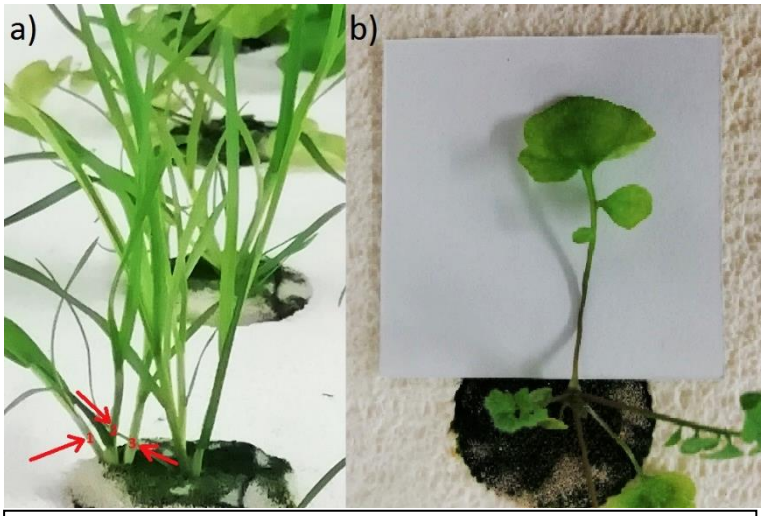

Figure 3. a) *P. annua* plants after 1 week in hydroponics, each plant produced three different sprouts that were counted as nodes. b) Picture *in vivo* of *C. hirsuta* leaf to calculate leaf area at the second week of culture.

considered at the second week, while and the largest and most developed leaf in the whole plant was taken into account at the fourth week. In *S. media* the third pair of leaves were analyzed at the second week, and the eighth pair of leaves after the cotyledons, at the fourth week.

To precisely calculate the leaf area, leaves pictures were taken using as a background cardboard square of known size (5 x 5 cm or 15 x 15 cm), the leaf was spread as much as possible on the cardboard in order to avoid measurement biases due to leaf wrinkles. Leaf's stalks were included in the pictures and considered in the calculation, the camera was vertically placed on the cardboard in order to maintain the same proportions between leaf and cardboard in the picture. At the end of the second week this procedure was performed *in vivo*, without removing the leaves from the plants (Fig. 3b), to prevent plant damages or hindering its growth.

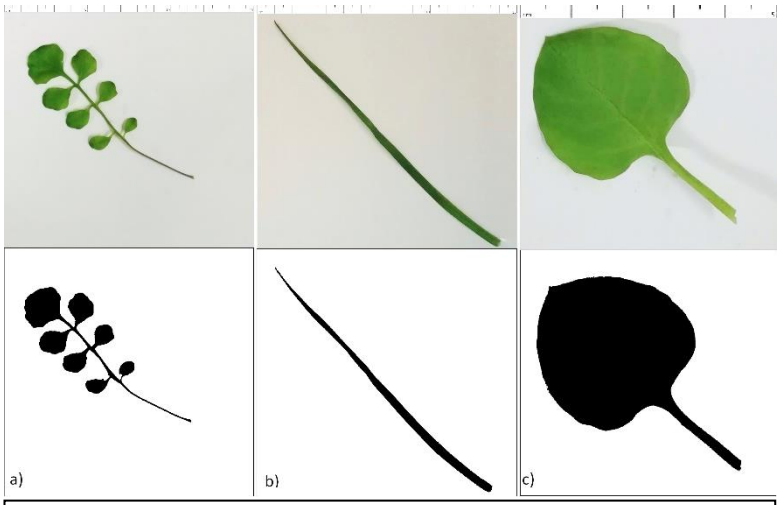

Figure 4. Original leaf pictures (top) and binary version (bottom) used to count pixel. a) *C. hirsuta*. b) *P. annua*. c) *S. media.*

To calculate leaf area, every picture was cut using Adobe® Photoshop software at the dimension of the cardboard square, setting the size of the image at 5 x 5 cm or 15 x 15 cm. After that, using the software ImageJ (https://imagej.net), all pictures were transformed to binary (only black and white pixels) to obtain a black leaf shape on a white background area (Fig. 4). After binary transformation, white and black pixels were counted using the tool *Analyze>Measure* available in the software toolbar. Leaf area was then calculated using the following proportion:

Black pixels : Total pixels = Leaf area : Square area

$$\text{Leaf area } (cm^2) = \frac{\text{(Black pixels) x (Square area)}}{\text{Total pixels}}$$

At the end of the cultivation period (week 4) fresh weight (FW), dry weight (DW) and amount of photosynthetic pigments were also measured. For the determination of dry weight, approximately 0.3-0.5 gFW aliquots of roots and shoots bulk powders were oven-dried at 60°C for 24 hours and weighted.

To quantify photosynthetic pigments, a modified method from Radwan et al. (2007) and Metzner et al. (1965) was used. 0.1 gFW of shoots grinded powders were extracted with 1.5 mL of 85% (v/v) acetone and mixed 2 times for 30 seconds, the samples were then centrifuged at 4°C, 2500 rpm for 5 minutes and the supernatant recovered. The supernatant was analyzed at three different wavelengths (663, 644 and 452.5 nm) and the obtained absorbance values were processed to give the pigment concentrations in mg/gFW with the following equations:

chlorophyll *a* = 10.3 x Abs_{663} – 0.98 x Abs_{644}
chlorophyll *b* = 19.7 x Abs_{644} – 3.87 x Abs_{663}
carotenoids = 4.2 x $Abs_{452.5}$ – [(0.0264 x chl-*a*) + (0.426 x chl-*b*)]

All the spectrophotometric analyses were performed with a VersaMax™ Microplate Reader (Molecular Devices, San Jose, California) spectrophotometer. A 96-wells plate was used to load samples on the instrument and for each sample 200 µL of acetone extract were placed in every well.

5.2.5. Data analysis

All the statistical analyses were performed using Rstudio software version 3.6.1. The differences in plant growth were evaluated among the three different species as well as among the different cadmium treatments. For each species a dataset with 18 variables (columns) and 30 observations (rows) was produced. Data were tested for normality using the Shapiro-Wilk normality test (data are normal if p>0.05), and for homogeneity using Levene's Test for Homogeneity of Variance (data are homogeneous if *p*>0.05) with default parameters from the package *Car* (https://CRAN.R-project.org/package=car). The Analysis of Variance ANOVA, followed by Tukey HSD test was used performed for all parametric samples group in order to detect significant differences between the analyzed groups (*p* value <0.05). While for the non-parametric data the Kruskal-Wallis test followed by Dunn's post-hoc test were used. Non-linear models were used to describe the trend of plant growth according to the different cadmium treatments. Principal Component analisys (PCA) was performed with the function prcomp, using default values. Graphical elaborations where performed using the R package ggpubr (https://CRAN.R-project.org/package=ggpubr).

5.3. Results

5.3.1. Poa annua

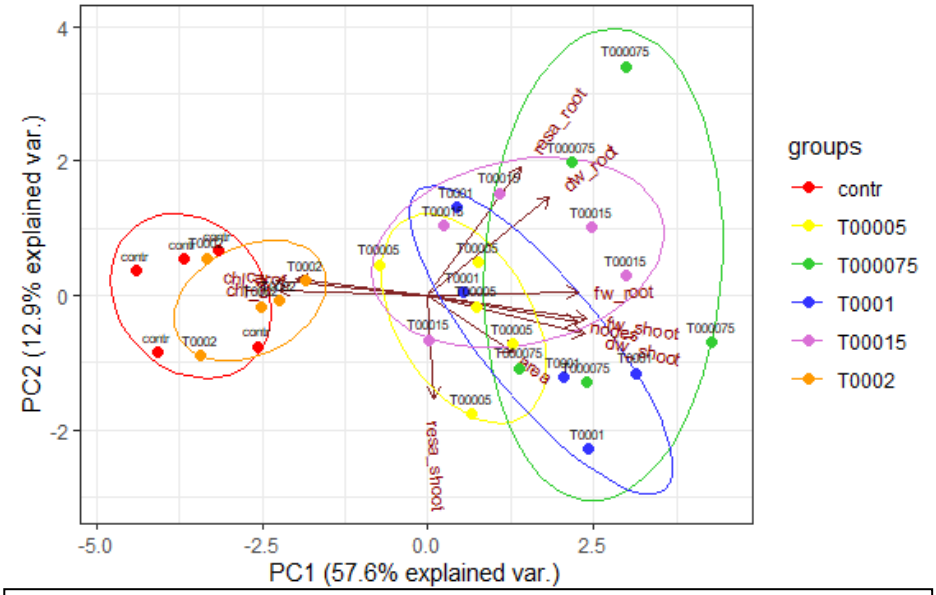

Figure 5. PCA showing the grouping of *P. annua* samples according to the treatment. The variables used in the PCA were: photosynthetic pigments (Chl a, Chl b, Carotenoids), shoot DW, FW and DW/FW, root DW, FW and DW/FW, nodes number, leaf area. Treatments: Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd.

Overall, this species showed an hormetic response to central Cd treatments (0.75 µM, 1 µM), with an increase in fresh and dry biomass of roots and shoots. This effects were not detected in control plants and high Cd dosages. The PCA analysis (Fig. 5) that took into account traits measured at 4 weeks, chlorophyll content, FW, DW and DW/FW, showed a clear separation of the treatments in two main groups: control and Cd 2 µM treated plants were very similar, in contrast with all the other Cd treated plants who showed mostly an increase in whole plant biomass. The variables that mainly allowed such a clear separation were shoots and roots fresh weight, and the number of nodes. These parameters were those mostly affected by different Cd treatments.

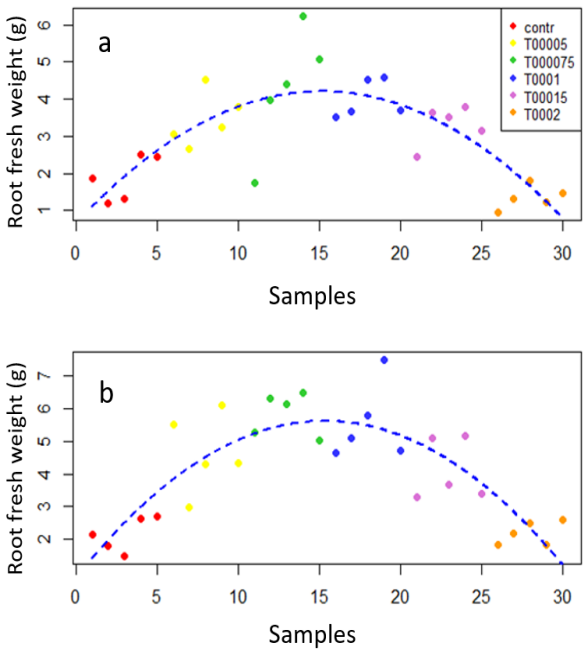

Figure 6. a) Root fresh weight in *P. annua*. b) Shoot fresh weight in *P. annua.* Samples: 0 to 5= 0 µM Cd (Contr), 6 to 10= 0.5 µM Cd (T00005), 11 to 15= 0.75 µM Cd (T000075), 16 to 20= 1 µM Cd (T0001), 21 to 25= 1.5 µM Cd (T00015), 26 to 30= 2 µM Cd (T0002). The blue dashed line show the trend of the treatments with a non-linear model ($y=x+x^2$).

Analysing more in detail the variable root fresh weight (Fig. 6a), it can be noticed that the control and the 2 µM Cd treatment showed a similar trend with an average root biomass of 1.61 gFW. These two treatments also resulted statistically different ($p<0.01$) from the group including plants treated with 0.5 µM, 0.75 µM, 1 µM, 1.5 µM Cd, which showed an average fresh weight of 3.76 g. The non-linear model ($p<0.01$, R=0.599) significantly described our data and showed an inverted U shape for the dose-response curve.

The shoot fresh weight variable (Fig. 6b), showed a similar grouping of data respect to root biomass fresh weight with 0.5 µM, 0.75 µM, 1 µM, 1.5 µM Cd treatments having an average weight of 5.03 gFW, significantly different ($p<0.01$) from the control and the 2 µM Cd treated plants (on average 2.18 gFW).

The non-linear model again well described our data ($p<0.01$, R=0.656). The inverted U shape dose-response curve showed, for both root and shoot biomass, the positive effect of intermediate Cd concentrations on these variables, on the contrary, the highest concentration (2 µM Cd) did not have the same effects performing similarly to the control treatment with no Cd.

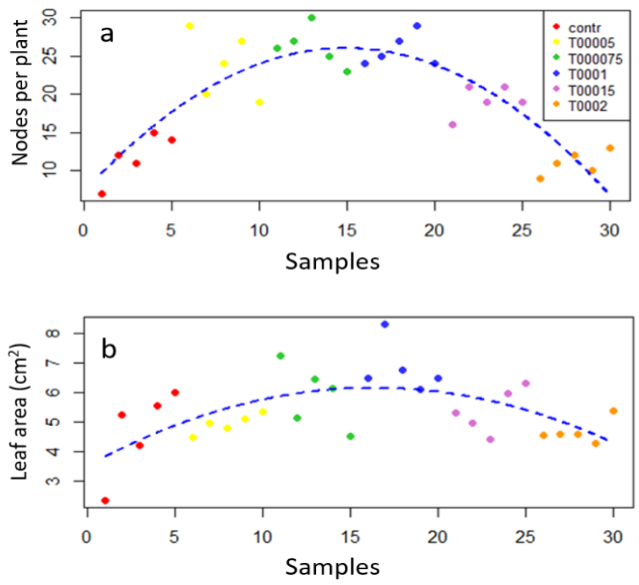

Figure 7. a) Number of nodes per plant in *P. annua* at the fourth week of cultivation. b) Leaf area in *P. annua* at the fourth week of cultivation. Samples from: 0 to 5= 0 µM Cd (Contr), 6 to 10= 0.5 µM Cd (T00005), 11 to 15= 0.75 µM Cd (T000075), 16 to 20= 1 µM Cd (T0001), 21 to 25= 1.5 µM Cd (T00015), 26 to 30= 2 µM Cd (T0002).

For all different treatments the DW/FW ratio was similar ($p>0.05$) (between 15-17 % in shoot and 2-5 % in root), showing the same trend in both shoot and root dry weights and fresh weights.

With respect to the number of nodes (Fig. 7a), it can be noticed that Cd treatments 0.5 µM,

0.75 µM, 1 µM, 1.5 µM were similar with an average of 23.7 nodes per plant, and significantly differed (p<0.01) from both the control and Cd 2 µM treated samples which showed an average of 11.2 nodes per plant. The data were again well described by a non-linear model (p<0.01, R=0.703), which showed that the intermediate Cd concentrations were able to increase the number of nodes produced by plants after four weeks of culture. This trend was already present and measurable at second week of cultivation when the number of nodes in control and 2 µM Cd treatment was on average 3.3 nodes per plant while, in other Cd treatments, was on average of 5.5 nodes per plant.

The variable leaf area measured after four weeks (Fig. 7b) did not show significant differences among treatments. Nonetheless, an inverted U-shape dose-response curve was shown by the non-linear model (p<0.01, R=0.324). In fact, the average leaf area of the 0.75 µM and 1 µM Cd treated *Poa* (6.37 cm^2) was 20 % higher if compared to the average of other samples (4.93 cm^2). The leaf areas, measured after two weeks, were not yet affected by Cd treatments.

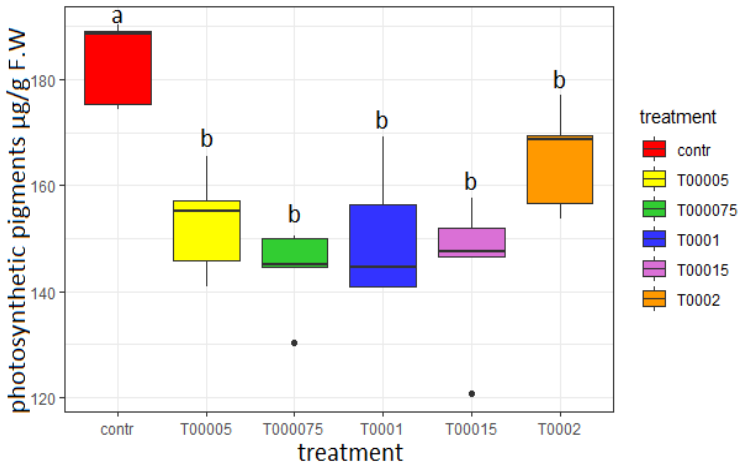

Figure 8. Photosynthetic pigments (sum of Chl a, Chl b, carotenoids) content in µg/ gFW of *P. annua*. Different letters indicate the statistically different samples. Treatments: Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd.

The analysis of photosynthetic pigments (Fig. 8) showed that only the control sample could be considered statistically different from the other treatments (p<0.01), despite a U shaped trend of this variable. . The average photosynthetic pigments (sum of Chl a, Chl b, carotenoids) content was 183.5 µg/gFW for the control plants, while all Cd treated samples treated had a similar pigment contents (p>0.05) with an average of 151.5 µg/ gFW.

5.3.2. Cardamine hirsuta

C. hirsuta like the previous species showed an hormetic response to central Cd treatments especially the 0.75 µM Cd, with an sensible increase in fresh and dry biomass of roots and shoots. This effects

were not detected in control plants and high Cd dosages. The PCA analysis (Fig. 9) that took into account traits measured at 4 weeks, chlorophyll content, FW, DW and DW/FW. This analysis (Fig. 9) pointed out that data from the control and the 2 µM Cd treated plants, overlapped showing therefore similar features. This trend was mainly associated with a lower biomass when compared to the other Cd treated samples. Shoot and root biomasses dramatically increased in the Cd 0.75 µM treatment compared to 1 µM and 1.5 µM Cd treatments, which were instead characterized by average values (7 g and 5.6 g respectively). The Cd 0.5 µM treatment was associated to an increase of chlorophylls.

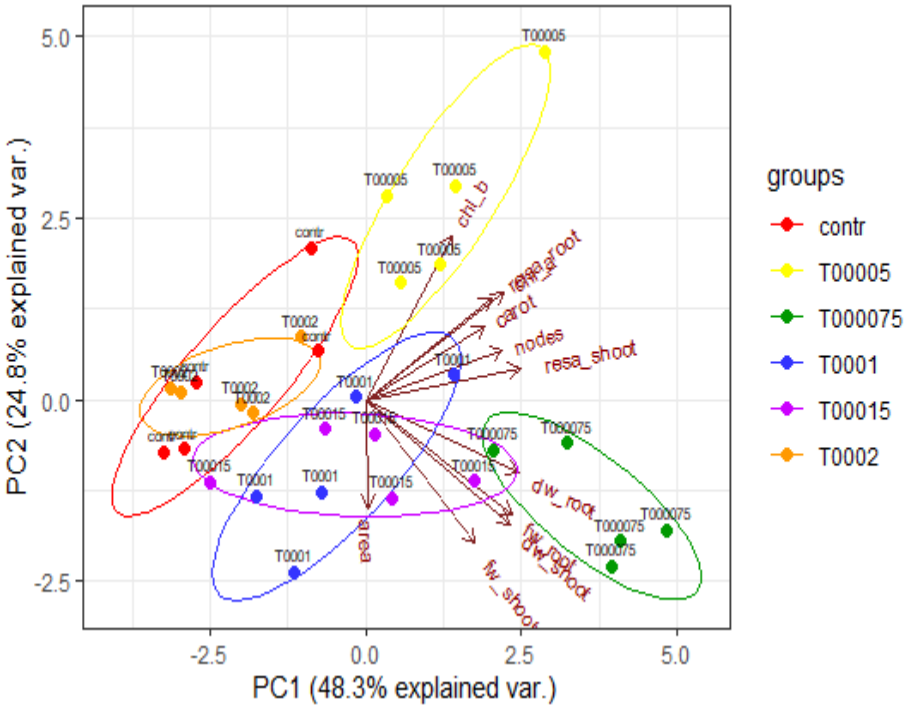

Figure 9. PCA showing the grouping of *C. hirsuta* samples according to the treatment. The variables used for the PCA analysis were: photosynthetic pigments (sum of Chl a, Chl b, carotenoids), shoot DW, FW and DW/FW, root DW, FW and DW/FW, nodes number, leaf area. Treatments: Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd.

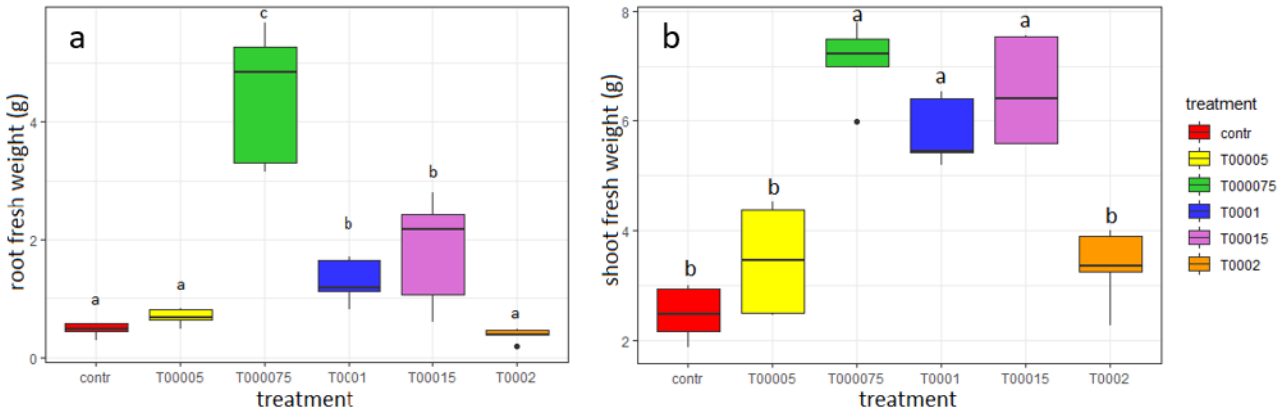

Figure 10. a) Root fresh weight of *C. hirsuta*. b) Shoot fresh weight of *C. hirsuta*. The letters indicate the statistically different groups. Treatments: Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd.

More in detail, the Cd 0.75 µM treatment showed a marked biomass increase of root fresh weight (Fig. 10a) with an average of 4.45 gFW, significantly different from other samples (*p<0.01*). Also an

increase of root biomass was pointed out in the 1 µM and 1.5 µM Cd treatments, which represented an independent group ($p<0.05$) with an average fresh weight of 1.55 g. The control and the 2 µM treatments were again similar and with an average root fresh weight of 0.51 g. The same trend could be noticed for shoot biomass fresh weight (Fig. 10b), where a significant difference ($p<0.01$) between the group composed by 0.75 µM, 1 µM, 1.5 µM Cd (average of 6.48 gFW) and the group composed by control, 0.5 µM and 2 µM treatments (average of 3.10 gFW) could be measured. Regarding the root DW/FW ratio (Fig. 11a), a significant difference was calculated between the Cd treatments 0.5 µM, 0.75 µM, that had an average of 2.2% ($p<0.01$), and the control 1 µM, 1.5 µM and 2 µM Cd treatments, having an homogeneous behavior with an average DW/FW ratio of 0.7%. The shoot DW/FW ratio (Fig. 11b) of the 0.75 µM sample was 14%, different ($p<0.01$) from the other treatment which showed a similar ratio of 11%.

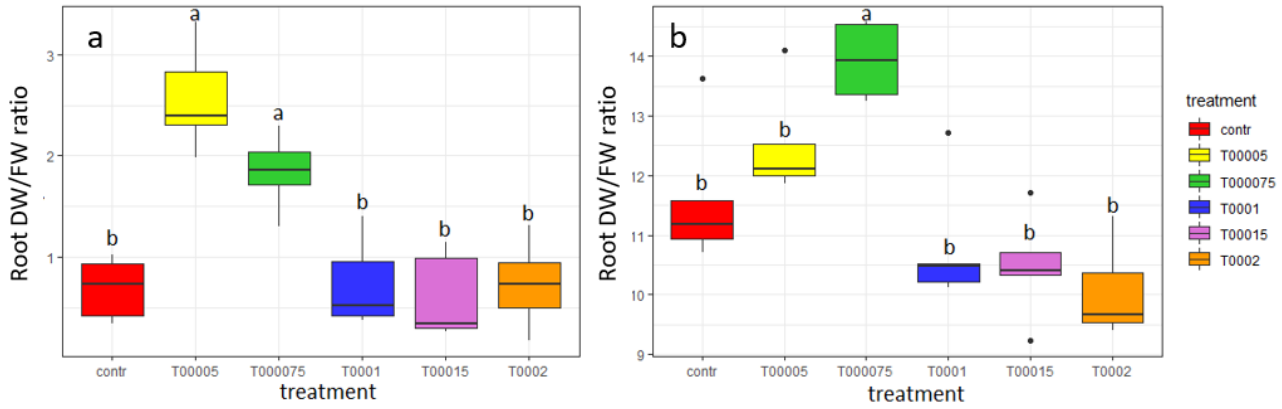

Figure 11. a) Root DW/FW ratio in *C. hirsuta*. b) Shoot DW/FW ratio in *C. hirsuta*. The letters indicate the statistically different groups. Treatments are: Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd.

As regards the number of nodes per plant at the fourth week of culture (Fig. 12a) the treatments 0.5 µM, 0.75 µM and 1 µM had an average nodes number of about 39 ($p>0.05$). This group was significantly different *($p<0.01$)* from the control and the Cd 0.2 µM treatment, which had a lower

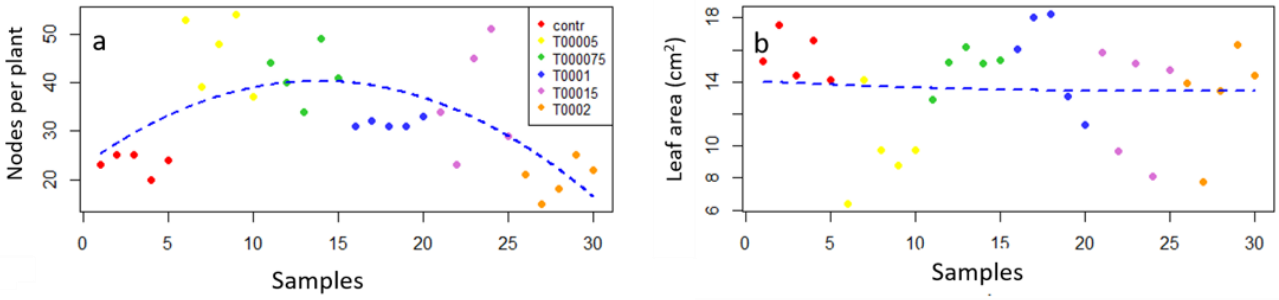

Figure 12. a) Number of nodes per plant in *C. hirsuta* at the fourth week of cultivation. b) Leaf area in *C. hirsuta* at the fourth week of cultivation. The blue dashed line describes the trend of the treatments with a non-linear model ($y=x+x^2$). Samples from: 0 to 5= 0 µM Cd (Contr), 6 to 10= 0.5 µM Cd (T00005), 11 to 15= 0.75 µM Cd (T000075), 16 to 20= 1 µM Cd (T0001), 21 to 25= 1.5 µM Cd (T00015), 26 to 30= 2 µM Cd (T0002).

number of nodes (22 nodes on average). Instead, data for the Cd 0.15 µM treatment were very variable, ranging between 20 to 50, for that reason no statistically significant differences with other plant data could be pointed out. For this variable, the non-linear model did not fit well since data were quite dispersed (p<0.01, R=0.3238), nonetheless a clear inverted U shaped curve was obtained (Fig. 12a). The data collected at two weeks of growth resulted instead similar (average of 7.5 nodes per plant) in all Cd treatments (p>0.05) therefore, for this trait the Cd effects were only measurable after four weeks. Cd treatments did not affect the leaf area neither after two nor after four weeks of culture (Fig. 12b), and no particular trend was evidenced. Also the ANOVA test did not highlight any statistically significant difference between treatments (p>0.05).

Similarly to leaf area, also for photosynthetic pigments no differences emerged among plant treatments except for the 0.5 µM Cd treatment, were photosynthetic pigments were slightly higher than in the other treatments (p<0.05) with 156.9 and 173.8 µg/g respectively.

5.3.3. Stellaria media

S. media did not show any hormetic response to Cd treatments, in fact was not possible to observe difference with regard to traits of biomass production, this can be observed also in the PCA graph (Fig. 13), that took into account traits measured at 4 weeks, chlorophyll content, FW, DW and DW/FW. The analysis showed that for *S. media* Cd treatments did not influence the different studied variables, since all the data groups were quite overlapped. In particular, the 2 µM Cd treated *Stellaria* was characterized by a decrease of biomass of roots an shoots and photosynthetic pigments amounts. The Cd 1.5 µM treatment resulted very

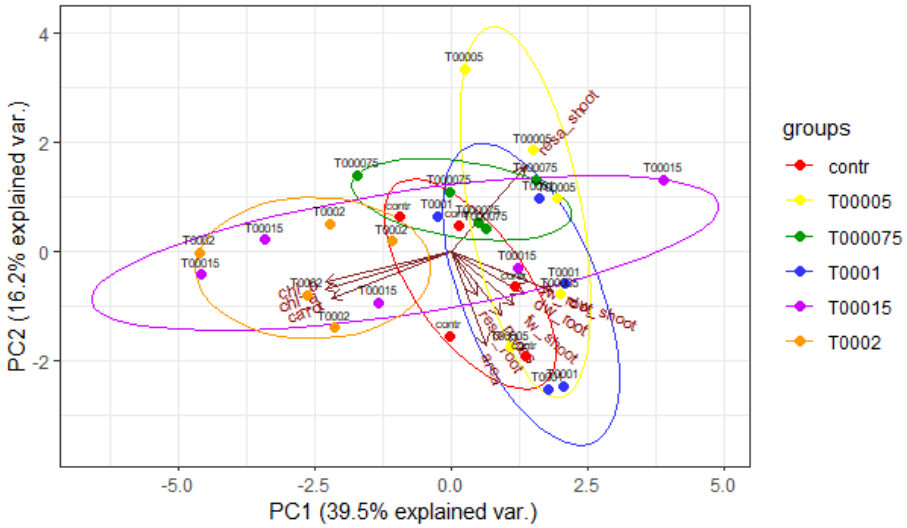

Figure 13. PCA showing the grouping of *S.media* samples according to the treatment. The variables used for the PCA analysis were: photosynthetic pigments (sum of Chl a, Chl b, carotenoids), shoot DW, FW and DW/FW, root DW, FW and DW/FW, nodes number, leaf area. Treatments: Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd.

variable and data overlapped with most of the other groups. The Cd 1 µM treatment was associated with higher biomass values.

The analysis of root fresh weight (Fig. 14a) showed that the Cd 1 µM treatment had a 52 % increase respect to the other treatments (p<0.01) with an average value of 11.2 gFW. The Cd treatments 0.5 µM, 0.75 µM, 1.5 µM had an average root fresh weight of 5.64 g, while the Cd 2 µM treatment had the lowest value (3.81 g). Shoot fresh weight (Fig. 14b) was similar for all Cd treatments (10.5 g on

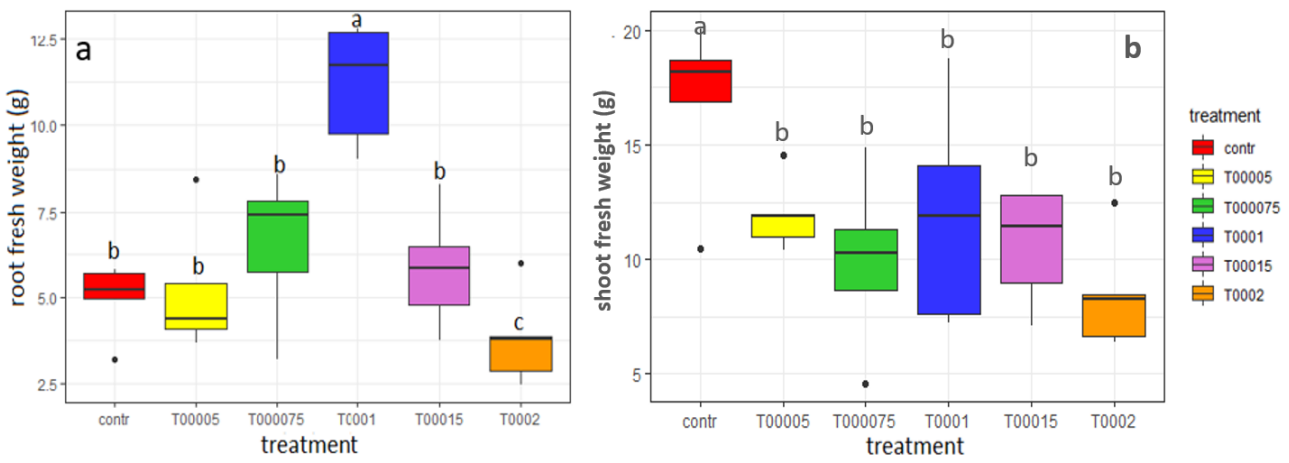

Figure 14. a) Root fresh weight of *S. media*. b) Shoot fresh weight of *S. media*. The letters indicate the statistically different groups. Treatments: Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd

average) and different from the control (p<0.05) (15.8 g on average). The lack of statistical differences among group of treatments (p>0.05), except for the control, was mainly caused by the high samples variability. Moreover, no hormetic curve is shown for shoot fresh weight.

The root DW/FW ratio (Fig. 15a) was very similar in all the treatments (p>0.05) because of the high

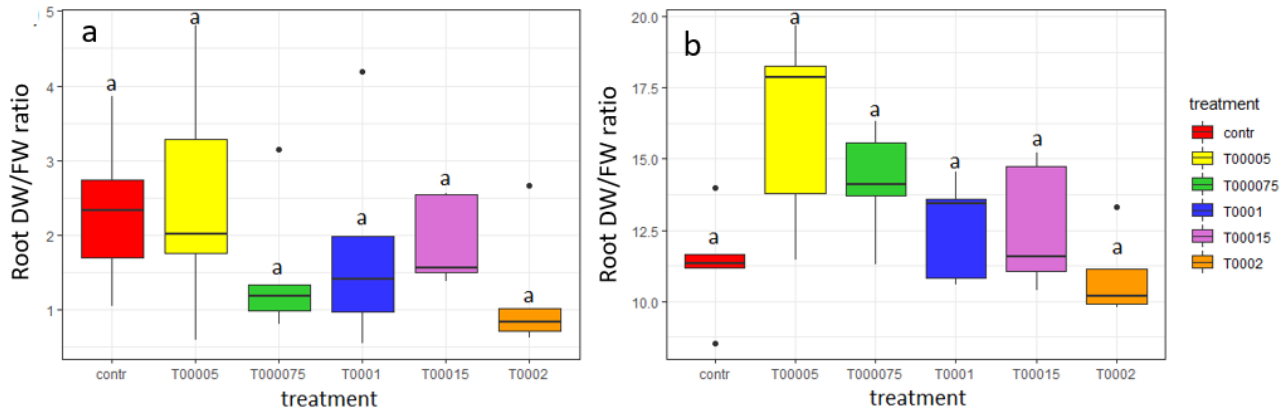

Figure 15. a) Root DW/FW ratio of *S. media*. b) Shoot DW/FW ratio of *S. media*. The letters indicate statistically different groups. Treatments: Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd.

dispersion of data, nonetheless as similarly shown by the PCA analysis (Fig. 13), the DW/FW ratio of Cd 2 µM treatment was lower, probably because of Cd toxicity.

The number of nodes per plant and the leaf area data at two and four weeks were not affected by none of the Cd treatments (*p>0.05*). The average values were 10 nodes per plant and 4.8 cm^2 of leaf area at 4 wees, and 5 nodes per plant and 2 cm^2 of leaf area at two weeks.

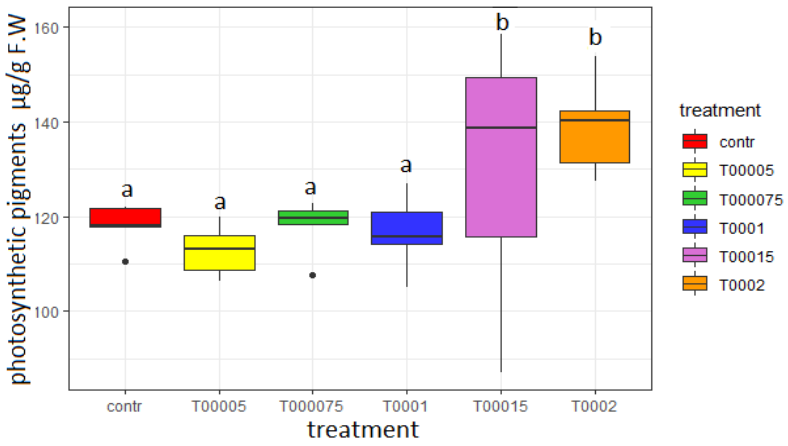

Figure 16. Total photosynthetic pigment content (sum of chl a, chl b, carotenoids) in shoots of *S. media.* The letters indicate statistically different groups Treatments Contr= 0 µM Cd, T00005= 0.5 µM Cd, T000075= 0.75 µM Cd, T0001= 1 µM Cd, T00015= 1.5 µM Cd, T0002= 2 µM Cd.

The results regarding the photosynthetic pigments levels (Fig. 16) showed two statistically different (*p<0.05*) groups of data: control, Cd 0.5 µM, 0.75 µM, 1 µM and 1.5 µM, had lower photosynthetic pigments content (84.9 µg/g FW) , while Cd 1.5 µM and 2 µM treatments, had an higher content (98.8 µg/g FW). Because all the parameters considered did not show an hormetic curve, we can consider *Stellaria media* not reactive to low Cd dosages.

5.4. Discussion

The present data demonstrated that micro-doses (from 0.5 to 2 uM) of Cd can induce hormesis at various degrees on the three studied species *P. annua, S. media* and *C. hirsuta,* grown in hydroponic culture (Figs. 5, 9, 13)

The hydroponic approach allowed to strictly control Cd concentration in the liquid culture media, avoiding biases due to metal sequestration or other reactions such as precipitation, sometimes happening when growing plants in soil.

Overall, plant biomass increase of both roots and shoots was the most glaring effect caused by cadmium. In *Poa annua*, a 130% gain in shoot fresh weight was observed (Fig. 6a, Fig. 6b) in the Cd treatments 0.5 µM, 0.75 µM, 1 µM, 1.5 µM if compared to the control and 2 µM Cd treatment. According to previously reported typical hormetic trend (Jia et al., 2015) showing an inverted U-shaped dose-response curve, the minimum biomass production was obtained at the highest and lowest Cd concentrations.

Previous data by Seth et al. (2007) on *Spirodela polyrhiza*, an aquatic plant of the Araceae family, analogously showed an increase in the total fresh biomass at Cd concentrations similar to those

used in the present research. The hormesis effect in *S. polyrhiza* started at 0.1 µM Cd, while the maximum biomass increase (+25%) was observed at 1 µM.

In *Poa annua* the root biomass increase was slightly higher if compared to shoot biomass increase (133.5% and 130% respectively) at hormetic Cd concentrations (5 µM, 0.75 µM, 1 µM, 1.5 µM) compared to the control.

The difference between root and shoot biomass increase, was much more marked in *C. hirsuta* in which, root biomass (Fig. 10a), increased of the 947 % in the 0.75 µM Cd treatment compared to the control and 2 µM treatment. Conversely, shoot biomass (Fig. 10b) increased of the 120%, for 0.75 µM, 1 µM, 1.5 µM Cd treatments, compared to control and 2 µM treatment.

These differences suggested that Cd effects were more marked on roots, probably as a result of a poor translocation towards shoots.

This fact was demonstrated, by Qiu et al. (2008), in a study on *Arabis paniculata* (Brassicaceae). In this plant, Cd concentrations in roots were three to five times higher than in shoots and the translocation factor was between 0.15 and 0.35. The effect of low Cd doses on the growth of *A. paniculata,* was also observed in hydroponic culture showing that, after three weeks of growth, the total fresh biomass increased from to 21% to 27% when treated with 22 to 89 µM of Cd respectively (Qiu et al. 2008). These data are in line with the Cd effects obtained in the present study despite for our species an icrease in biomass was shown with Cd doses around 0.75 µM.

The root fresh weight of *S. media* (Fig. 14a) followed a similar trend of the one observed for *P. annua* and *C. hirsuta* (with a slight increase at 1 µM Cd concentration). However, the shoot fresh (Fig. 14b) did not show any specific trend caused by Cd dosages.

These results, clearly demonstrated that some species are more sensitive than others to the presence of Cd in nutrient solutions, showing therefore different responses. Further data on the uptake and accumulation of Cd in these species can help in a better understanding of these different reactions.

Analogously to the present data, variable responses in different angiosperm species were detected by Xiong and Peng (2001) when investigating Cd stimulation of pollen germination and the pollen tube growth. In this study, only some species (*Pisum sativum* and *Plantago depressa*) showed the hormetic dose-response to Cd, while other species (*Vicia tetrasperma* and *Medicago hispida*), were not affected.

Shoot DW/FW ratio for *P. annua* was equal (about 16%) for all plant treatments with no Cd effect on this parameter, while a different situation was observed for roots. In fact, for *P. annua* the group

of treatments whith a higher biomass production (0.75 µM, 1 µM, 1.5 µM Cd) also showed a root DW/FW ratio (4.3%) when compared to control, 0.5 µM and 2 µM Cd treatments (DW/FW of 1.5%). For *C. hirsuta* the shoot DW/FW ratio (Fig. 11b) showed a sharp difference between the 0.75 µM Cd treatment (14%) and the other groups (on average 11%). Similarly, the root DW/FW ratio (Fig. 11a) in the 0.5, 0.75 µM Cd treatments, had a higher value (on average 2.2%) if compared to control and 1, 1.5, 2 µM Cd treatments (average of 0.7%). In *S. media* no significant difference between DW/FW ratio of Cd treatments and control samples was measured.

The shoot and root DW/FW ratio, could also give an important indirect information about carbon fixation capacity and water balance in the plant. In fact for *P. annua* and *C. hirsuta,* the increase in biomass, as consequence of Cd hermetic effect, seems to be driven by a major carbon fixation (higher DW/FW ratio) and not by a higher water retention (lower DW/FW ratio). In accordance to present results Hajiboland et al. (2013) reported that hormetic Al concentration (300 µM) induced in *Camellia sinensis* a biomass growth more pronounced in roots(74%) than in shoots (27%) as consequence of higher Al levels in roots than shoots. Also the net CO_2 assimilation rates increased following the rise of stomatal conductance and the transpiration rate caused by Al. In the present research, the quantification of Cd accumulation in different plant tissues is still in progress, but, despite that, the biomass data support the thesis of a differential distribution of Cd between root and shoots (root>>shoot for *C. hirsuta*, root> shoot for *P. annua,* root> shoot for *S. media*) as similarly reported by Hajiboland et al. (2013).

This study suggested that positive Cd effects on *C. hirsuta* and *P. annua*, can be obtained within a narrow range of concentrations strictly around 0.75 µM Cd. These hormetic concentrations cannot be considered constant, but vary widely among different metals and plant species. For instance, hormetic Cd concentrations between 0.1 to 1 µM, also enhanced *S. polyrhiza* growth (Seth et al., 2007) as similarly observed for *P. annua, C. hirsuta* and *S. media*, while in *A. paniculata,* this range is much higher (between 22 and 89 µM) (Qiu et al., 2008). Instead, other metals like Al which is less toxic for plants, showed beneficial effects when tested at higher concentrations (from 50 µM to 300 µM) on *C. sinensis* (Hajiboland et al., 2013).

Another positive effect of Cd was the increase in growth speed of *P. annua* and *C. hirsuta*. In fact, the number of nodes per plant in the treatments 0.5, 0.75, 1 and 1.5 µM Cd were double if compared to the control and 2 µM treatments (Fig. 7a, Fig. 12a), after 4 weeks of cultivation. Treated plants produced more nodes, hence more leaves and stems. Despite few studies have previously investigated nodes production, some other traits were instead quantified to demonstrate hormesis. Xiong and Peng. (2001) measured pollen germination and pollen tube length of different species.

They observed that, at concentrations of 0.0001 µg/ml and 1.58 µg/ml Cd, *Pisum sativum* and *Plantago depressa* pollen germination and pollen tube length increased respectively of 2-9% and 10-80%. Durenne et al. (2018) observed that root elongation stimulatory effect in *Brassica napus* was induced by increasing Cd treatments between 5 to 15 µM.

In the present study, Cd did not affect the photosynthetic pigments content of *C. hirsuta* and *S. media*, but had a negative effect on the production of chlorophylls a, b and carotenoids in *P. annua* (Fig. 8). We speculated that, despite the better performance in *P. annua* biomass production of Cd treated samples, photosynthetic pigments content decrease was caused by Cd toxicity. This effect was also detected in *Spirodela polyrrhiza* (Seth et al., 2007), where a reduction in photosynthetic pigments after 0.5 µM Cd treatment, was detected. Similary, Muszyńska et al. (2019) reported a chl *a* and *b* decrease of about 13-14% in the shoots of a non-metallicolous population of *Silene vulgaris,* cultivated for four weeks in the presence of 33 µM Pb. The reduction in photosynthetic pigments was detected only in *P. annua,* which is a metal-sensitive plant, while *C. hirsuta* (as many other *Brassicaceae* species) is to some extent tolerant to HMs. For *Stellaria media*, the effect of Cd did not negatively impact photosynthetic pigments content but instead was more reflected on shoot biomass (Fig. 14b). Nonetheless, even in the presence of a damage, the hormesis effect can still be observed. In fact, hormesis is the consequence of an over-compensatory response, which does not necessary recover completely all plant damages. This concept was also stated by Calabrese (2015a), who asserted that the plant response to a damage in one part may influence the growth of other sections. This means that, even if the plant fails in restoring a specific damage (i.e. plant photosynthetic pigments), the action undertaken could benefit other plant functions (Calabrese, 2015a).

The processes at the basis of this phenomenon are multiple, but in the case of HMs the dominant hypothesis involves the activation of the antioxidant system. Some scientists speculated that the low oxidative stress caused by HMs, can trigger hormesis. In fact, the production of ROS and the consequent production of antioxidant-related metabolites in a low-stress regime, could cause the over-compensatory effect (Poschenrieder et al., 2013; Hajiboland et al., 2013; Seth et al., 2007). In support of this hypothesis, a study conducted by Lin et al. (2007) on *Triticum aestivum* cultivated in presence of low Cd levels, showed stimulated plant growth but also reduced level of oxygen-derived free radicals in plant cells mainly due to an hyper-efficiency of the ROS scavenging system. In this study the enhanced activity of antioxidants enzymes was mainly detected for superoxide dismutase (SOD), catalase (CAT,), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR).

5.5. Conclusion

Cadmium is one of the most hazardous and ubiquitous soil and water contaminants generated mainly by human activities, nonetheless, if present at low concentrations, can have stimulatory effects on plant growth. In the present study on three weeds species grown in hydroponic culture, Cd concentrations between 0.5 to 1.5 µM were the most effective and in particular the 0.75 µM treatment showed the highest impact on plant growth.

The multi-species test carried out in the present study, demonstrated that hormesis effect is not only dose-dependent but also species-dependent. In fact, hormesis features were significantly evident only in two species (*P. annua* and *C. hirsuta*) out of three, since for *S. media* no significant changes of the measured parameters were observed. Therefore, hormetic reactions can be considered species-specific and could vary in intensity according to the species and metal used.

In standard growth conditions of light and temperature, low Cd concentrations led to an increase of biomass production and growth speed (calculated as number of nodes), showing at least for *P. annua* and *C. hirsuta,* the classical hormesis dose-response feature, for most of the investigated variables, and represented by an inverted U-shaped curve (Fig. 17).

Several practical applications could be foreseen for the hormesis effect from medicine to cell culture to food production, without neglecting the possible adverse effects of this metal. For this reasons further studies are definitely necessary to better unfold the mechanism of this complex phenomenon.

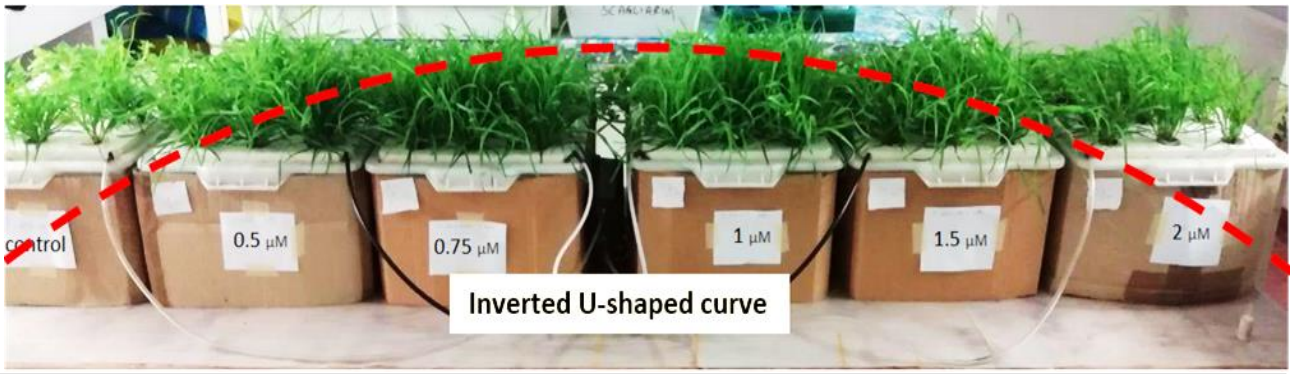

Figure 17. *P. annua* after four weeks of culture in hydroponic system at different low Cd concentrations. Plants treated with 0.75 to 1.5 µM showed a clear hormesis stimulatory effect. The red line represents the U-shaped curve that described the function shoot biomass in *P. annua.*

5.6. References

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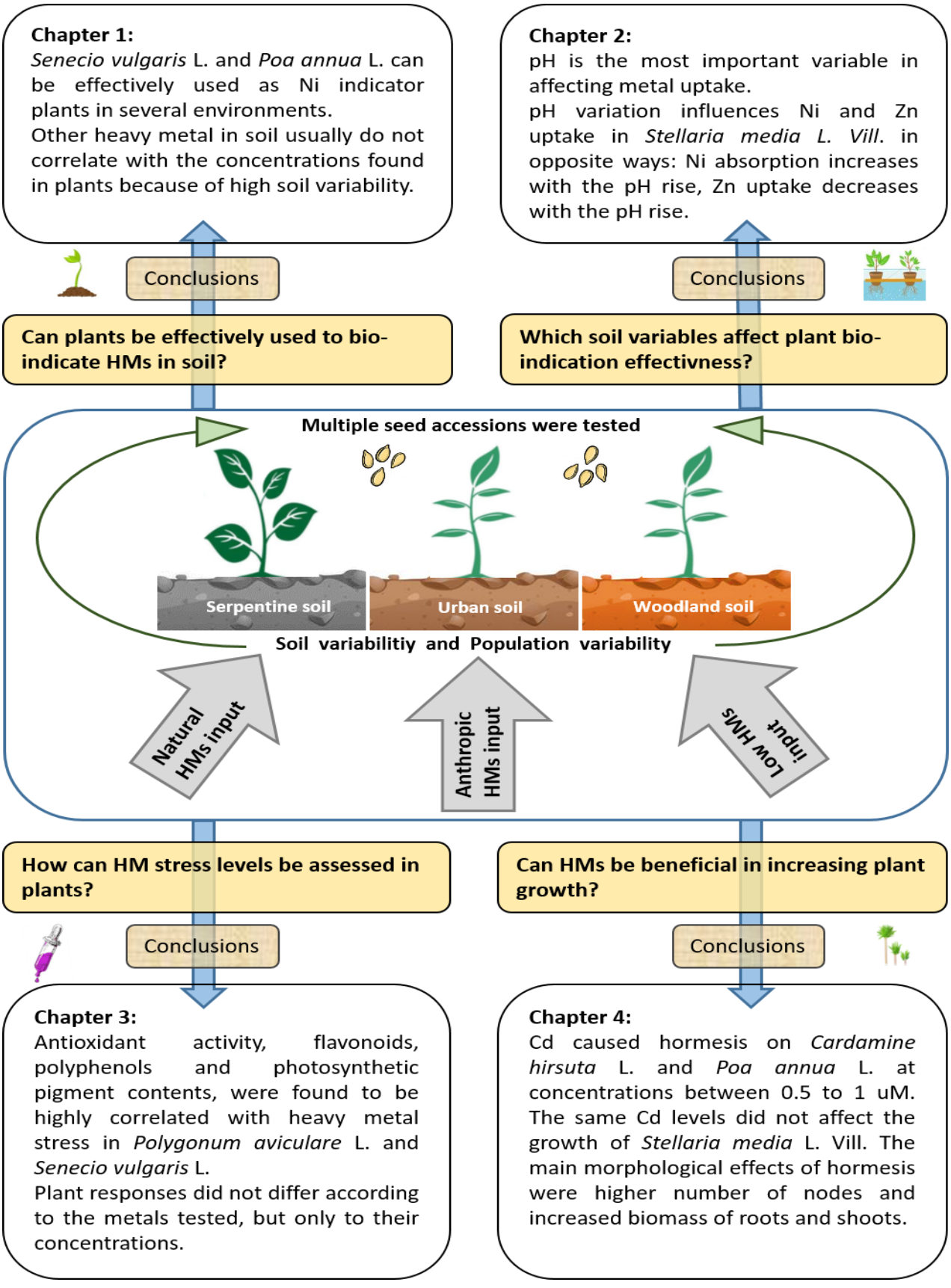
6. Final conclusions and future perspectives

The conceptual map below shows the former scientific question that guided each studied and the main conclusions we drew after the analysis of our results.



Within the present thesis, several aspects related to plants dealing with HMs demonstrated to be deeply interconnected. The four articles/chapters took into consideration only common weeds (*S. vulgaris, P. aviculare, P. annua, C. hirsuta, S. media*), capable of growing in a wide range of environments, from the most disturbed, like cities, to the least contaminated, like woodlands.
In the first chapter, our results showed that concentration of the six studied metals in soils seems in general not correlated with that in the shoots when plants are grown under natural conditions, with the only exception of Ni in *Poa annua* and *S. vulgaris*. This fact highlighted how soil properties and plant translocation capacity, are essential in determining the final shoot metal concentration, even more than the total metal concentration present in the soil.
Among the different factors influencing metal uptake from substrate to plant, pH seemed to be one of the most important. In the second chapter, by means of an hydroponic approach, *S. media* plants were subjected to variable pH values, keeping the concentration of Zn and Ni constant in the growing solution. The experiment results confirmed the primary importance of this parameter in determining the concentration of these two metals in *S. media* shoot and root tissues. In fact, pH was positively correlated with Ni uptake in hydroponic culture, conversely, Zn uptake was negatively correlated with pH. Results led to conclude that this variable must be taken into account during bioindication studies in order to avoid biases caused by a reduced or enlarged pool of available metals in the soil. When Zn and Ni were highly available to the plant, *S. media* also presented several toxicity symptoms and a correlated increased production of antioxidant substances (like flavonoids and polyphenols), which was proportional with Zn and Ni concentrations in shoots. On the contrary, biomass production and photosynthetic pigment content were supressed by increasing concentrations of metals. These correlations were the main subject of study in the third chapter, that aimed at verifying the possibility to evaluate and quantify HM-related stress and toxicity in plants using antioxidant metabolites, photosynthetic pigments and biomass as reliable markers. For this purpose *P. aviculare* and *S. vulgaris* were used as model species.
The study results demonstrated that these two species showed a linear uptake of Cd, Cr, Cu, Ni, Pb and Zn, when cultivated in hydroponic conditions in the presence of increasing metal concentrations, for this reason they can be considered indicator species, despite their behaviour on natural soil is strongly affected physical an chemical parameters. Morover, *P. aviculare* demonstrated to be more tolerant than *S. vulgaris* to high HMs levels suggesting its possible use for phytostabilization of HM polluted lands. In this study five different populations (2 urban, 2 woodland, 1 serpentine) for each species were tested, the differential HM accumulation and the

production of some antioxidant metabolites and of photosynthetic pigments among these populations, demonstrated (especially for *P. aviculare*) that some urban ecotypes seemed to show a better pre-adaptation to urban polluted soils.

Nonetheless, the main results of the third chapter demonstrated that antioxidant activity, flavonoid, polyphenol, photosynthetic pigment production and plant biomass were mostly correlated with the metal contents in plant shoots. In conclusion, these parameters could effectively be used as markers of HM stress in the tested plant species.

On the other hand, it has to be taken into account that oxidative stress caused by heavy metals is quite generic, and that the only presence of antioxidant metabolites cannot give information neither about the type of metal that caused the stress nor regarding the concentration of the stressor. In fact, a high dosage of poorly toxic metals may give similar effects than low doses of highly toxic metals.

Despite the deleterious effect that HMs produce on plants, (chlorosis, reduction in biomass, oxidative stress, etc.,) when present at very low concentrations in the growing medium, they could also exert beneficial effects. Some of them are in fact micronutrient for plants like Cu, Zn and Ni thus essential for the development of these organisms. Some other are non-essential nutrients, nevertheless, if present at low concentration the can enhance plant growth, by causing the so-called hormesis effect. In the fourth chapter, we demonstrated that Cd micro-doses (between 0.5 to 1.5 µM) were able to induce hormesis in *P. annua* and *C. hirsuta* cultivated in hydroponic conditions. Results demonstrated that hormesis effect was not only dose-dependent but also species-dependent. In fact, hormesis features were significantly evident in *P. annua* and *C. hirsuta,* but absent in *S. media* under the same growth conditions.

In general, low Cd concentrations led to an increase of biomass production and growth speed, showing, at least for *P. annua* and *C. hirsuta,* the classical hormesis dose-response feature represented by an inverted U-shaped curve. Several practical applications could be foreseen for this effect, from plant cell culture to increased plant production.

All the investigations carried out in this thesis contributed a little to the advancement of the state of knowledge related to plant dealing with HMs, but mostly demonstrated how complex and interweaved are the relations in the system soil-plant, with regard to which heavy metals are only a little component of the environment. The awareness of the infinite amount of relations and equilibriums that plants have developed with all the environmental features surrounding them (Fig. 1) must be the driving force for future studies aimed at furtherly unfolding this mysterious world.

Figure 1. An artistic installation by Michael Pederson in Sydney, who delights in creating humorous and thoughtful signs to highlight hidden elements of urban landscape like plants, in this picture a dandelion. *Image source* : https://www.thisiscolossal.com/2015/10/sydney-street-signs/

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