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PhD Thesis

Use of tree bark to study the distribution and concentration of trace elements in the atmosphere

XXX Cycle

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1. Introduction

1.1. Pollution: an old/new problem

Pollution has been seen as a critical problem since the introduction of the first industrial productions; examples of such concern can be found in the Alkali Act (1862) and the Rivers Pollution Act (1876) emitted by British Parliament in order to control the increasing level of pollution. These two Acts anticipated the Clean Air Act, emitted by UK Parliament in 1956, following the famous "Great Smog" of London in 1952. The deterioration of different ecosystems became critical especially during the second decade of the twentieth century, when the continuous development of the industrial processes led to the emission of continuously "new" or "emerging" contaminants, increasing the concern for environmental damages and for human health. Even though organisms were able to adjust, to a certain extent, to the presence of pollutants in their living environment, being able to control the uptake or to limit possible damages, this protecting effect is reduced in respect of trace elements (Kabata-Pendias, 2010). As a consequence of this concern, a drastic increase in studies about toxicity, both acute and chronic, of pollutants has been observed.

The term "pollutant" identifies a large number of substances which are released by human activities into the environment, where are commonly absent or present at levels far below those known to cause harmful effects. Holdgate (1980) proposed the following definition of "pollution": "*the introduction by man into the environment of substances or energy liable to cause hazards to human health,*

harm living resources and ecological systems, damage to structure or amenity, or interference with legitimate uses of the environment".

It is possible to identify two types of pollutants: *primary pollutants,* which are able to negatively affect the environment and have harmful effects in the form in which they enter the environment itself, and *secondary pollutants* which are a result of chemical and/or physical processes on precursors usually less harmful than the resulting products (Alloway and Ayres, 1997). The contaminants of anthropogenic origin enter the biogeochemical cycles within the environment but, having different characteristics (e.g. mechanism of transport, residence time, etc.) for each ecosystem, usually they have been studied separately according to the ecosystem interested (soil, water and air) (Alloway and Ayres, 1997).

Among pollutants, trace elements can be naturally present in the environment, but for many of them human activities have a significant impact on their concentrations, distribution, oxidation state and bioavailability. This type of influence can produce as a result a negative impact on the environment and on the human health, leading to the necessity of a serious monitoring programme to continuously study concentration and distribution of such pollutants. For this reason, this thesis project was focused on the study of the concentrations and of the distribution of trace elements, considering the particular relevance of their impact on human health and on the environment.

1.2. Atmospheric pollution and the importance of monitoring air quality

The atmosphere is mainly composed by nitrogen, oxygen, argon and carbon dioxide, along with other elements and compounds, some of them at trace level. The atmosphere has a strong influence on living organisms, absorbing UV radiations from sun, transporting water from seas to land and providing essential elements (i.e. oxygen). Unfortunately, the industrial development (especially those sectors related to incineration and smelting processes) and the increasing urbanization along with the constant demand of energy has led to increasing emission of various chemical substances that can react in the atmosphere producing a great variety of pollutants. The emission sources of pollutants can be essentially distinguished between *point* and *non-point* sources, meaning respectively an emission by a single source (e.g. the emission from an incinerator) and an emission from a diffuse source (e.g. the emissions from a highroad).

Airborne particles are in general referred to as aerosol; the dimensional range of those particles is between 0.0005 and 100 μm with an average diameter of about 1 μm. The particle dimensions are a key factor for residence time in the atmosphere, which can be up to 30–40 days; a longer residence time means that particles can be spread over long distances (Seinfeld and Pandis, 2016). The two main patterns, with which the trace elements present in the atmospheric aerosol are transferred to the ground, are essentially the *wet deposition* and the *dry deposition*. With wet deposition, the trace elements are encapsulated in the nuclei of forming drops within clouds and then transferred to the ground with rain, snow, etc. Dry deposition depends essentially on Stokes law for sedimentation and thus is

strongly related to particle dimensions; wind strength and direction also play an important role in dry deposition. This effect of transport over long distances was only taken into account after 1960s when it was realized that industrial and urban emission of pollutants negatively affected also remote areas far from anthropogenic source of pollutants (Bargagli, 1998).

Atmospheric particulate (referred as PM) is harmful for living organisms and, more important, for human beings. These harmful effects usually tend to be of a chronical form instead of an acute form, following a persistent exposure to low levels of complex mixtures of pollutants. Among all chemical contaminants, trace elements, and especially metals, play an important role in the outbreak of cardiovascular and pulmonary diseases (R. Chen et al., 2016) and in increasing cancer mortality (X. Chen et al., 2016; Pope et al., 2002). Moreover, they can interact with gaseous pollutants catalyzing chemical reactions, generating new pollutant species: as an example, Mn is able to catalyze the phase oxidation of SO_2 to SO_4^{2-} while Cu, Zn and Mo are able to increase the production of HNO_2 from NO_2. It is therefore important to be able to control the emission of possible harmful compounds and, in case, to adopt drastic actions if the concentration of such substances reaches high levels. Toward this goal several normative and reduction emission policies have been adopted all around the world. In addition to the well-known contaminants, the emission of new pollutants presents a continuous challenge in controlling air pollution and air quality, which indeed is a crucial factor for human beings (WHO, 2013).

1.3. Bioindicators: an effective tool for air pollution monitoring

Nowadays air quality is assessed by means of monitoring stations, located in specific points within the area of interest. However, a relatively new (the first studies trace back to the early ’60s) and effective tool for air pollution monitoring is the use of “*bioindicators*”. This term can assume several meanings, but for the purpose of this study, we refer to bioindicator as an organism, or a part of it, in which the concentration of a specific trace element reflects the concentration of the same trace element in the environment. Bioindicators can be divided into two categories according to their origin:

i) Animal bioindicators (such as fishes, mussels, sponges, etc.)

ii) Vegetal bioindicators (such as lichens, mosses, plants, etc.)

Among vegetal bioindicators, it is possible to identify different typologies of bioindicators according to the mechanism of the uptake of pollutants:

i) Excluders: are able to prevent the absorption of specific ions thanks to avoidance mechanism, thus are not properly suitable for monitoring purposes (Bargagli, 1998).

ii) Indicators: are able to take advantage of different mechanisms, such as chelation, chemical inactivation and localization, to accumulate trace elements. Bioindicators of this type are extremely useful for biomonitoring purposes, as the concentration of trace elements in the organism reflects the bioavailability of the same element in the environment (Bargagli, 1998).

iii) Accumulators: are able to accumulate and tolerate high concentrations of trace elements, regardless of the physiological role and environmental abundance (Bargagli, 1998).

Thus, biomonitors are able to reflect the stress and the quality of the environment. This feature is the basis on which biomonitoring campaigns are afforded: to verify the quality of the environment in order to grant good environmental quality for people life.

The continuous use of biomonitors in environmental studies has led to the identification of the main characteristics that a good biomonitor should have to be effectively used:

i) Widespread in the geographical area of interest
ii) Abundant in the area of the study
iii) Easy to find and to sample
iv) Adequate for the determination of the selected pollutants
v) Stable to environmental changes
vi) Present, at least, during a year-long period
vii) Able to accumulate pollutants in such a way that it reflects their atmospheric concentrations

Among vegetal bioindicators, lichen and mosses have been extensively used and studied for biomonitoring purposes.

Lichens are a symbiotic association of fungi and green or blue-green algae; the algal component of the lichen is called *photobiont*, while the fungal part is called *mycobiont.* The symbiotic association is essential for lichen life in various

environments, included those characterized by extreme conditions (i.e. alpine and polar conditions). The fungus receives from the algal partner all the essential nutrients (e.g. carbohydrates) for growth and reproduction, but provides itself essential nutrients (nitrogen, phosphorous, etc.) taken from the environment; moreover, the fungal hyphae are able to protect the algal partner against the extreme environmental conditions, such as light, heat and low temperatures. Lichens are characterized by the absence of waxy cuticles and organs for the absorption of water and minerals, thus they largely depend on gaseous exchange and on deposition (both wet and dry) from the atmosphere for nutrient absorption (Bargagli, 1998).

The lichen uptake mechanisms are essentially three:

i) Extracellular ion exchange: takes place mainly in the thallus thanks to rapid passive process depending on reversible binding to anionic sites in cell wall and surfaces in the plasma membrane.

ii) Intracellular accumulation: mainly due to selective uptake and carrier mediated uptake.

iii) Particulate trapping: due to lichen ability of trapping PM in the intercellular spaces of medulla (an intermediate layer between the cortex and the photobiont zone).

Given the widespread diffusion of lichens it is possible in most cases to sample them directly in the area of study, but it is also possible to adopt the technique of *transplant*, which means that lichens are sampled in a non-polluted area or in an area with very low and known concentrations of pollutants and transferred to the

area of interest for biomonitoring. Moreover, it is also important to know the background concentration of trace elements in lichen in order to be able to really identify the amount of trace elements originating from anthropogenic emissions.

Bryophytes, (e.g. mosses), differ from higher plants because of the absence of roots and ways of storing water internally, thus they can be considered poikilohydric (Bargagli, 1998). As for lichens, mosses depend on wet and dry deposition for the absorption of nutrients and the main uptake processes are ion exchange, chelation and complex formation. Moreover, the lack of proper conducting tissues along with the neglectible absorption of trace elements from the substrate, due to the absence of roots, makes mosses viable bioindicators (Onianwa, 2001). Given the enormous differences among moss species it is very important to carefully consider species-specific peculiarities in order to choose the correct bioindicator.

Along with lichens and mosses, specific parts of higher plants, especially leaves and bark (both from trunk and from branches), have recently been used for biomonitoring.

Leaves are directly exposed to wet and dry deposition, but their characteristics play an important role for the accumulation processes. Leaves characterized, as far as possible, by large surface area, horizontal presentation and rough hairy cuticles should be preferred as biomonitors if compared to leaves with other characteristics (Bargagli, 1998).

One concern of using tree leaves is the possible contamination by soil-derived trace elements; in fact, higher plants can absorb trace elements from the roots and then translocate them to the above ground parts, including leaves. Thus, it is very

important to know the contribution of the root uptake and the contribution of soil-derived trace elements, which should be subtracted from the total concentration measured. Moreover, the possibility of dust removal from leaves due to heavy rain is also an aspect that should be taken into account when working with leaves.

Tree bark is composed by suberized dead cells, forming a multilayer tissue; every year a new layer of dead cells is added isolating the previous one from the external environment. Even if the uptake mechanism of trace element absorption has not been fully understood, dry deposition and impact of windborne particles have been hypothesized as the main mechanisms. Moreover, tree bark does not present the problem of the root uptake, or it can be considered negligible (Catinon et al., 2008, 2011); this characteristic limits the possible soil contamination and allows to measure directly the concentrations of atmospheric trace elements.

The above examples of bioindicators show that it is possible to use an alternative method for air quality assessment along with common monitoring stations. The advantages of the use of bioindicators are a bigger number of collectible samples, the widespread diffusion, both in urban and industrial sites, which allows a better spatial resolution, the ease of the sample collection and the cost saving analytical technique. Thus, it is possible to integrate the data collected by monitoring stations with those obtained from bioindicators in order to better characterize the studied area and to achieve a more detailed knowledge of the distribution and concentration levels of pollutants.

For this PhD project, different bioindicators for the determination of a number of trace elements were selected:

i) Tree trunk and tree branches bark of holm oak (Q*uercus ilex* L.*)* sampled in the city of Genoa, north western Italy;

ii) Tree trunk bark of *Jacaranda mimosifolia D. Don*, collected during a three month stay as visiting PhD student, in the city of Lisbon (Portugal);

iii) The lichen *Cetraria islandica* L. for a study on trace element translocation in herbal preparation.

2. Urban and industrial contribution to trace elements in the atmosphere as measured in holm oak bark

This chapter refers to the published article: *Urban and industrial contribution to trace elements in the atmosphere as measured in holm oak bark.*
Giuliana Drava, Daniele Brignole, Paolo Giordani, Vincenzo Minganti. Atmospheric Environment 144 (2016) 370-375.

2.1. Introduction

As reported by the WHO, air quality plays an essential role in human well-being and thus continuous efforts towards its improvement are pursued.

Different types of chemicals are an integral part of the bigger class of air pollutants; among them metals play an important role in disease outbreak and have quickly become a serious health threat, even at trace level. Thus their presence in the atmosphere, due to natural and anthropogenic sources, has been widely investigated (Nriagu, 1979; Pacyna and Pacyna, 2001).

Nowadays Particulate Matter (PM) has raised more attention, given the already proven adverse effects that it can have on human health; thus monitoring programmes have been increased and the monitoring networks implemented. Unfortunately, not all the monitoring station are equipped with suitable instruments for PM analysis or are able to asses PM chemical composition; this situation could lead to a reduced quality of the information provided by the monitoring network because it is not possible to highlight which component of PM, i.e. trace elements, NO_x, etc., has the bigger impact on the human health and the environment. On the other hand, bioindicators, exposed to different contaminants, can provide an efficient complementary and/or alternative strategy to monitoring stations.

Many studies have proven the usefulness and the effectiveness of tree bark as a bioindicator, capable of providing information on the levels of trace elements in the atmosphere (Cucu-Man and Steinnes, 2013; Faggi et al., 2011).

In this project, samples of tree bark of holm oak (*Quercus ilex* L.*)* were collected in different areas of the city of Genova (N.W. Italy) and analyzed to study the concentrations of As, Cd, Co, Cu, Fe, Mn, Ni, Pb, V and Zn.

In the city of Genoa, holm oak is commonly widespread both along roads and in urban green areas, thus its sampling allows obtaining samples which are effectively representative of the whole city.

The choice of the trace elements was made according to their impact on human health and the possibility to achieve good accuracy and reproducibility from the analytical method; moreover, the sensitivity of the analytical equipment was also taken into account.

Given the peculiar characteristic of the presence of industrial settlements nearby residential houses in some districts of Genova, the main objective of this project was to obtain information about the trace element deposition in different urban environments, in order to highlight the impact that industrial activities may have on people living in the surroundings of industrial facilities and to differentiate between residential and industrial emission sources.

2.2. Materials and methods

2.2.1. Holm oak bark as a bioindicator

Tree bark has been extensively used as a bioindicator for biomonitoring purposes (Berlizov et al., 2007; Böhm et al., 1998; Schelle et al., 2008). The bark of holm oak, *Quercus ilex* L. (Fagaceae), was chosen as a bioindicator for this study.

Holm oak, an evergreen tree with a massive, rounded crown, is commonly widespread in the Mediterranean region and can be found over a large altitudinal range (from 0 to 2000 m a.s.l.), depending on the latitude. Moreover, other than being present as a native tree in mixed forests, it is also common in urban environments, along streets and in parks. Holm oak is characterized by a finely square-fissured tree bark, produced by the activity of a secondary meristem, which leads to the production of a protective and multilayer tissue formed by dead suberized cells (cork) (Minganti et al., 2016). Since that the layers are considered non-shedding, they can be considered persistent for several years (Quilhó et al., 2014).

2.2.2. Sampling campaign

The samples (n = 56) were collected from the trunks of trees in the city of Genoa (N.W. Italy). The city lies on a tight coastal belt (approximately 30 km long), delimited to the north by hills with an average height of 750 m a.s.l., situated between 6 and 10 km from the sea. Genoa is one of the most important ports of the Mediterranean and ranks 15th in Europe, with over 2 million containers and 52 million tons of goods exchanged in 2014.

The sampling area was planned in order to cover the whole city, which has been divided, for the purposes of this study, in two different areas: the western part and the central-eastern part.

The western part of the city (34 samples) is characterized by the proximity of the residential area to some important industrial activities. In fact, this area includes a power plant (coal and heavy fuel oil, 295 MWatt) and it is characterized by intense port activities, due to oil and cargo terminals and ship construction repairs. Moreover, electrical engineering, electronics and petrochemicals industrial facilities, together with the related transport infrastructures (railway, highways, and airport) are also present in this area.

The district of Cornigliano, in which in the 1950s a steel plant was built, is an integral part of the western area of the city. Following the steel crisis, the coke oven and the blast furnace were respectively closed in 2002 and 2005. From 2006 to 2011, the plant was partially dismantled. Simultaneously with the closure of the industrial facility several studies regarding pollution in that area (Prati et al., 2000) and its effects on human health (Parodi et al., 2005) were performed.

The central-eastern part of the city (22 samples) is mainly characterized by residential, office and commercial settlements and there are no important industrial activities in the area; a part of the port is also present but the maritime traffic takes place in the western part.

The map in Figure 2.1 shows the sampling sites and the main industrial and port activities present in the sampling area.

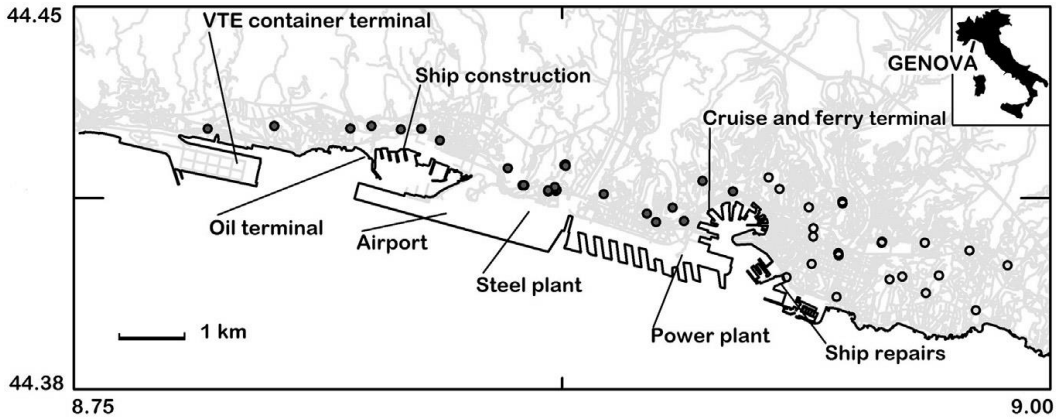

Fig. 2.1. Sampling sites of holm oak bark in Genoa. Filled circles correspond to industrial-residential sites; empty circles correspond to residential sites. The main industrial activities are also indicated.

The tree bark, with an average thickness of 4 mm, was removed at a height ranging between 1.5 and 2.0 meters from the ground, both along roads (less than 5 m from road edge) and in urban green areas. The height from the ground is an important variable to be considered because of the possibility of soil dust contamination (Schelle et al., 2008). For each tree, the geographical coordinates were recorded with a GPS system, to be able to exactly associate the position of the sample to the related area. The estimated age of the trees, based on the method of Panaïotis et al. (1997), which considers the diameter of the stem at the height of the bark sample collection as a factor in a second order polynomial equation, was ranging between 50 and 100 years.

2.2.3. Sample preparation

The samples collected were taken to the laboratory and any extraneous material was manually removed; each sample was freeze-dried and homogenized in 25 mL Teflon grinding jars with a 10 mm zirconium oxide grinding balls using a MM 2 Mixer Mill (Retsch, Germany). Removing water is an important step of the sample

preparation, given the high variability in water content observed; bark collected during dry period showed a mean water content of 10%, while samples collected during a rainy period had 33% of mean water content; the range was from 4% to 54%. For this reason, concentrations are reported as µg g^{-1} dry weight (d.w.). For each sample, given the wide availability of trunk bark, an adequate amount of dry matter (all samples analyzed in duplicate or triplicate) was obtained and analyzed without any further treatment.

2.2.4. Chemical analyses

About 0.10-0.15 g of the grinded samples were mineralized using 5 mL of 65% (m/m) nitric acid (for trace metal analysis from Scharlau, Spain) in closed Teflon PFA vessels by means of a MDS 2000 (CEM Corporation, USA) microwave digestion system. After a cool down period of about one hour the solutions were transferred in 25 mL volumetric flasks and diluted to volume with ultra-pure (>18 MOhm cm) water (Elgastat UHQ, Elga). All the glassware material used was accurately cleaned with 3 M nitric acid and rinsed twice with ultra-pure water.

The concentrations of the trace elements were measured by atomic emission spectrometry with an inductively coupled plasma source (ICP-OES) using an iCAP™ 7000 Series (Thermo Scientific, U.K.). The ICP-OES instrumentation mounted a concentric nebulizer, where the expanding gas carrier (argon) converts the liquid in an aerosol and brings it into a cyclonic nebulizer chamber. The droplets of correct dimension are taken to the plasma torch, while the others are discharged from the bottom of the chamber (Fig. 2.2). For achieving a better sensitivity, axial plasma view was selected; in this instrumental configuration, the

plasma is viewed along the analyte channel, resulting in a higher viewing volume if compared to radial viewing. Furthermore, this configuration improves the signal/background ratio and allows to detect lower concentrations (Nölte, 2003).

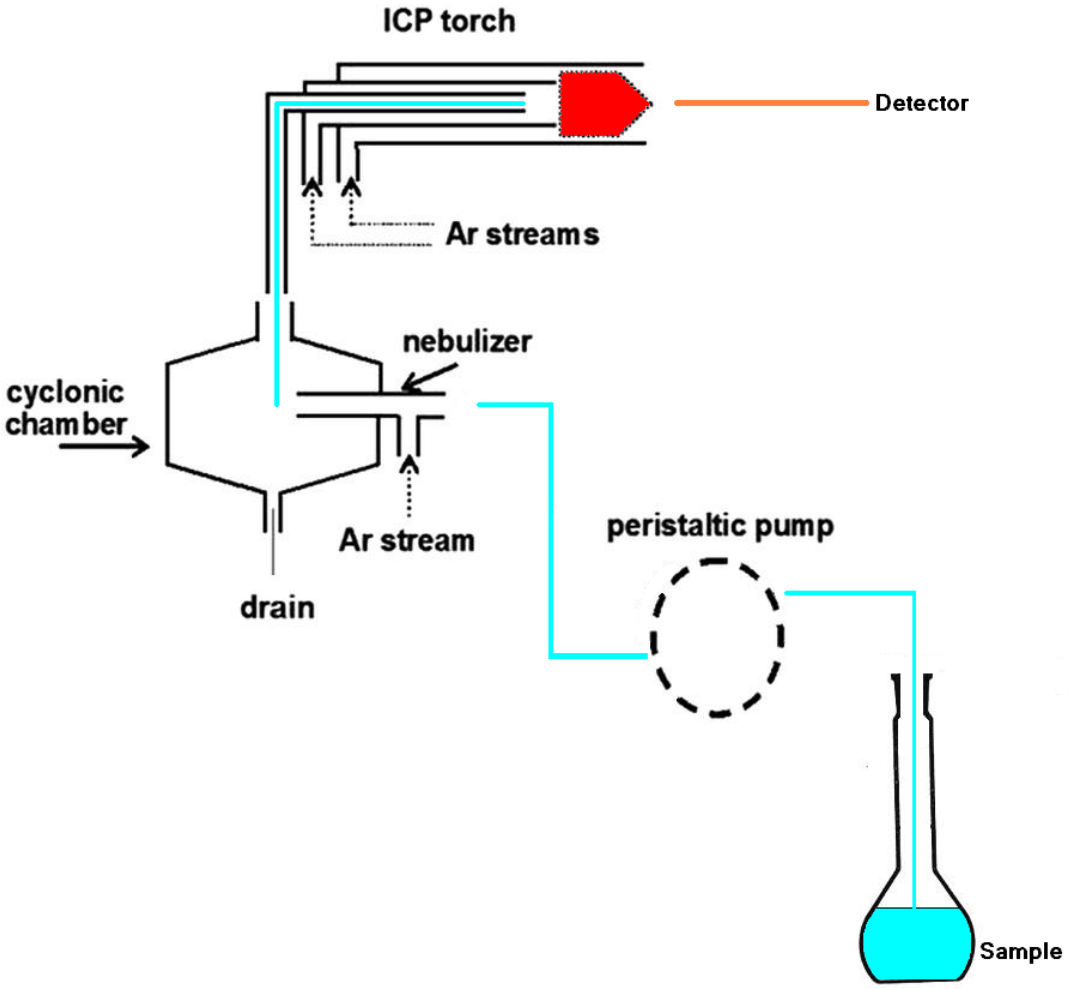

Fig. 2.2. Scheme of ICP-OES instrumentation with horizontal plasma torch and axial viewing (modified from Royal Society of Chemistry).

2.2.5. Method development

Calibration was carried out using aqueous standard solutions in 3 M nitric acid with scandium at 4 µg mL^{-1} as internal standard. The use of standard solutions is based on the matrix matching theory, thus matching the standard solutions to the sample solutions; this helps in reducing non-spectral interferences. Given the impossibility to obtain an exact match between the standards and the samples, an internal standard was also added. The use of the internal standard is crucial for the correction of the non-spectral interferences, since it is possible to use the effect of non-spectral interferences on the internal standard as a correction factor for the analyte signals. Usually an element which is supposed not present in the samples, soluble in sample solution and calibration solution and not causing spectral interferences, is chosen as internal standard and added during sample preparation (Nölte, 2003). Moreover, it is worth noting that also the concentration of the acid solution used could have an influence on the aerosol characteristics, the sample uptake rate and, in the end, on the intensity of the peaks (Todolí and Mermet, 1999). For each trace element investigated, the adequate wavelengths were selected according to the information reported in literature. Moreover, the final wavelength selection was made based on the following criteria:

i) A small Background Equivalent Concentration (B.E.C.), considering that the smaller the value of the B.E.C. the bigger the sensitivity for the selected analytical line.

ii) The intensity of the analytical line chosen, for this parameter the bigger the value the bigger the sensitivity (Nölte, 2003).

The concentration values obtained were then reported as a mean value of the results obtained. For each run (10 samples), two blanks were analyzed in order to track any possible contamination.

2.2.6. Quality control for chemical analyses

Quality control (QC) has proven to be a key factor in monitoring industrial processes but it has been extensively used also at laboratory level, to monitor chemical processes and analytical procedures.

For this study, the accuracy was assessed by analyzing a certified reference material (CRM), the CRM 482 (Lichen), certified by the European Commission – Joint Research Centre – Institute for Reference Materials and Measurements (IRMM). The results of the quality control process are reported in Table 2.1.

Table 2.1. Quality assurance. The results obtained for the Certified Reference Material CRM 482 (European Commission – Joint Research Centre – Institute for Reference Materials and Measurements, IRMM), expressed as µg g^{-1} d.w., are compared with the certified or reference concentrations. The values found are reported as mean ± standard deviation (n = 7).

Element	Certified µg g^{-1} d.w.	Found µg g^{-1} d.w.
As	0.85±0.07	0.90±0.17
Cd	0.56±0.02	0.57±0.01
Co[a]	0.32±0.03	0.35±0.02
Cu	7.03±0.19	7.19±0.35
Fe[a]	804±160	734±33
Mn[a]	33.0±0.5	29.3±0.8
Ni	2.47±0.07	2.49±0.09
Pb	40.9±1.4	40.0±1.7
V[a]	3.74±0.61	3.73±0.21
Zn	100.6±2.2	103.6±0.6

[a] No certified data available and the reference concentrations are reported.

The quality control process has been performed for all the three years, so quality charts have been drawn to monitor the recovery profile for each trace element analyzed. Figures 2.3 and 2.4 report as an example the charts for Cd and Fe, and for V and Zn, respectively. Control charts, developed by Dr. Walter A. Shewhart in 1920, are a graphic visualization of the quality of the characteristic measured. In Shewhart QC charts the limits of the quality analysis are defined as follows:

i) Upper Control Limit (UCL)= +3s

ii) Lower Control Limit (LCL)= -3s

The analysis is considered acceptable and corrected if the results fall in the range defined by UCL and LCL; a single value outside the defined range is still acceptable, considering it as an outlier.

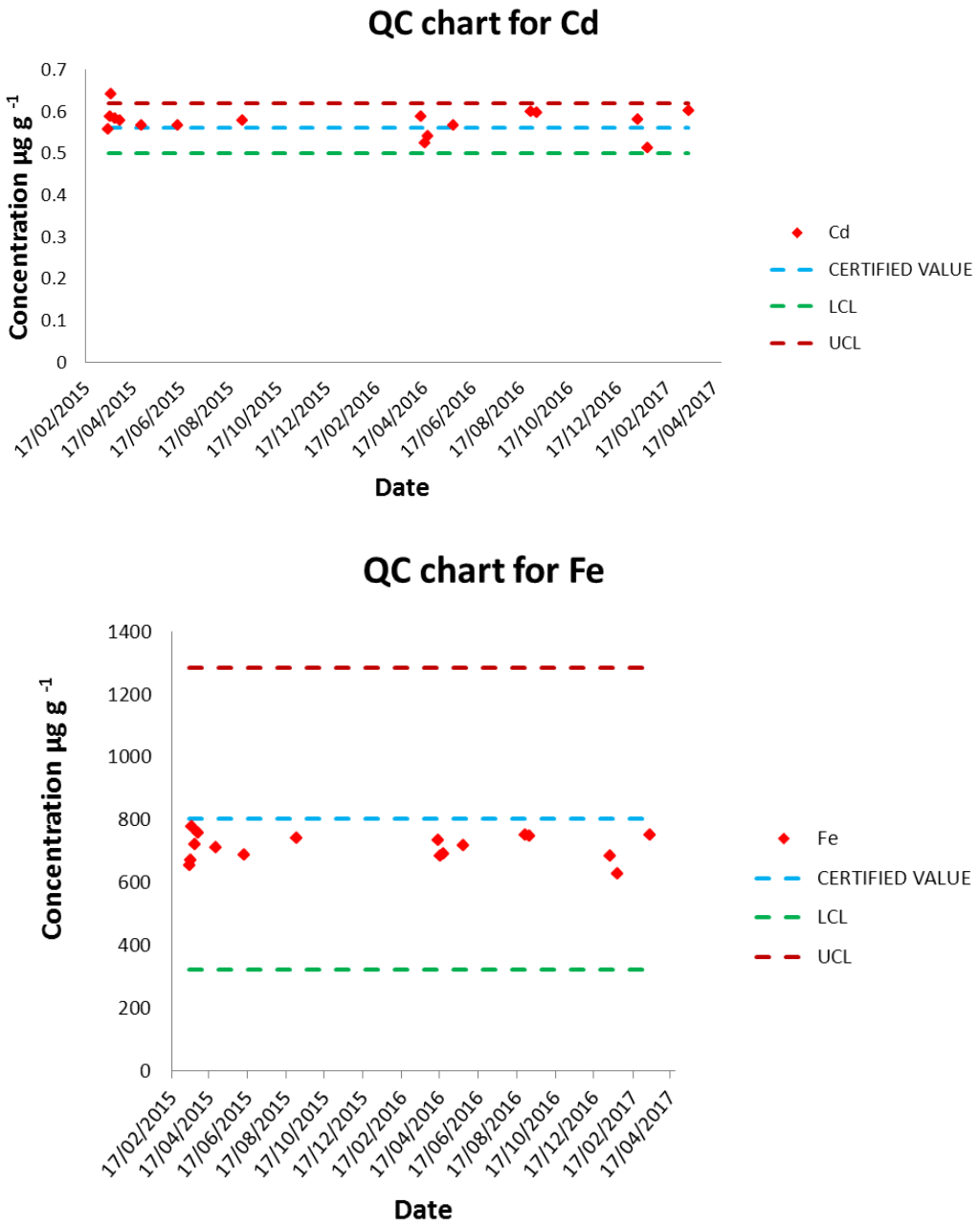

Fig. 2.3. Quality control charts for Cd (top) and Fe (bottom).

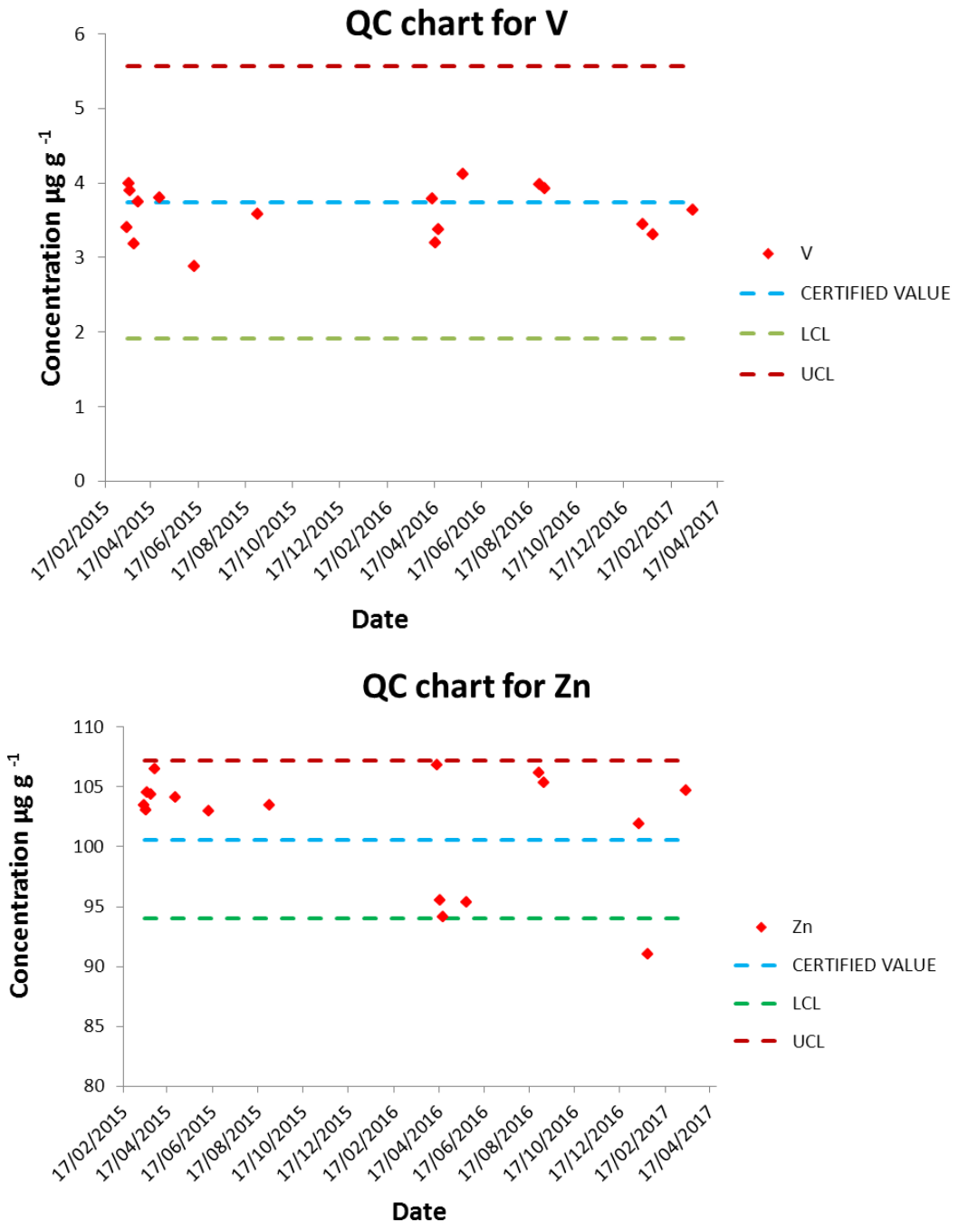

Fig. 2.4. Quality control charts for V (top) and Zn (bottom).

From the above graphs it is possible to see the good accordance between the certified values and our experimental results, obtained from the analysis of the certified material; all the results were inside the range defined by the upper control level and the lower control level. The experimental values for Zn and for Cd were

more spread around the certified value, this is due to the difficulty that arises because of the contamination problems related to the laboratory air quality; such problem could only be solved by working in a laboratory with an adequate air filtering equipment. Nevertheless, even though one value was outside of the range, the results were considered acceptable, following what previously reported.

2.2.7. Data analyses

Since the samples were collected both in urban parks and along roads, the data obtained were analyzed for any significant effect of tree location applying a t-test. Moreover, a t-test was applied to study any relevant distinction between the two areas of the city, western part and eastern part, in terms of trace element concentration and distribution; when the difference between the two areas was significant, the ratio of the concentration was calculated. A Principal Component Analysis (PCA) (Jackson, 2005) was performed as exploratory multivariate statistical technique in order to evaluate the presence of outliers and/or groups of similar samples.

2.3. Results and Discussion

Table 2.2 shows a summary of the results (mean value, µg g^{-1} d.w., standard deviation and range) for each trace elements analyzed. Samples were collected both along roads (n= 43), and inside urban green areas (n= 13); the t-test performed to study any possible effect of tree location, showed non-significant difference ($p<0.01$) between trees sampled in green areas and along roads. A PCA analysis was run on the same data set, highlighting the presence of two outliers, which were consequently removed.

Tab. 2.2. Descriptive statistics (mean ± standard deviation and range, expressed in µg g^{-1} d.w.) of trace element concentrations in holm oak, bark (*Quercus ilex* L.) collected in Genoa, Northwestern Italy.

Element	Concentration µg g^{-1}d.w.
As	0.58 ± 0.35 (0.10–2.10)
Cd	0.47 ± 0.29 (0.08–1.23)
Co	1.07 ± 0.63 (0.17–2.90)
Cu	44.06 ±33.86 (11.41–164.25)
Fe	1697 ± 1477 (160–6730)
Mn	131.6 ± 95.3 (15.6–380.5)
Ni	8.78 ± 6.11 (1.01–31.38)
Pb	64.4 ± 61.8 (4.7–344.9)
V	6.03 ± 4.25 (0.41–23.93)
Zn	120.6 ± 93.8 (18.4–466.2)

One of the aim of this project was to study any significant difference in the concentration of the trace elements analyzed between the two main areas in which the city was divided. When a significant difference was founded (t-test, $p < 0.01$), the ratio of the concentrations measured was calculated (Fig. 2.5).

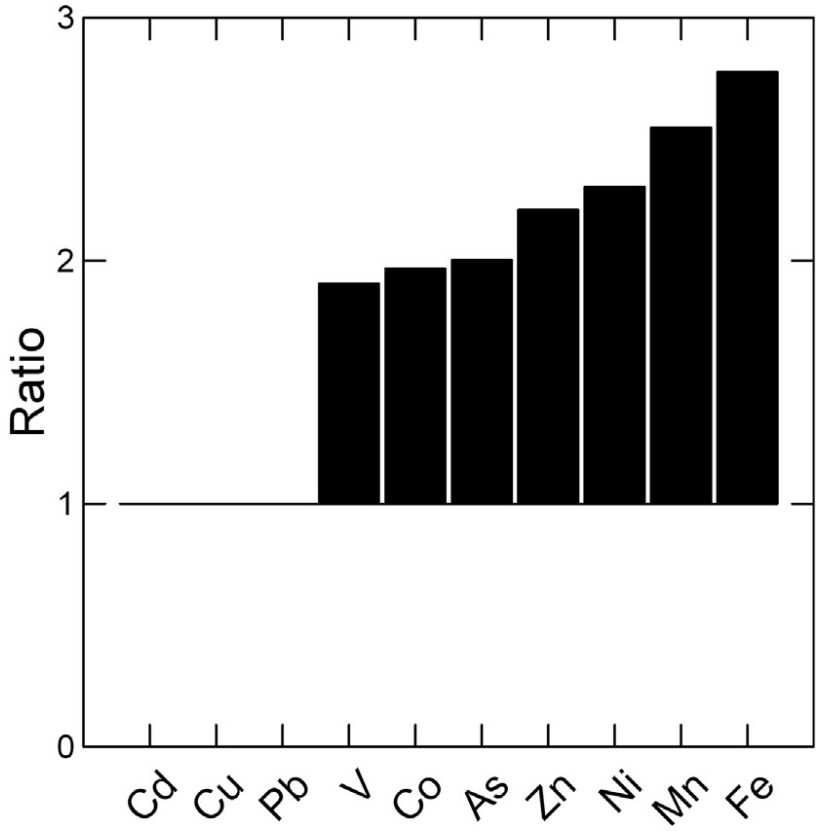

Fig. 2.5. Ratio between the mean trace element concentrations measured in the western area and those measured in the central-eastern area. For non-significant differences at $p < 0.01$, the ratio is reported as 1.

The concentration of As, Co, Fe, Ni, Mn, V and Zn measured in the western part of the city resulted to be twice (from 1.9 to 2.8 times higher) than those found in the central-eastern part. No significant distinction in the concentration levels of Cd, Cu, Pb was detected from the analyses performed.

The presence of Pb can be related to the use of leaded fuels and thus to traffic emissions (Werkenthin et al., 2014), although Pb has been removed from fuels since 2001. Several authors (Werkenthin et al., 2014; Zechmeister et al., 2005) have also related the emission of Cu to traffic and vehicular emissions; thus, considering the two areas of the city comparable in terms of vehicular traffic, it seems logical that no difference occurs for these element concentrations.

For Cd there are divergent opinions on considering it a traffic related element or not; Tanner et al. (2008) related Cd emission to the wearing of brakes and diesel engines, while Galal and Shehata (2015) found no correlation between the distance from a highway and Cd content measured in soils and weed samples.

In our study, no differences between the two areas were identified, thus this could suggest the absence of any industrial emission of Cd. Furthermore, no significant difference (t-test, $p < 0.01$) was found between the concentration of Cd in Genoa and those measured in a reference site (Minganti et al., 2016).

The widespread use of tree bark as a bioindicator allows comparisons between the data found in literature and the data obtained in this work; the data in literature were considered for comparison, only if the texture of the bark of the selected tree species was similar to holm oak.

Higher Pb concentrations were only found by El-Hasan et al. (2002), but this is probably due to the use of leaded fuels in Jordan during 2002; while lower concentrations of Pb, if compared to our data, were found even in big cities (Faggi et al., 2011; Perelman et al., 2010).

Cd and Cu concentrations reported in literature (El-Hasan et al., 2002; Faggi et al., 2011) were comparable to our data.

The use of tree bark as a bioindicator is a useful tool for drawing maps, which can provide information on trace element distribution with high spatial resolution.

Figure 2.6 shows the distribution of concentrations of As, Fe and Zn in the sampling area; very high values were founded near the steel plant. These high levels can be explained by the steel plant activity; in fact, the concentrations of Fe and Zn measured in the samples collected near the steel plant (n=13) were roughly

twice the concentrations found in the other parts of the city (Drava et al., 2016). The high concentration of As, usually associated to coke production, can be explained by the presence of a blast furnace which was operating in the plant until 2002. Moreover, the concentrations of As are in agreement with the study of Suzuki (2006).

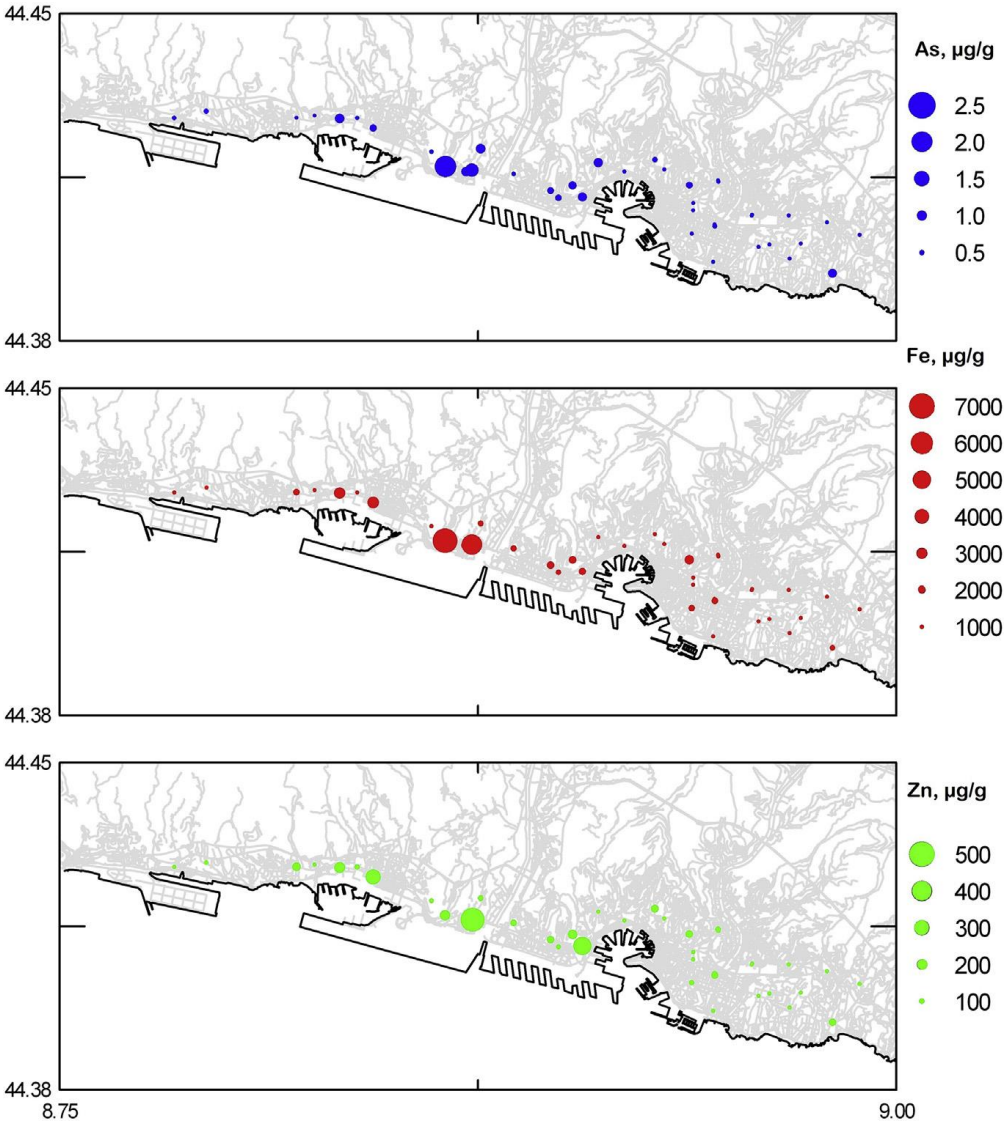

Fig. 2.6. Maps of the distribution of As, Fe and Zn concentrations in holm oak bark (µg g^{-1}dry weight). Circle size is proportional to element concentration.

Cobalt, Mn and Ni showed a more widespread distribution in the western part of the city, but it is difficult to clearly identify on the map a point source for these

trace elements (Fig. 2.7). Mn showed higher concentration values than those reported in other studies (El-Hasan et al., 2002; Faggi et al., 2011; Perelman et al., 2010) but this could mainly be due to the specific characteristic of the area analyzed (Minganti et al., 2016). Regarding Ni, as for Mn, higher concentration values were founded in the samples analyzed than those reported in previous studies (El-Hasan et al., 2002; Faggi et al., 2011; Perelman et al., 2010; Suzuki, 2006). The distribution of the concentration for the other elements analyzed (Cd, Cu, Pb, even if not significantly different in the two urban areas, and V) is shown in Figure 2.8.

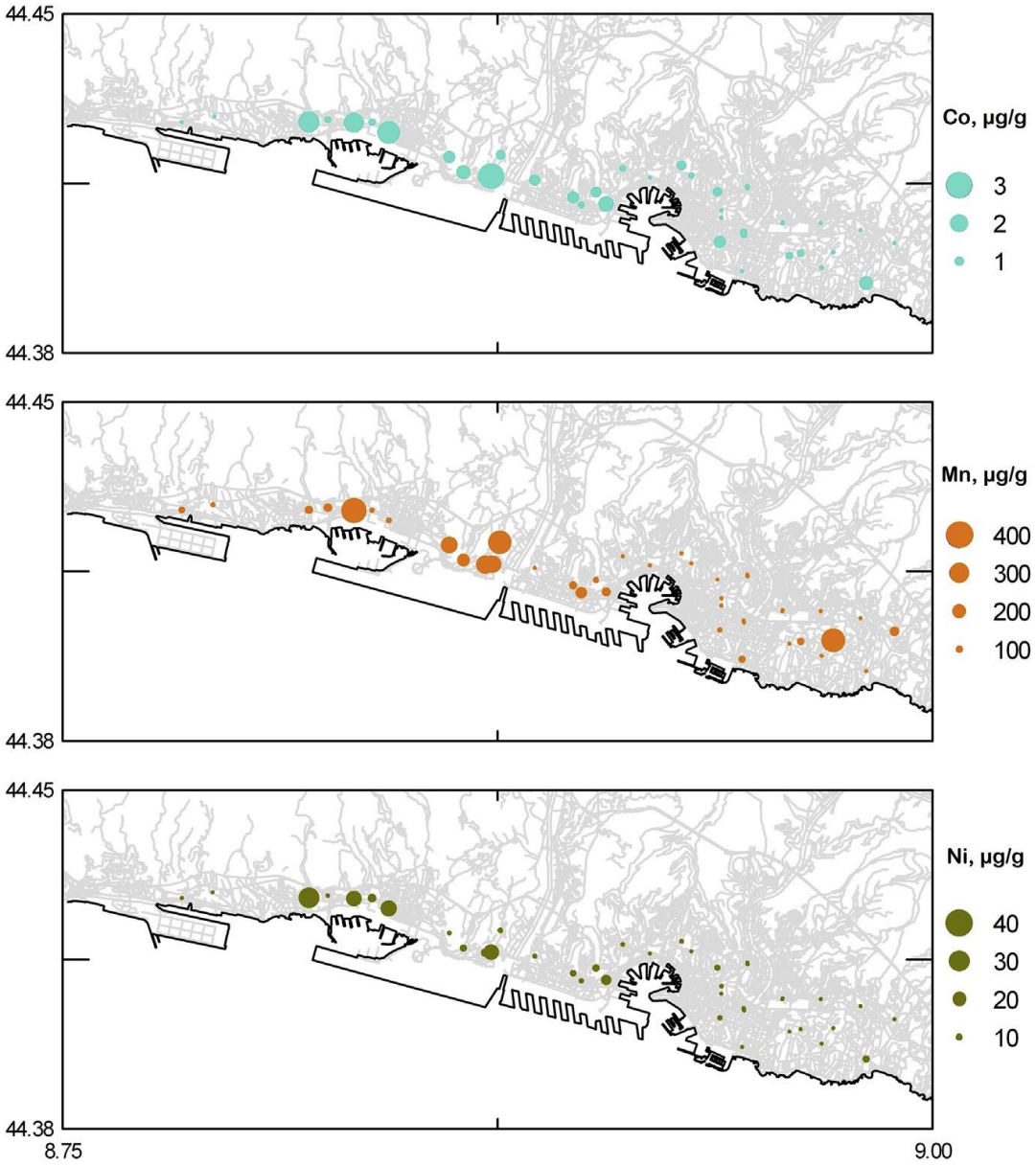

Fig. 2.7. Maps of the distribution of Co, Mn and Ni concentrations in holm oak bark (µg g^{-1} dry weight). Circle size is proportional to element concentration.

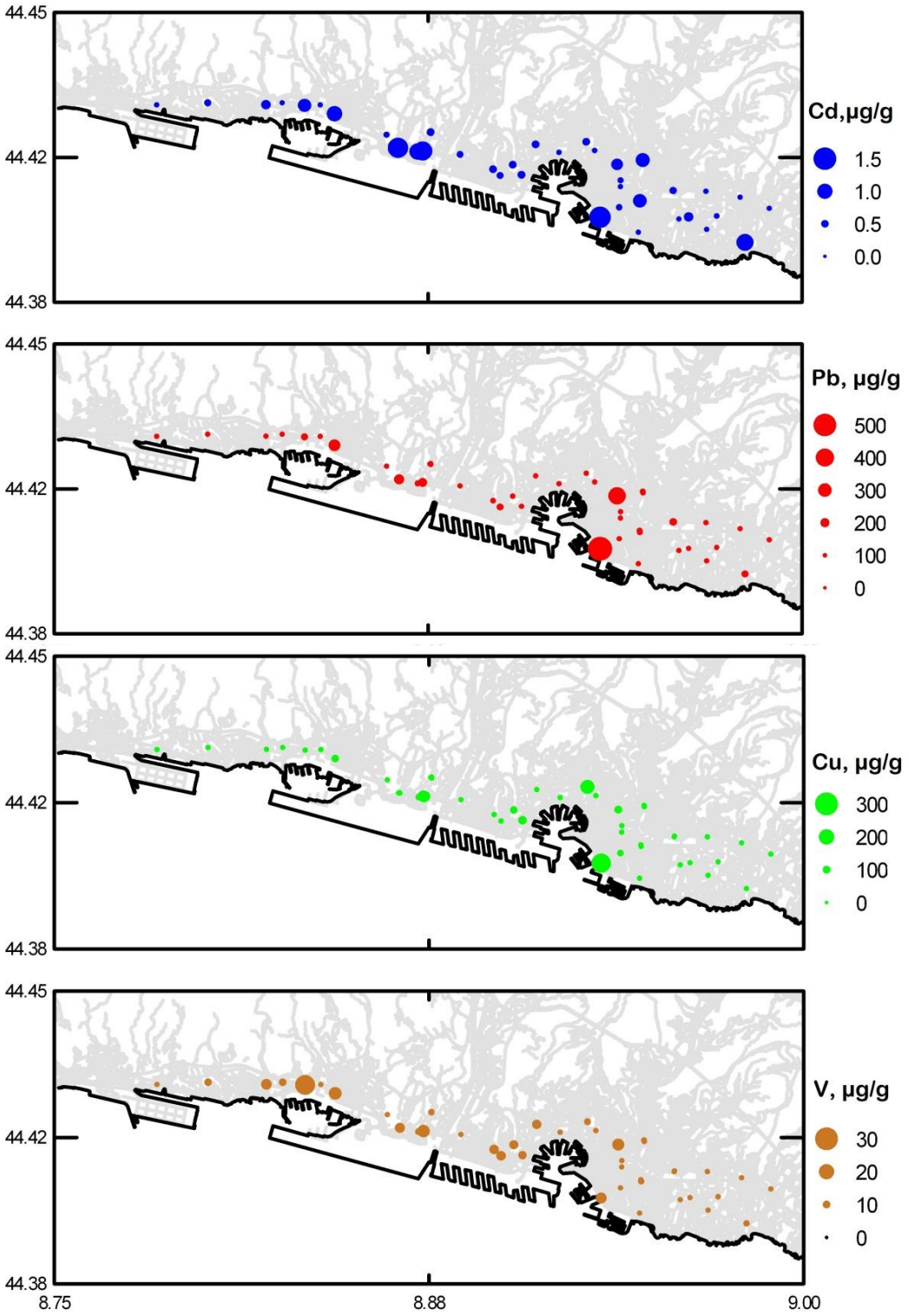

Fig. 2.8. Maps of the distribution of Cd, Pb, Cu and V concentrations in holm oak bark µg g^{-1} dry weight). Circle size is proportional to element concentration.

2.4. Conclusions

Trace elements within PM (Particulate Matter) play a key role in any assessment of air quality, thus an exhaustive information on their concentrations and distribution is advisable.

Monitoring stations are usually applied for routine monitoring studies of air quality in urban environments; this type of stations, generally, are few in number and are placed in selected areas of the city, due to their cost and to maintenance related problems.

On the other hand, the use of holm oak tree bark to study trace element concentrations and distribution can provide spatial information with high density of sampling, covering a wider area. This approach allows drawing detailed maps of trace element distribution, which can highlight any point source and/or point out the most critical areas.

This high density of sampling can be achieved thanks to a good distribution pattern of holm oak trees in all the different city areas (parks, residential areas, industrials areas, roads); this is helpful in giving an indication of the exposure levels of the people living in different areas of the city.

This research project shows that in urban environments people living in areas characterized by industrial activities are exposed to metals in atmosphere at significantly higher levels than those living far from any industrial settlement. Thus, being able to evaluate the exposure time of the tree bark to the pollutants is important for assessing the exposure of the people to the same pollutants; this aspect will be further evaluate in the next chapter.

3. The bark of the branches of holm oak (*Quercus ilex* L.) for a retrospective study of trace elements in the atmosphere

This chapter refers to the published article: *The bark of the branches of holm oak (Quercus ilex L.) for a retrospective study of trace elements in the atmosphere.* Giuliana Drava, Daniele Brignole, Paolo Giordani, Vincenzo Minganti. Environmental Research 154 (2017) 291–295.

3.1. General introduction and temporal variability

Tree bark has been extensively studied and used (Berlizov et al., 2007; Böhm et al., 1998; Cucu-Man and Steinnes, 2013; Drava et al., 2016; Faggi et al., 2011; Fujiwara et al., 2011; Harju et al., 2002; Minganti et al., 2016) as an effective biomonitor for its ability to accumulates trace elements, both via wet and dry deposition, due to its exposition to atmospheric pollutants for several years.

However, time trends and/or time changes in pollutants are usually important aims in environmental studies; the bark collected from the trunk of trees is not useful for achieving this goal, because the exact exposure time and the accumulation rate cannot be determined. Thus, the possibility to trace back in time any pollution trend could be an important achievement. One common source for retrospective studies are the natural archives such as herbaria, layers of snow, ice and sediment cores, ombrotrophic peat bogs and tree rings (Blais et al., 2015). Nevertheless, the disadvantages could be a reduced sample amount and a limited geographical distribution; in the worst scenario, the unavailability of these archives led to the inability of a retrospective study.

On the other hand, while the bark of tree trunk is not adequate for time trend studies, the bark collected from the annual segments of tree branches can be related to a defined period of time. In fact, starting from the apical bud, which is

related to the growing section of the current year, it is possible going back of one year at a time every time an old scar of the terminal bud is encountered (Fig. 3.1). Since the growth of tree branches is annual and continuous it is possible to trace back the presence of pollutants in a time span of about two decades (Drava et al., 2017a); thus, it is possible to monitor, with an annual resolution, the time pattern of different pollutants even in absence of any monitoring programmes.

For this study the branches of holm oak, *Quercus ilex* L., were chosen, given the widespread presence of this tree species both along roads and in parks. The use of tree branches as a viable tool for detecting time trends of pollutants was already proven by Drava et al. (2017a) with a study on the emission of hexavalent chromium from a point source. In the present work, a wider number of trace elements were analyzed from samples collected in an urban environment and thus exposed to different possible sources of emission. A comparison with deposition data reported in literature was also done, in order to validate all the process. Given the wide distribution of trees both in industrial and urban areas and the simple operational process, sample collection, preparation and analysis, this monitoring method is easily affordable.

As for tree trunk bark, the concentrations of As, Cd, Co, Cu, Fe, Mn, Ni, Pb, V and Zn were measured by atomic emission spectrometry with an inductively coupled plasma source (ICP-OES) using an iCAP™ 7000 Series (Thermo Scientific, UK).

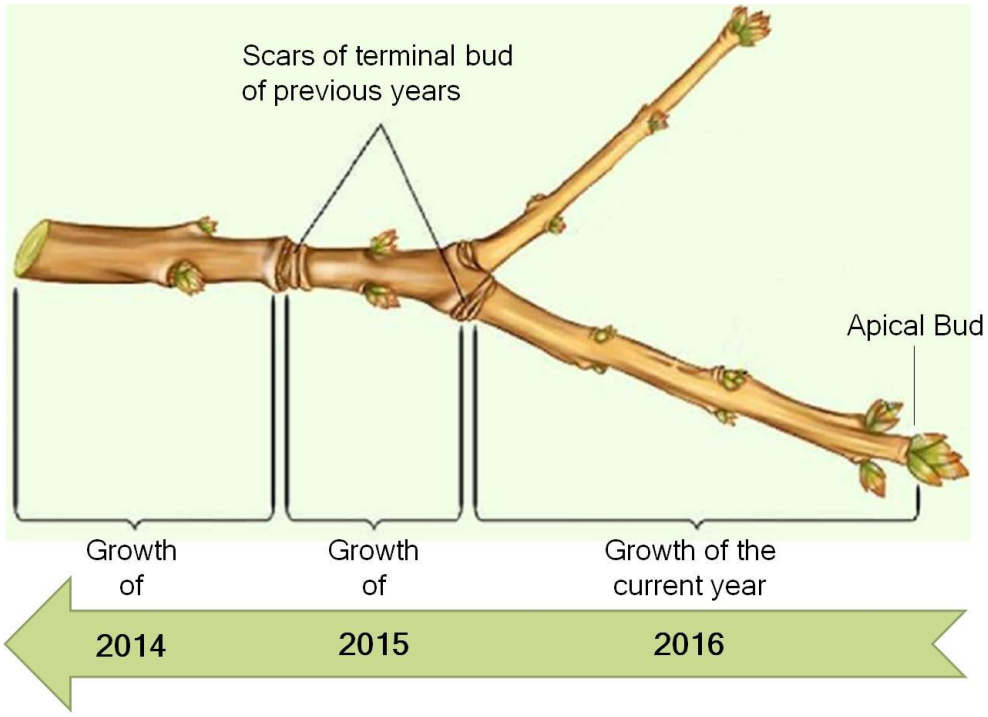

Fig. 3.1. Segments of a tree branch and related year of growth. From poster presentation Brignole et al. (2016).

3.2. Materials and Methods

3.2.1. Sampling campaign

This study was carried out in the western part of the city of Genoa (for site description please refer to section *2.2.2* of chapter *2*). Seven trees were selected in four sites in an area of about 0.3 km^2 and a total of nine branches were collected from trees located both in parks and along roads. Each branch was cut and divided in segments and each segment was assigned to the correct year. A total of 94 samples of branch bark, manually removed from each segment, were obtained; the time span covered was from 2001 to 2013. The samples were prepared according to the process described in section *2.2.3 "Sample preparation"* of chapter 2.

3.2.2. Chemical analyses

A defined amount of grinded material was mineralized with 5 mL of 65% (m/m) nitric acid (for trace metal analysis from Scharlau, Spain) in closed Teflon PFA vessels using a MDS 2000 (CEM Corporation, USA) microwave digestion system. The solutions obtained, were then diluted to 25 mL of volume and analyzed, for the trace elements selected, with an inductively coupled plasma source (ICP-OES) using an iCAP™ 7000 Series (Thermo Scientific, U.K.). Please refer to section *2.2.4 "Chemical analyses"* of the chapter 2 for a detailed description.

3.2.3. Data analyses

Our data set contains from 4 to 9 samples per year, thus a normality test raises some criticality; moreover, it is more suitable the use of non-parametric test if data are not supposed to follow a specific probability distribution (Gilbert, 1987). The visual inspection of the data set frequently highlighted the presence of skewness or the presence of outliers, even if the result of the Shapiro-Wilk test allowed to consider the normal distribution suitable to describe our data; this situation is frequent for small data sets like ours. For this reason, trend analysis was run by means of non-parametric Mann-Kendall test, testing the hypothesis of no trend against the hypothesis of an upward of a downward trend. Moreover, the non-parametric Kruskal-Wallis test was run in order to study the presence of any difference between the measured variables during the years monitored, as a distribution free analog of one-way ANOVA (Drava et al., 2017b). The presence of any differences or trends was considered significant at $p < 0.05$.

3.3. Results and discussion

The results of the analyses, reported as the median concentration of the trace elements (μg g^{-1} d.w.), are shown in Table 3.1. Along with the results, a summary of descriptive statistics (median, mean, standard deviation, minimum and maximum value) is also reported at the bottom of the table.

Table 3.1. (part 1.) Median concentration of trace elements (μg g^{-1} d.w.). Each annual segment refers to a specific year of growth.

Calendar Year	N	As	Cd	Co	Cu	Fe	Mn	Ni	Pb	V	Zn
						μg g^{-1} d.w.					
2001	4	0.69	0.29	0.48	16.53	956	50.1	3.81	13.7	2.02	49.0
2002	5	0.58	0.24	0.42	16.50	763	52.8	3.53	11.3	1.54	47.5
2003	6	0.78	0.21	0.39	18.64	481	57.9	3.77	9.7	1.56	40.2
2004	6	0.72	0.20	0.37	17.30	451	55.4	3.44	8.5	1.63	39.2
2005	8	0.88	0.28	0.46	20.52	752	69.9	4.28	12.6	1.93	46.8
2006	9	0.52	0.19	0.35	20.12	683	64.8	4.02	10.1	1.71	52.8
2007	9	0.78	0.21	0.40	19.32	631	54.9	3.67	8.5	1.86	46.9
2008	9	0.45	0.21	0.46	30.87	834	51.1	4.56	8.2	1.80	39.0
2009	9	0.80	0.18	0.32	15.64	511	47.8	3.09	6.7	1.46	36.9
2010	9	0.39	0.17	0.39	19.29	679	54.4	3.22	5.7	2.04	32.5
2011	8	0.76	0.18	0.40	16.35	713	54.1	4.04	5.1	2.25	33.9
2012	6	0.51	0.17	0.41	17.42	855	59.0	4.00	5.7	2.37	33.9
2013	6	0.63	0.18	0.47	17.34	639	59.9	4.29	5.6	2.35	39.4

Table 3.1. (part 2.) Summary descriptive statistics (median, mean, standard deviation, minimum and maximum value) are also reported for the whole data set.

Calendar Year	N	As	Cd	Co	Cu	Fe	Mn	Ni	Pb	V	Zn
						μg g^{-1} d.w.					
Median	94	0.63	0.20	0.40	18.34	673	55.2	1.65	7.6	1.83	43.1
Mean	94	0.64	0.21	0.46	25.68	816	68.0	3.63	9.1	1.99	56.2
Std. dev	94	0.03	0.01	0.02	2.02	136	3.9	4.15	0.5	0.49	4.9
Min	94	0.11	0.10	0.16	10.89	132	16.9	1.80	2.7	0.54	21.6
Max	94	1.61	0.39	1.67	116.87	3123	161.9	11.29	25.2	5.35	337.0

To study within-tree variability (i.e. two branches of the same tree) and between-tree variability (i.e. branches of different trees) a Principal Component Analysis was run. No significant within-tree variability was detected, while, regarding between-tree variability, one tree was observed to be different from the others, due to a higher Mn concentration; thus, branches collected from this tree were considered separately when Mn was considered for data analysis.

Since one of the advantages of using tree branches in bio-indication is the possibility to detect time trend and/or temporal variations, two different methods, non-parametric Mann-Kendall statistic test and Pearson's coefficient (obtained from median value for the concentrations of each year), were applied to data to check for either time trend or temporal variations.

The results showed a downward time trend for Cd (Mann-Kendall p=0.00, Pearson's coefficient r =−0.74, p=0.00), Pb (Mann-Kendall p=0.00, Pearson's coefficient r =−0.89, p=0.00) and Zn (Mann-Kendall p=0.01, Pearson's coefficient

r =−0.67, p=0.01). Only V showed an upward time trend (Mann-Kendall p=0.01, Pearson's coefficient r =0.65, p=0.01) (Fig. 3.2).

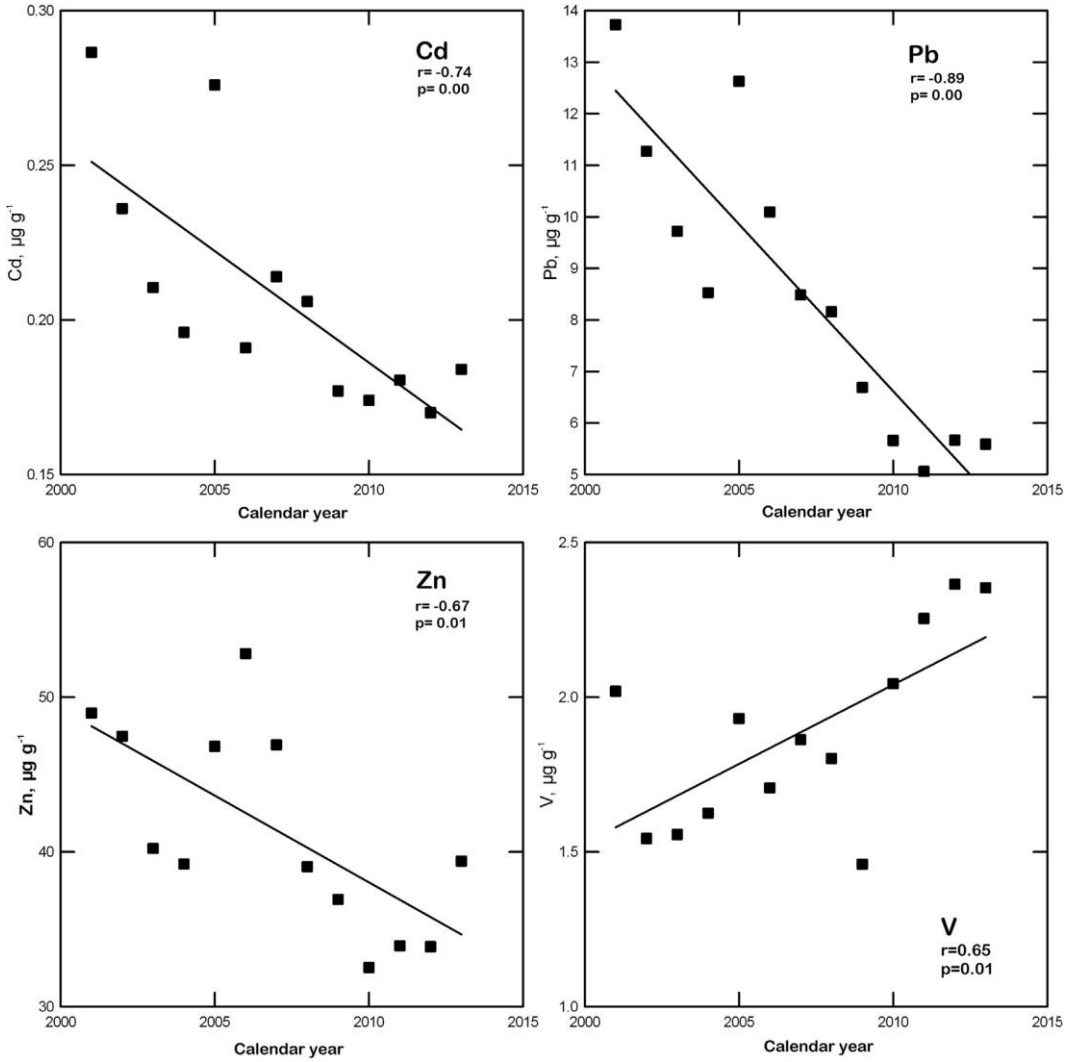

Fig. 3.2. Time trends for trace element concentrations (µg g^{-1} d.w.) in bark of holm oak branch annual segments.

No significant and appreciable time trend or time variation (Kruskal-Wallis p= 0.13–1.00; Pearson's coefficient r= 0.04–0.28) was detected for the remaining trace elements analyzed (As, Co, Cu, Fe, Mn and Ni). This result may suggest that the accumulation of those trace elements is not proportional to the duration of the exposure.

In order to have a more detailed study of the problem, climatic data were also retrieved (DICCA, 2016) and analyzed for any possible correlation with our data. The results showed no correlation (Pearson's coefficient r =0.06-0.39) between trace element concentrations and temperature, nor rainfall (Pearson's coefficient r=0.04–0.40).

The validity of the results obtained was verified by means of a research about trace elements in the atmosphere both in literature and in databases in order to perform a comparison with our data. It is very difficult, however, finding proper information for a good correlation, first of all because of the lacking of articles regarding temporal variation of trace elements in the atmosphere, but also because the published studies (Adamo et al., 2011; Aničić et al., 2011; Gerdol et al., 2014) usually differ in terms of matrix used for the analyses (lichens, mosses, tree leaves, filters, etc.) and in terms of sampling areas with different types and levels of pollution (reference, urban and industrial areas).

Only for Pb and Cd suitable information can be obtained, and the most complete dataset is issued in the framework of the European Monitoring and Evaluation Programme (EMEP, 2016), with data going back in time up to early 1970s (Tørseth et al., 2012). In literature, a general and significant reduction of the concentration of Cd and Pb is reported, and a summary of this downward trend is provided by the European Environment Agency (EEA, 2016a). In order to compare our data with the ones in the EEA summary, the concentrations measured in tree branches were plotted against the ones retrieved from EEA (Fig. 3.3). Moreover, a Pearson correlation analysis was also carried out and the correlation coefficients obtained were r=0.77 (p=0.02) for Cd and r =0.91 (p=0.00) for Pb.

This result strengthens the hypothesis that branch bark can be a viable and effective indicator for the temporal variation of these trace elements.

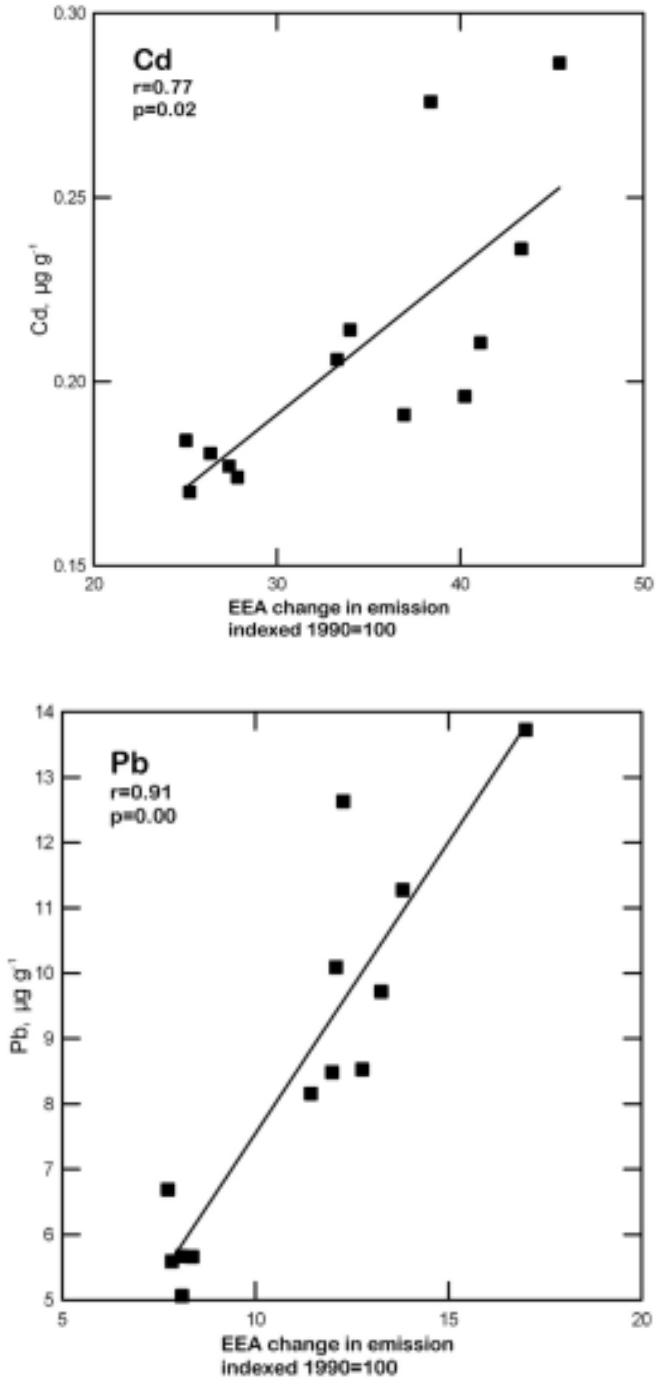

Fig. 3.3. Concentrations of Cd and Pb (µg g^{-1} d.w.) in bark of holm oak versus the percent reduction in the atmosphere as reported by the European Environment Agency (EEA), indexed 1990=100.

The trace element concentrations measured between 1986 and 2008 by Heimbürger et al., (2010) in the north-western part of the Mediterranean region were in agreement with our data, confirming a reduction of Cd, Pb and Zn, while showing a stable situation for Co, Cu, Fe, Mn and Ni. Moreover, in the time span covered by our study a decrease of approximately 54% for Pb and 30% for Cd and Zn was detected.

3.4. Conclusions

The bark of the annual segments of tree branches has proven to be a powerful natural archive and an effective tool to monitor time trend and temporal variations of pollutants. The use of tree branches allows the "*a posteriori*" reconstruction, with annual resolution, of atmospheric trace elements, with a relative easy and cost saving methodology. Moreover, the possibility for "*a posteriori*" tracking of pollutants can help in gaining information in areas not covered by monitoring programmes or in areas not easily accessible for positioning static monitoring stations. It is also worth noting that the ability of tracking time trend could be used to obtain information about "emerging contaminants" not included in previous monitoring programmes, such as Platinum Group Elements or Rare Earth Elements

3.5. A parallel project: Trace elements and PM_{10}

The term Particulate Matter (PM) usually refers to a heterogeneous mix of solid and liquid particles suspended in the atmosphere. The reduced dimensions of those particles (see classification below) can increase the residence time in the

atmosphere, leading to an increased risk of being transported over long distances and to an enhanced risk for human health.

Particulate Matter, is frequently classified according to particle dimensions in:

i) PM_{10}: for particles less than 10 μm in aerodynamic diameter

ii) $PM_{2.5}$ for particles less than 2.5 μm in aerodynamic diameter

iii) $PM_{0.1}$ for particles less than 0.1 μm in aerodynamic diameter

As an integral part of PM, trace elements play an important role in air and environmental pollution, exposing to serious health treat the people living nearby main emission sources. Among all pollutants, a main point is to be able to assess the proper chemical composition and concentration of trace elements, including those not mentioned by the legislative reference framework for air quality standards.

For this reason, a new project has been started, and is still on-going, as it aims to verify the correlation between the concentration of trace elements found in tree branches bark and the PM_{10} data provided by classic monitoring stations.

Thus, a sampling campaign was performed in selected areas in the city of Genova, nearby automatic monitoring station to be able to perform the statistical analyses. Branch samples (n=18) were collected and prepared as reported in chapter 3, section 3.2.1, and subsequently analyzed with an ICP-OES instrumentation in order to determine the concentration of the following trace elements: As, Cd, Co, Cu, Fe, Mn, Ni, Pb, V and Zn.

Each branch section could be related to a specific year in the past (see Paragraph 3.1) and thus the concentration of all the selected trace elements was compared

with the data collected by monitoring station and provided by the Regional Agency for Environmental Protection (ARPAL) for each year.

Among the pollutants considered for the analysis, only Cd, Ni, Pb and Zn, usually considered as produced by vehicular emission and which can have an influence on human health, showed significant ($p<0.05$) correlation with PM_{10} (Fig. 3.4).

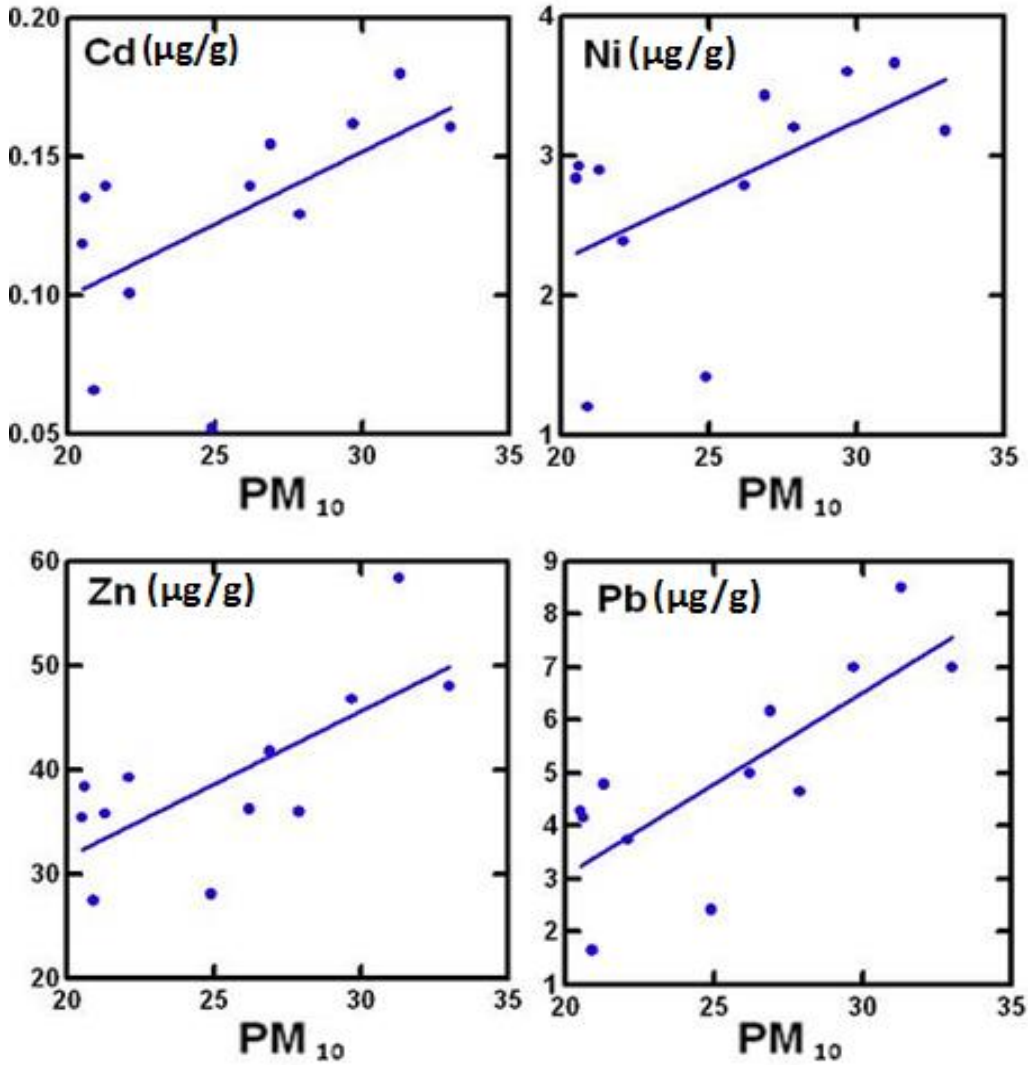

Fig. 3.4. Linear correlation between PM_{10} measurements recorded by 4 monitoring stations in Genoa and element concentrations (expressed in µg g^{-1}) in the bark of tree branches collected along the streets of the city.

Since the ban of the leaded fuels, Pb it is no more emitted from vehicle exhausts, but there could be other emission sources related i.e. to brakes, road dust or tire

wear (Denier van der Gon and Appelman, 2009), which in turn could positively influence the Pb concentration and thus providing a significant correlation with PM_{10}. Considering traffic emission as the main sources of trace elements and the main contributor of PM, the significant correlation obtained for the other trace elements may be due to this hypothesis.

Considering the wide applicability of the use of tree branches as biomonitor and the good correlation achieved comparing data from different approaches on different measurements (between the direct measurements of atmospheric particulate matter and chemical determinations on a bioindicator), this may be view as a new starting point for a more extended network of monitoring programmes using tree branches as an interesting parallel and integrating approach to classic monitoring station.

4. The bark of *Jacaranda mimosifolia* tree: a visiting PhD project across Europe

This chapter refers to the two experiences abroad, as a visiting PhD student, in Lisbon (Portugal) and Antwerp (Belgium); the two periods were respectively three months and three weeks long. The study period in Lisbon was funded by the European Union with the Erasmus+ project. The results of this study have been published in the article: *Chemical and magnetic analyses on tree bark as an effective tool for biomonitoring: A case study in Lisbon (Portugal).*
Daniele Brignole, Giuliana Drava, Vincenzo Minganti, Paolo Giordani, Roeland Samson, Joana Vieira, Pedro Pinho, Cristina Branquinho. Chemosphere 195 (2018) 508-514.

4.1. The Erasmus+ grant

The Erasmus project was created in 1987 allowing students and people from all over Europe to travel and study, increasing personal skills and knowledge and improving partnerships among countries and institutions. The year 2017 marked the 30th anniversary of the programme foundation.

The Erasmus+ programme was launched in 2014 and it will cover the period from 2014 to 2020 integrating and updating the seven previous programmes (The Lifelong Learning Programme, The Youth in Action Programme, The Erasmus Mundus Programme, Tempus, Alfa, Edulink, Programmes of cooperation with industrialized countries in the field of higher education) with three new Key Actions focused on: the learning mobility of individuals (Key action 1), the cooperation for innovation and the exchange of good practices (Key Action 2) and the support for policy reform (Key Action 3) (Erasmus+ annual report, 2014).

The budget for the considered period is of €14.7 billion, which will allow about 4 million people to travel and study abroad.

4.2. Visiting PhD student at ULisboa

During the period from 8th January 2016 to 8th April 2016, my research activity was carried out at the Center of Ecology, Evolution and Environmental Changes of the Faculty of Science at the University of Lisbon. During the stay, I was involved in the programmes of the center along with the research activity planned in my traineeship learning agreement. The project included a sampling campaign in the city of Lisbon, after a careful study of the proper bioindicator, the analyses of the collected samples and the subsequent preparation of a scientific article. During the three month stay, in order to strengthen the data obtained with chemical analyses, it was planned a second study period abroad at the University of Antwerp, in order to analyze, with magnetic techniques, the samples collected in Lisbon. The whole dataset, chemical and magnetic data, was used for the preparation of the scientific article.

4.3. Visiting PhD student at UAntwerpen

During the period from the 20th of June 2016 to the 14th of July 2016, my research activity was carried out at the Bioengineering department of the University of Antwerp. During the stay, I applied several magnetic techniques (Saturation Isothermal Remanent Magnetization, Susceptibility, etc.) to the samples collected during the study period in Portugal. The objective of this study was to verify whether bark magnetic properties can be used as a good proxy of metal loads, in order to apply magnetic techniques along with classical chemical analyses to better characterize pollutants impact and distribution. The application of both techniques

on tree bark samples was an innovative approach since no references were found in literature.

4.4. Introduction

The increasing risk of lung cancer (Pope et al., 2002; Raaschou-Nielsen et al., 2016), the outbreak of cardiovascular and pulmonary diseases (R. Chen et al., 2016) associated to air pollution, has risen more attention on monitoring programmes, aiming to provide information with high spatial resolution about temporal patterns of contaminants and to relate their concentration with the possible adverse effects on human health. Toward this goal, regulatory policies are planned and implemented in order to reduce emissions, and the decrease of several atmospheric contaminants is continuously monitored (WHO, 2013).

Even though the importance of trace element levels has been highlighted in the European policy for air pollution reduction, a reduced number of monitoring stations are monitoring metals through Europe (EEA, 2016b), affecting, due to reduced spatial cover, the accuracy of high-resolution model. Moreover, the differences among urban areas, due to microclimatic conditions (e.g. local winds), the presence or absence of green spaces or different road characteristics such as uphill or downhill roads, with respect to the location point of monitoring stations, play an important role in influencing both the spatial information and the air quality data. Therefore, in order to obtain wide-ranging information, other tools to monitor atmospheric pollutants are needed. Bio-monitors of air pollution (e.g. lichens, tree bark and leaves) are effective tools for assessing the effects of pollution on the biotic component of ecosystems, providing complementary

information with respect to traditional chemical-physical monitoring (Nimis et al., 2002) and are able to provide data with high spatial resolution.

For this project, tree bark was chosen as bioindicator, given its fully proved ability of assessing air pollution, thanks to trace elements accumulation, both through wet and dry deposition (Cucu-Man and Steinnes, 2013; Drava et al., 2016; El-Hasan et al., 2002; Minganti et al., 2016). Metals emitted from anthropogenic activities, exposed to several physical and chemical processes occurring in the atmosphere, may be accumulated in different form that in turn may affect the magnetic fingerprint of living organisms. The possibility of take advantage of this magnetic fingerprint to study metal distribution has led to an increasing application of magnetic techniques, i.e. Isothermal Remanent Magnetization (IRM), Saturation Isothermal Remanent Magnetization (SIRM), Magnetic Susceptibility (χ), for biomonitoring purposes. The main field of application of magnetic analyses are mainly on leaves (Hofman et al., 2014; Kardel et al., 2012; Maher et al., 2008) dust (Sipos et al., 2014; Zhang et al., 2012) soil samples (Lourenço et al., 2012) or mosses and lichens (Salo and Mäkinen, 2014; Vuković et al., 2015), but scarce data are available in literature about the application of these techniques to tree bark samples (Kletetschka et al., 2003)

4.5. Magnetic techniques for environmental monitoring

4.5.1. Magnetic Susceptibility (χ)

It is used for studying the "magnetisability" of a selected material, and can provide information about the structural characteristic of the sample (Dearing, 1994). It measures the magnetic behavior of a material to an applied magnetic field, and it is

defined as the ratio between such behavior (M) and the external magnetic field (H) (Liu et al., 2012)

$$\chi = dM/Dh$$

The magnetic behaviour depends on the configuration and interactions of all the electron motions; so, different possible behaviour are identified (ferromagnetic, ferromagnetic, canted antiferromagnetic, diamagnetic and paramagnetic) (Dearing, 1994).

The measure could be related to the volume or the mass of the material obtaining respectively the volume and the mass specific susceptibility; moreover, it is possible to measure the magnetic susceptibility at two different magnetic intensities leading to the low field (or low frequency) and high field (or high frequency) susceptibility.

4.5.2. Isothermal Remanent Magnetization (IRM) and Saturation Isothermal Remanent Magnetization (SIRM)

The term *remanent magnetization* is referred to the property of a sample to show permanent magnetization without an applied external magnetic field; this property is typical of materials which exhibit hysteresis. More in detail IRM is the remanent magnetization acquired by a sample when exposed to a magnetic field at a fixed temperature (Hunt et al., 1995). IRM increases with the increase of the intensity of the magnetic field applied and when the intensity of the field is high enough to reach the saturation of the response of the sample then the SIRM occurs (usually at 1T as magnetic field intensity).

4.6. Aims

The novelty of this project was the comparison of the concentration data of trace elements measured on tree bark and the data obtained from the magnetic analyses on those samples.

The study was planned to test whether:

i) Magnetic intensities are a good proxy of metal loads in tree bark

ii) The methods used are able to discriminate trees located in different types of urban (green spaces, small roads and large roads) site and thus being able to have a detailed overview of the pollutant distribution in the selected areas.

The concentrations of selected trace elements (As, Cd, Co, Cu, Fe, Mn, Ni, P, Pb, V and Zn) were measured in bark samples of *Jacaranda mimosifolia* collected from trees in different areas of the city of Lisbon (Portugal). The trace elements were selected according to the following criteria:

i) The possible adverse effect on human health.

ii) The sensitivity of the instrumentation.

iii) The possibility to reach good accuracy and reproducibility.

Magnetic analyses (SIRM, IRM and χ) were also performed on the tree bark samples to measure their magnetic properties.

4.7. Materials and methods

4.7.1. Choosing the right bioindicator

Among all the possible bioindicators, it was decided to choose the tree bark as the preferred bioindicator for the project. Among all the possible tree species, we selected *Jacaranda mimosifolia* tree; the selection was made taking into account the presence of such specie both in urban green areas and parks but also along road (both small and large ones). Moreover, the roughness of the bark was considered comparable with the one of *Quercus ilex* L. previously used for biomonitoring purposes by our group with good results. In addition, the choice of rough bark texture is in line with the hypothesis that such typology of bark is more able to retain pollutants than smooth texture.

4.7.2. Sampling plan and sampling campaign

The samples were collected in Lisbon, the capital city of Portugal (Fig. 4.1). The city lies on the banks of the estuary of the river Tagus; the metropolitan area has a population of about 2.8 million people. Lisbon is one of the most important ports on the Atlantic Ocean and around 20 million of passengers are served by its airport. After a precise survey on the distribution of *Jacaranda mimosifolia* trees in the sampling area, bark samples were collected from trees located in 34 sites (3 trees per site, except for two sites for which no more than one tree was available) in January and February 2016; for the selection of the sampling sites a stratified random sampling was applied. Given the lack of any proper information about the descriptors of air pollution (or monitoring station), an adequate proxy variable for

traffic intensity was chosen; therefore, the sampling sites were classified, according to their land use, in:

i) Large roads (two or more lanes).

ii) Small roads (one lane).

iii) Green spaces (presence of green areas/urban parks even if very small).

The tree bark was collected at a height between 1.5 and 2.0 meters and all around the tree circumference. The bark samples obtained were stored in a plastic bag and taken to the lab. Before the homogenization process using a MM 400 Mixer Mill (Retsch, Germany), they were freeze-dried to remove water. For each sample, an amount ranging from 0.2 to 1.1 g of dry matter was obtained and analysed without any further treatment.

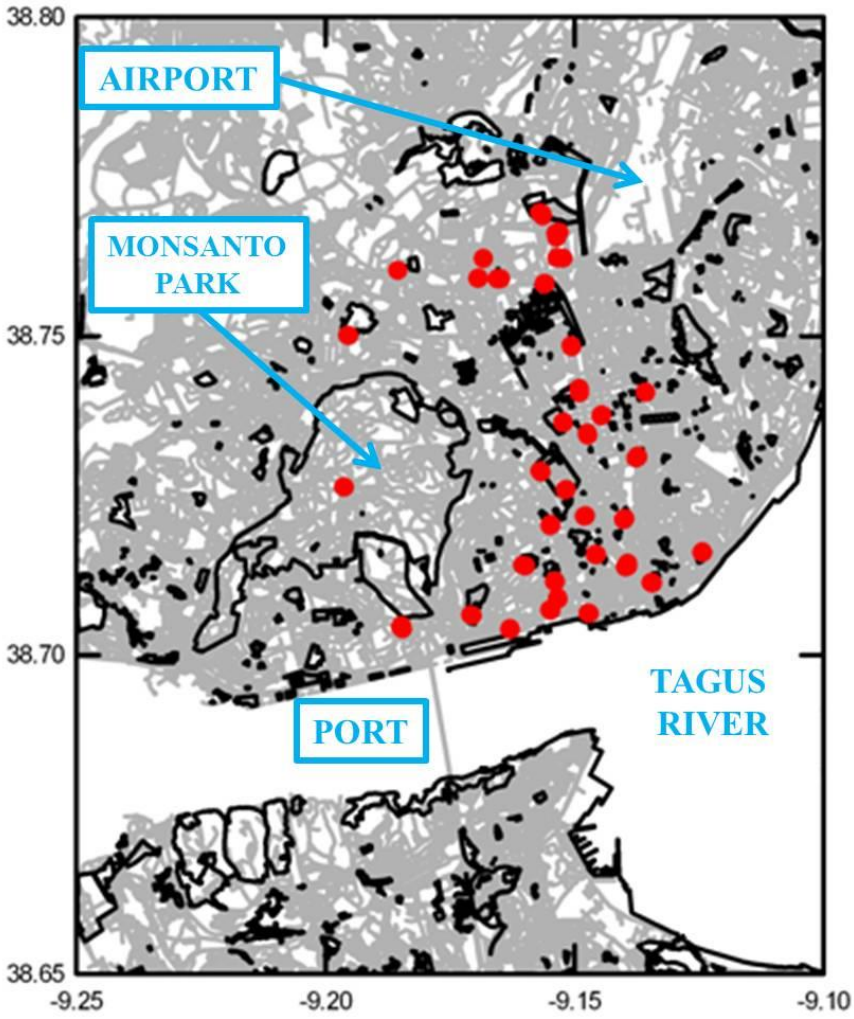

Fig. 4.1. Map of sampling site in the city of Lisbon

4.7.3. Magnetic analyses

An amount of about 0.85-0.9 g d.w. of grinded bark was used for the magnetic analyses; since all the magnetic techniques are non-destructive, the same sample was used for all the measurements. The powder was wrapped in a fixed amount of cling film to protect the instrumentation and the samples from contamination.

The samples were then put in a 10 cm^3 sampling pot and analyzed for Magnetic Susceptibility (χ), both at low frequency (χLF) and at high frequency (χHF), Isothermal Remanent Magnetization (IRM at 0.05 T and 0.2 T) and Saturation

Isothermal Remanent Magnetization (SIRM at 1 T). All the values obtained from magnetic analyses were normalized for sampling pot volume and for sample dry mass. A MS2 Magnetic Susceptibility System (Bartington Instruments Ltd., U.K.) with a MS2B type dual frequency sensor, with a resolution of 2 × 10-6 SI was used to measure magnetic susceptibility. The frequencies used by the MS2B sensor were 0.465 kHz (χLF) and 4.65 kHz (χHF) ± 1%. Before measuring the samples, the instrument was calibrated for both frequencies with a sample containing a small ferrite bead. The correction for air drift fluctuations was applied to all the measurements and calculations removing the background drift (Dearing, 1994).

The Frequency Dependence Susceptibility was then calculated as:

$$\chi_{fd\ (\%)} = [(\chi_{LF} - \chi_{HF})/\ \chi_{LF}]\ 100$$

A Pulsemag DS4 magnetizer (Molspin Ltd., U.K.) was used to magnetize the samples at selected intensities (0.05 T, 0.2 T and 1 T), then a triple-shielded, annular fluxgate, Minispin magnetometer (Molspin Ltd, U.K.), was used to read the magnetized samples to measure IRM and SIRM. To avoid any errors each sample was read twice and the instrument was recalibrated every ten measurements with the provided rock specimen.

4.7.4. Chemical analyses

A defined amount of grinded material was mineralized with 5 mL of 65% (m/m) nitric acid (for trace metal analysis from Scharlau, Spain) in closed Teflon PFA vessels using a MDS 2000 (CEM Corporation, USA) microwave digestion system.

The solution obtained were the diluted to 25mL of volume and analyzed, for the trace elements selected, with an inductively coupled plasma source (ICP-OES) using an iCAP™ 7000 Series (Thermo Scientific, U.K.). Please refer to section *2.2.4 "Chemical analyses"* of the chapter 2 for a detailed description.

4.7.5. Data analyses

A Pearson correlation analysis was run on the magnetic and chemical data for the 98 samples, applying the Bonferroni correction to the p values calculation. Hierarchical cluster analysis, with Pearson correlation coefficient as dissimilarity measure and single linkage as a clustering algorithm, was used to detect groups of correlated variables. The median values of trace element concentrations and magnetic intensities for the 34 sites were submitted to a Principal Component Analysis (PCA). Moreover, an ANOVA analysis was run on the scores of the PCA, to test the possibility of a significant distinction in land use (large roads, small roads and green spaces). Data analysis was performed in R environment (vers. 3.1.2, R Core Team 2014) using FactoMineR package vers. 1.34 (Lê et al., 2008), and Systat for Windows Version 13 (Systat Software Inc., U.S.A.).

4.8. Results

Table 4.2 shows descriptive statistics of trace element concentrations and magnetic intensities for the samples collected in 34 sampling sites, classified for land use.

The data obtained from the analysis of 98 samples were submitted to Pearson correlation analysis. Cobalt, Cu, Fe and Zn were highly correlated with all the magnetic variables ($r > 0.7$, $p < 0.001$); As, Cd, Mn, Ni, P were less correlated

(0.68 > r > 0.48, p < 0.001); only Pb and V showed low correlation with all the magnetic parameters (r < 0.48); furthermore, V showed non-significant correlation with magnetic susceptibility measurement.

The results obtained from cluster analysis showed the magnetic parameters grouped together and highly correlated (Fig. 4.2); a good similarity was also found between the group of the magnetic variables and the cluster including Cu, Fe.

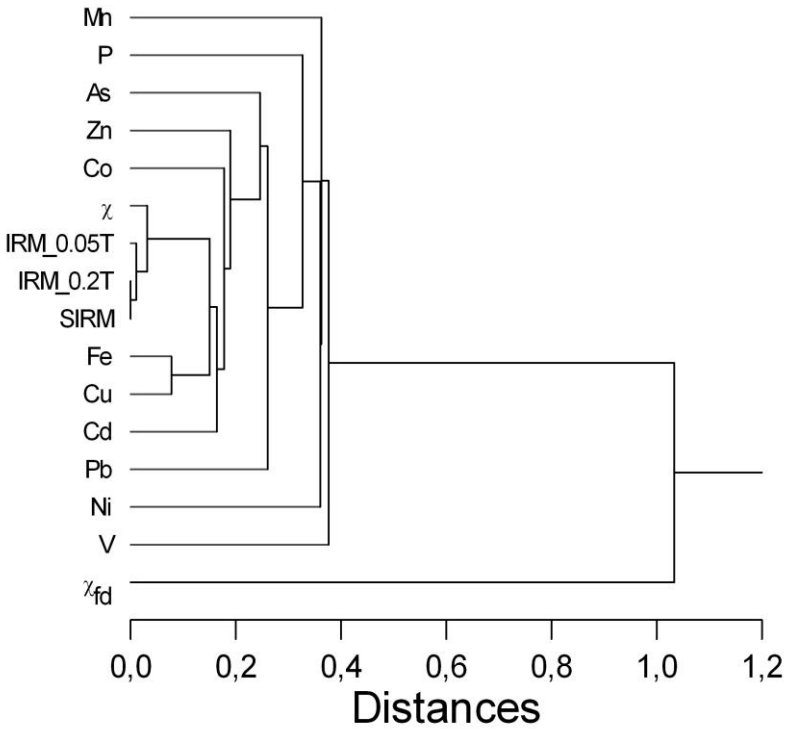

Fig. 4.2. Dendrogram of magnetic intensities and trace element concentrations (Pearson correlation coefficient as dissimilarity measure and single linkage as agglomeration method; n=98).

Table 4.1. (part 1). Descriptive statistics (median, mean and min-max range) of trace element concentrations and magnetic parameters based on land use for 34 sampling sites.

LAND-USE	STAT	As	Cd	Co	Cu	Fe	Mn	Ni	P
		μg g^{-1} d.w.	μg g^{-1} d.w.	μg g^{-1} d.w.	μg g^{-1} d.w.	μg g^{-1} d.w.	μg g^{-1} d.w.	μg g^{-1} d.w.	μg g^{-1} d.w.
LARGE ROADS	Median	1.11	0.20	1.31	57.70	1517	28.5	3.88	534
N = 16	Mean ± S.D.	1.21 ± 0.55	0.3 ± 0.32	1.20 ± 0.44	75.96 ± 55.8	1844 ± 1295	30.3 ± 11.1	4.05 ± 2.68	583 ± 207
	Range	0.40-2.75	0.09-1.39	0.42-2.18	26.6-254.28	301-5290	12.5-49.9	1.10-11.89	372-1233
SMALL ROADS	Median	0.72	0.09	0.80	31.05	643	29.8	1.68	519
N = 9	Mean ± S.D.	0.73 ± 0.20	0.09 ± 0.04	0.86 ± 0.34	31.58 ± 6.44	651 ± 340	26.5 ± 10.9	1.76 ± 0.64	535 ± 96
	Range	0.40-1.01	0.05-0.18	0.42-1.44	20.68-42.49	269-1290	11.3-43.2	0.80-2.70	369-687
GREEN SPACES	Median	1.03	0.15	0.85	40.18	863	24.9	1.81	432
N = 9	Mean ± S.D.	1.09 ± 0.29	0.19 ± 0.15	0.89 ± 0.26	40.98 ± 21.22	789 ± 393	26. ± 10.0	2.59 ± 1.67	462 ± 96
	Range	0.72-1.59	0.06-0.54	0.48-1.28	12.82-87.08	223-1464	12.8-44.4	0.89-5.98	303-609

Table 4.1. (part 2). Descriptive statistics (median, mean and min-max range) of trace element concentrations and magnetic parameters based on land use for 34 sampling sites

LAND-USE	STAT	Pb μg g^{-1} d.w.	V μg g^{-1} d.w.	Zn μg g^{-1} d.w.	IRM_0.05T 10^{-4}A m^2 kg^{-1}	IRM_0.2T 10^{-4}A m^2 kg^{-1}	SIRM 10^{-4}A m^2 kg^{-1}	χ $10^{-8}m^3$ kg^{-1}	χ_{fd} %
LARGE ROADS	Median	25.3	4.36	115.4	10.94	23.82	25.25	31.46	3.14
N = 16	Mean ± S.D.	60.5 ± 64.1	4.89 ± 2.77	114.9 ± 52.6	12.24 ± 7.69	26.91 ± 16.96	28.42 ± 17.87	33.03 ± 20.42	6.82 ± 8.82
	Range	3.4-185.4	1.23-12.27	42.5-237.7	1.02-27.45	2.48-54.52	2.62-58.23	3.2-75.28	-1.19-27.50
SMALL ROADS	Median	7.0	2.23	30.7	3.59	8.01	8.45	6.62	3.64
N = 9	Mean± S.D.	10.6 ± 10.6	2.62 ± 1.04	37.3 ± 22.7	4.70 ± 2.96	9.64 ± 5.54	9.99 ± 5.86	10.15 ± 6.63	19.08 ± 48.78
	Range	1.3-32.7	1.14-4.66	15.8-89.0	1.10-8.33	2.52-17.25	2.68-17.97	2.68-20.61	-6.90-147.27
GREEN SPACES	Median	16.3	3.07	72.1	5.27	11.24	11.77	11.17	14.25
N = 9	Mean ± S.D.	20.5 ± 17.2	4.02 ± 2.55	79.2 ± 42.7	4.89 ± 2.58	10.39 ± 5.42	10.99 ± 5.72	9.51 ± 4.92	16.81 ± 15.27
	Range	2.7-57.2	1.77-9.78	29.2-178.6	1.19-9.41	2.79-19.87	2.99-20.96	2.82-14.98	-2.13-44.40

4.8.1. Magnetic results

Figure 4.3 shows the SIRM profile for the median values of the selected intensities according to land use. Small roads have a lower SIRM profile if compared to green spaces and large roads. Surprisingly the small roads showed a slightly lower SIRM profile than green spaces. A good separation between different types of urban land use is evident. The results of the measurements of magnetic susceptibility follow a similar pattern, with the values for small roads clearly lower than those for large roads and slightly lower than those for green spaces.

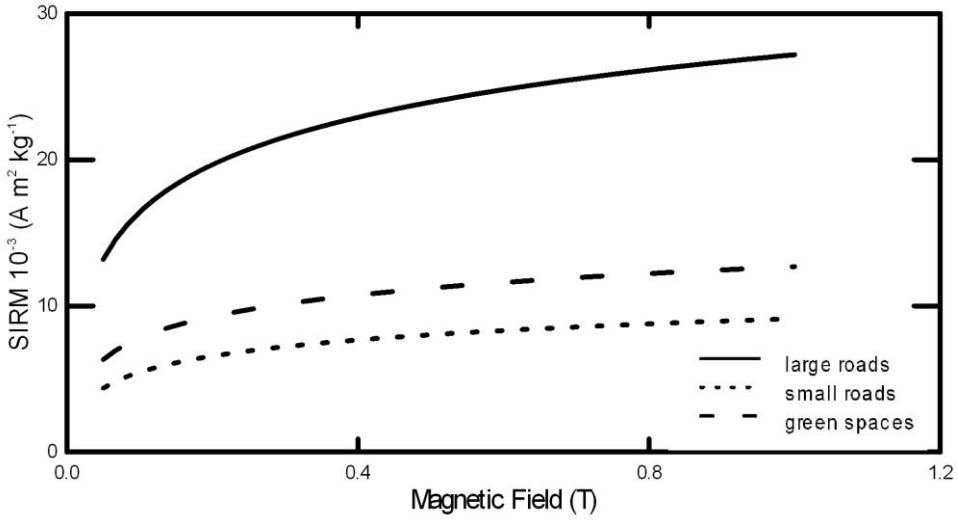

Fig. 4.3. SIRM profiles for land use classification.

4.8.2. Principal Component Analysis results

The medians of chemical and magnetic data for 34 sampling sites were submitted to PCA. The total variance explained by the first two components was 78.6% (Fig. 4.4), 66.1% of the total variance explained by the first component and 12.5% from the second one. The first component is associated to an increase of trace element concentrations and magnetic intensities, while along the second component the magnetic and the chemical

variables are clearly separated. Only χfd (%) is not correlated with the trace element concentration and magnetic intensities.

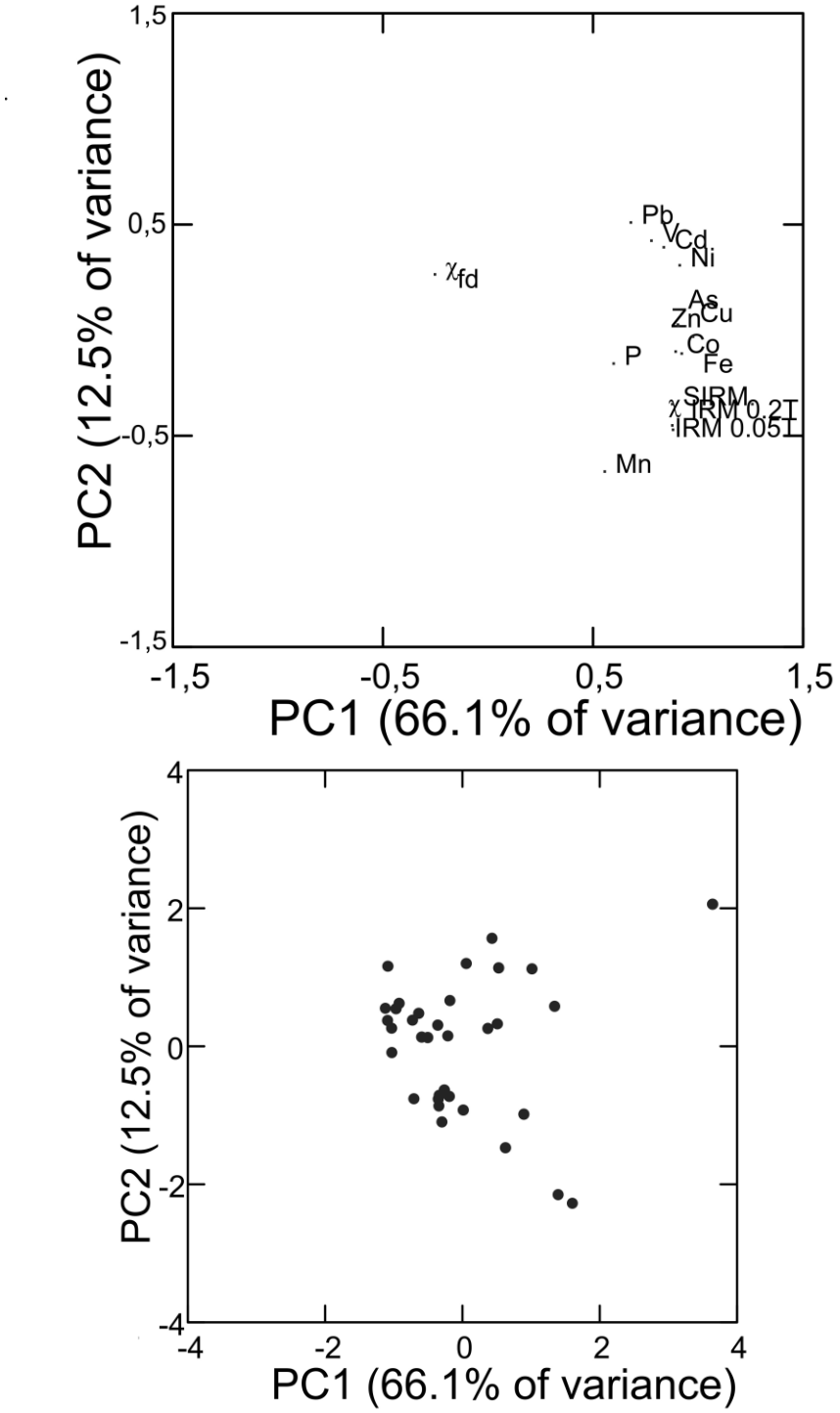

Fig. 4.4. Results of PCA: loading plot (top) and score plot of the 34 sites (bottom) of trace element concentrations and magnetic intensities.

4.8.3. Analysis Of Variance (ANOVA) results

An ANOVA analysis was run on the scores of PC1 and PC2 to study the ability of the components of the PCA in discriminating among trees in different typology of urban sites. The results of the ANOVA analysis (Fig. 4.5) showed no significant differences in discriminating land use for PC2, while for PC1 significant differences (p=0.004) were obtained. The results from a post-hoc test (Tukey test) for PC1 showed a significant difference between green spaces and large roads (p=0.039) and between small roads and large roads (p=0.006).

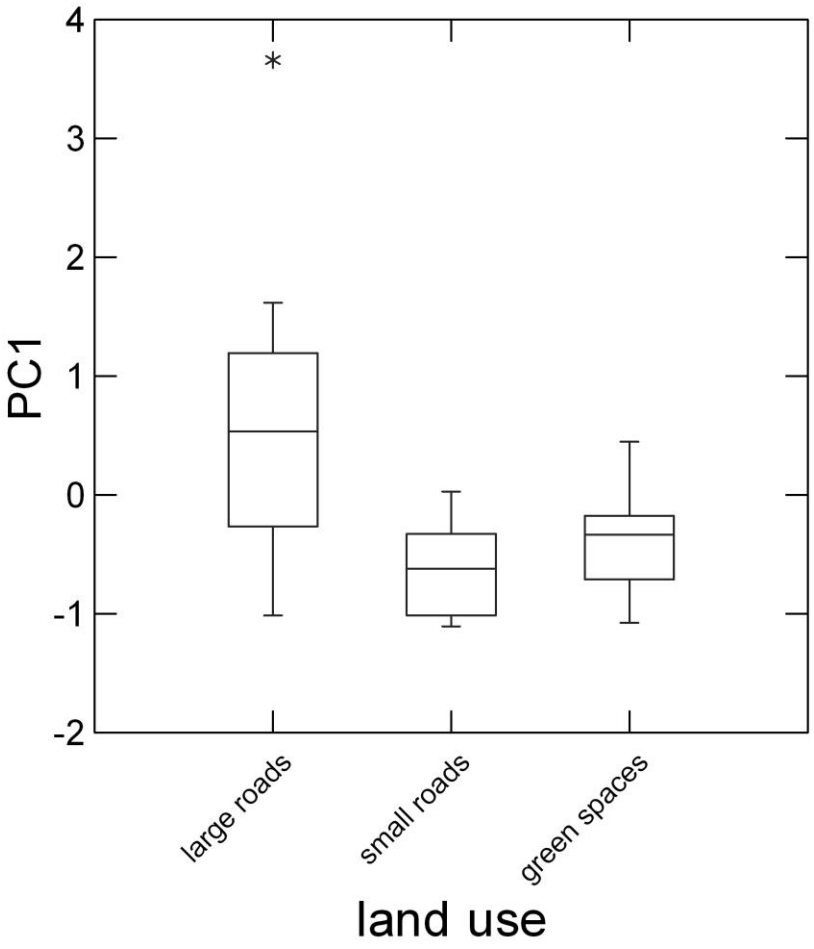

Fig. 4.5. Box plot of land use classification according to PC1 from PCA analysis on magnetic intensities and element concentrations.

4.9. Discussion

Magnetic analyses in tree bark can be used as a proxy of metal loads in urban areas.

Several studies have proven the reliability of tree bark as a bioindicator able to provide information about trace element concentrations in the atmosphere. In this study, a good correlation between magnetic and chemical data (trace element concentrations and magnetic intensities) was observed. A common origin or a causal relation between magnetic particles and trace elements may be hypothesized based on this correlation. Several authors (Baghdadi et al., 2012; Lu and Bai, 2006) formulated the hypothesis that this correlation could be related to the incorporation of trace elements onto the surface or in the lattice structure of pre-present magnetic particles. In line with this hypothesis, in this study a significant correlation was observed between the major traffic related elements and the magnetic parameters. These results are in agreement with the results found by Lu et al. (2005) in the magnetic analyses of automobile particulate emission. In the present study, the poor correlation found between Pb concentration and magnetic parameters are in contrast with the findings of Lu and Bai (2006) and Maher et al. (2008). Probably, such difference in the results could be related to the different structure of the sampling area considered and to a reduced resuspension from roads (Pb is no more emitted since 2001 in most of European countries). For the other trace elements analyzed (As, Cd, Co, Mn and P), a good correlation with the magnetic parameters was found. This is probably due to the high correlation among them (probably due to a common emission source, domestic or traffic) and to their good correlation with Fe. Arsenic is frequently related to coke production (Drava et al., 2016); even though the concentrations found in our samples were comparable to those reported for an industrial

site (Drava et al., 2016), such activities are not reported in the sampling area. Cadmium emission has been associated both with the wearing of the brake (Tanner et al., 2008) and with traffic levels (Khan et al., 2011; McKenzie et al., 2009), thus given the absence of other evident point sources, Cd emission in Lisbon is probably traffic related and the relation with magnetic particles could be a result of the breaking process and/or combustion process in vehicles exhaust. El-Hasan et al. (2002) related the emission of Co and Mn to automobile emission and to the corrosion of automobile parts, thus these two causes could be reasonably the main sources of the two trace elements in Lisbon.

Large roads, considered as a possible source of particles, can be effectively distinguished from green spaces and small roads.

Figure 4.4 points out how, being absent in the city specific industrial emission sources, large roads in Lisbon are the most critical site in terms of atmospheric pollution, as indicated by tree bark samples exhibiting a magnetic profile and a chemical composition different with respect to the samples collected in green areas and small roads. The higher traffic volume in large roads determines an increased automobile exhaust emission; the presence of stop lights and of uphill and downhill roads may also contribute to create local variability in chemical and magnetic composition and profile. Gautam et al. (2005) and Santos et al. (2017) showed in their research how green areas are able to reduce the dust effect produced by roads; consequently, in the inner part of the green spaces the concentration of trace elements is lower and so is the magnetic profile (Kardel et al., 2012). One possible explanation for the lower magnetic profile and trace element concentration of small roads compared to green spaces may be associated with the dimensions of the green space itself. Large green spaces are clearly different from

streets, but with the decrease of the dimensions of the green area, the similarity with small roads in terms of chemical and magnetic profile increases. Considering that green areas could actively decrease the dust impact at respirable height (Gautam et al., 2005), it is important an effective planning of new green areas in high traffic/polluted roads to decrease dust particle emission.

4.10. Conclusions

A high spatial resolution is a key factor for air monitoring studies in big cities, given the wide variability that may occur in the different areas of the city, mainly related to local situation of wind, road directions (uphill, downhill), resuspension and other factors. The results obtained highlighted how the use of biomonitors, i.e. tree bark, is an effective way to obtain such resolution, being possible the collection of a representative number of samples. Moreover, the project has pointed out how the non-destructive magnetic techniques can be efficiently used in biomonitoring programs, as a good proxy of the overall metal loads. The combination of the two techniques (chemical and magnetic) proved to be an efficient tool in discriminating the effects of atmospheric pollution on different urban areas.

5. A parallel project: Is there a risk of trace element contamination in herbal preparations?

This chapter refers to the published article: *Is there a risk of trace element contamination in herbal preparations? A test study on the lichen Cetraria islandica.* Paolo Giordani, Vincenzo Minganti, Daniele Brignole, Paola Malaspina, Laura Cornara, Giuliana Drava. Chemosphere 181 (2017) 778-785.

5.1. Introduction

"Lichens are the symbiotic phenotype of nutritionally specialized fungi that live as ecologically obligate biotrophs in symbiosis with algal and/or cyanobacterial photobionts" (Giordani et al., 2017). These organisms, thanks to their colonizing ability, can be found in terrestrial habitats characterized by extreme environmental condition (Nash, 1996). One of the important results of this symbiotic interaction is the production of extracellular secondary metabolites, which can be used as a source of products with biological activity for human use (Huneck and Yoshimura, 1996; Ranković and Kosanić, 2015). Almost only the lichen *Cetraria islandica* ("Iceland moss") is still used across Europe in several pharmacopeias, as single ingredient or as a component of combination of products combination products (in coated tablets, hard capsules or herbal teas) (Committee on Herbal Medicinal Products, 2014). A decoction or infusion preparation of *C. islandica* can be used as a remedy for pharyngeal irritation associated with dry cough and, in some cases, for the treatment of temporary loss of appetite (Crawford, 2015). Naphthoquinones, depsidones (e.g. fumarprotocetraric, protocetraric and cetraric acid) and polysaccharides (esp. glucans, e.g. lichenin and isolichenin) are among the products of *C. islandica* potentially implicated in its antimicrobial activity (Bradley, 2006; Gülçin et al., 2002; Zarabska-Bożejewicz et al., 2015). Moreover, extracts of *C.*

islandica were tested for antiviral and immunomodulation activity (Olafsdottir et al., 1999; Stübler and Buchenauer, 1996) and were observed to be active against *Mycobacterium aurum* (Ingólfsdóttir et al., 1998).

Surface complexation, bio-mineralization, and physical trapping of dust and soil particulates are among the main bioaccumulation mechanisms through which lichens can absorb both essential and non-essential elements (Purvis and Halls, 1996). Given the absence of protective cuticles they are exposed to trace element bioaccumulation, and *C. islandica*, as foliose lichen, is more subjected to accumulation because of the wider surface area exposed to the atmosphere. Minganti et al. (2014) used herbarium specimens of *C. islandica*, collected from 1981 to 2007 in Italy, to reconstruct trends in trace element deposition, pointing out the variation caused by the impact of human activities. The main sources of *C. islandica* for pharmaceutical use are the natural stands of this lichen principally founded in northern and eastern European countries (Committee on Herbal Medicinal Products, 2014). Since that there is the possibility that even lichen thalli growing in remote areas could be exposed to environmental pollution (Giordani et al., 2014), it is extremely important an effective a-posteriori quality control of the material collected. Moreover, in order to avoid any harmful effect of high levels of contaminants, the concentrations for some metals in foodstuffs have been regulated by several international institutions; as an example, the European Union has decided a limit value of 3.0 mg kg^{-1} for Pb (European Community, 2008) and a limit value of 1.0 mg kg^{-1} for Cd (European Community, 2014). One important factor, which is expected to modify the availability of elements ingested, resulting in a possibly out-breaking of health problems due to acute or chronic poisoning, is the procedure used for the preparation of herbal products. In a decoction, cold water is poured onto the herbal

material, grinded or cut to a proper size, and then heated until the boiling point; the preparation is then let boiling for a period of time depending on the dimension and the type of the herbal material (Committee on Herbal Medicinal Products, 2010). Thus, decoction can extract trace elements from the plant material, transferring them to the herbal preparation (Malik et al., 2013).

This project aimed to study the possible trace element contamination of *C. islandica* herbal preparations, hypothesizing that different raw materials and preparing procedures may have an influence on the potential toxicity of the final product. In order to achieve this result, samples of *C. islandica* collected in different areas of Europe and supplied by several companies were compared for their content of several trace elements both in dried material and in their decoctions.

5.2. Materials and methods

5.2.1. Sample collection

Samples of *C. islandica* herbal preparation (n=14) were bought from different sources on the European market (Table 5.1).

Table 5.1. Characteristics of the analyzed samples.

Sample	Provider	Origin	Country
1	Shop	Northern Europe	Estonia
2	Shop	Northern Europe	Iceland
3	Shop	Balkans	Bulgaria
4	Shop	Balkans	Bosnia
5	Shop	Undeclared	-
6	Shop	Undeclared	-
7	Shop	Undeclared	-
8	Company	Northern Europe	Poland
9	Company	Balkans	Montenegro
10	Company	Balkans	Montenegro
11	Company	Balkans	Romania
12	Company	Balkans	Bulgaria
13	Company	Balkans	-
14	Company	Balkans	-

For all the samples, the material obtained was more than 100 g, with the amount varying based on the selling terms of conditions. The samples were divided in two categories, one for the samples (n=7) provided by different companies (Provider = “Company”) which were packaged and with a quality assurance report; the other (Provider = “Shop”) for the samples (n=7) directly bought un-packaged in herbalist shops or on-line and without quality assurance report.

The origin of the herbal material was divided in two main groups as follow:

i) Northern Europe: Iceland, Estonia and Poland
ii) Balkans: Montenegro, Bosnia, Bulgaria, Romania and sample indicated as Eastern Europe

For three samples, it was not possible to know the origin as it was undeclared.

5.2.2. Chemical analyses

The chemical analyses were performed according to the procedures described in the section *2.2.4 "Chemical analyses"* of the Chapter 2. Please refer to it for a detailed description.

5.2.3. Decoction preparation and analysis

For the preparation of the decoction 100 mL of ultrapure water was poured on 5 g of dried thalli, heated to boil and maintained boiling for 3 minutes. Following a cooling period, 5 mL of the decoction were put in 25 mL volumetric flasks, with air-cooled condenser and added of 5 mL of nitric acid. The solution obtained was digested for 3 hours at 160 °C. After the digestion, the solution was brought to volume and analyzed for the trace elements selected with the same method used for the dried material.

5.2.4. Data processing

In order to study any significant differences in the concentration of trace elements according to several categorical predictors, a factorial ANOVA was performed.

The categorical predictors were the following:

i) Origin (Northern Europe, Balkans and undeclared origin)

ii) Provider (company vs. shop)

iii) Preparation (exsiccata vs. decoction).

The model was computed considering also the interaction among the predictors; moreover, Tukey HSD post hoc tests were performed to check any significant pairwise difference between categories.

The ANOVA analysis and all the calculations were performed with the software package Statistica Version 8.0 (StatSoft, Tulsa, OK, USA).

A PCA analysis was also performed on auto-scaled data, to better visualize all the information in the dataset of the dried lichens and of the corresponding decoctions. The analysis and graphs were performed with Systat for Windows Version 13 (Systat Software Inc., USA).

5.3. Results and Discussion

The effects of the considered factors, namely Provider, Preparation and Origin, plus the effect of the interaction of Provider and Preparation on elemental concentration in *C. islandica* samples, were evaluated by means of an ANOVA test (Table 5.2). The factor "Provider" was found to have a significant effect on the concentration of Co, Fe, P and V. Their concentrations were lower in the products provided by companies than those from shops. The factor "Preparation" (exsiccata vs. decoction) was found to be extremely significant ($p < 0.01$) for all the elements except for Mn, Ni and Zn; in general, their concentrations were lower in decoctions than those in exsiccata.

No significant effect was found, except for Mn and P, for the factor "Origin", and even though Mn and P showed significant differences, no specific geographic pattern was found.

The effect of interaction of the factors "Provider" and "Preparation" was found significant only for Cd, Co, Fe, Pb and V.

Table 5.2. Factorial ANOVA univariate tests on the single and interactive effects of Provider, Preparation and Origin on the concentration of 11 elements in *Cetraria islandic*a herbal products. Statistically significant F-statistics are in bold (*p < 0.05; **p < 0.01). Total degrees of freedom for each univariate test = 27..

Element	Intercept	Provider	Preparation	Origin	Provider×Preparation
As	**117.052****	3.858	**21.499****	1.960	0.000
Cd	**73.157****	2.093	**56.368****	0.375	**4.712***
Co	**92.731****	**10.492****	**49.368****	1.651	4.503*
Cu	**280.753****	2.571	**374.336****	0.623	1.876
Fe	**201.535****	**11.481****	**244.697****	0.518	**11.511****
Mn	**21.437****	2.157	1.035	**6.494****	0.134
Ni	**139.179****	0.133	0.861	1.745	2.737
P	**441.822****	**9.415****	**13.646****	**5.618***	0.852
Pb	**48.362****	3.652	**57.720****	1.045	**5.432***
V	**328.088****	**7.927***	**268.602****	0.765	**15.441****
Zn	**282.201****	1.416	2.222	0.040	2.371

A Tukey HSD post-hoc test pointed out more complicated and element-specific relationships (Table 5.3). In exsiccata, significant differences in elemental concentrations between the samples from shops and those provided from companies

were found; the concentrations of Cd, Co, Fe, Pb and V were significantly higher in samples from shops. The differences in terms of elemental concentrations between exsiccata and decoctions were, in most cases, independent from the provider of the sample. No differences were found between samples from Shops and those from Companies, within decoction samples.

Table 5.3. Mean and standard deviation (mg g^{-1}) of the element concentrations detected in exsiccata and decoctions of *Cetraria islandica* provided by different sellers. Letters represent homogeneous groups ($p > 0.05$) after Tukey HSD post-hoc test.

Element	Exsiccata, Company	Exsiccata Shop	Decoction Company	Decoction Shop
As	0.39±0.13bc	0.44±0.18c	0.18±0.06a	0.23±0.09ab
Cd	0.16±0.05b	0.28±0.12c	0.03±0.01a	0.03±0.02a
Co	0.12±0.06b	0.20±0.07c	0.04±0.01a	0.05±0.01a
Cu	1.99±0.38b	2.31±0.42b	0.03±0.01a	0.05±0.02a
Fe	228±71b	352±56c	11±8a	15±13a
Mn	54.7±31.3a	47.7±55.3a	46.8±29.4a	31.1±31.5a
Ni	0.47±0.17a	0.69±0.16a	0.52±0.29a	0.49±0.23a
P	370±82ab	441±117b	294±61a	314±72a
Pb	3.1±1.1b	5.7±2.7c	0.2±0.1a	0.2±0.1a
V	0.79±0.21b	1.21±0.18c	0.12±0.04a	0.12±0.04a
Zn	24.3±3.7a	32.8±11.9a	24.5±4.8a	24.6±3.5a

In order to better visualize the results obtained, a Principal Component Analysis (PCA) was performed on auto-scaled data and on the two data-set separately (exsiccata and decoctions).

The bi-plot (scores of samples and loadings of variables) of Principal Components 1 and 2 for exsiccata (Fig. 5.1 top) and for decoctions (Fig. 5.1 bottom) allowed visualizing the

different origins among the considered samples. According to the graphs the only sample clearly distinct from the others is the one from Iceland (sample 2), while for the others no clear separation is possible based on the origin. This sample shows the lowest concentration of As, Cd, and Pb and the highest concentration of Co and P among exsiccata samples.

Even though the decoctions seem more similar among them than the exsiccata used for their preparation, the decoction of the sample from Iceland is clearly distinct from the other preparations (Fig. 5.1 bottom) and it is characterized by high concentrations of Co, Cu, Fe, P and Zn and low concentrations of Cd, Ni and Pb.

The PCA plot in Figure 5.2 (top) allows visualizing the different providers for the exsiccata samples, while the one in Figure 5.2 (bottom) shows the decoctions prepared from the corresponding exsiccata samples. Higher element concentrations for exsiccata were shown for shops and online samples, compared to those from company (Fig. 5.2 top); however, no “Provider” separation is possible for decoctions (Fig. 5.2 bottom).

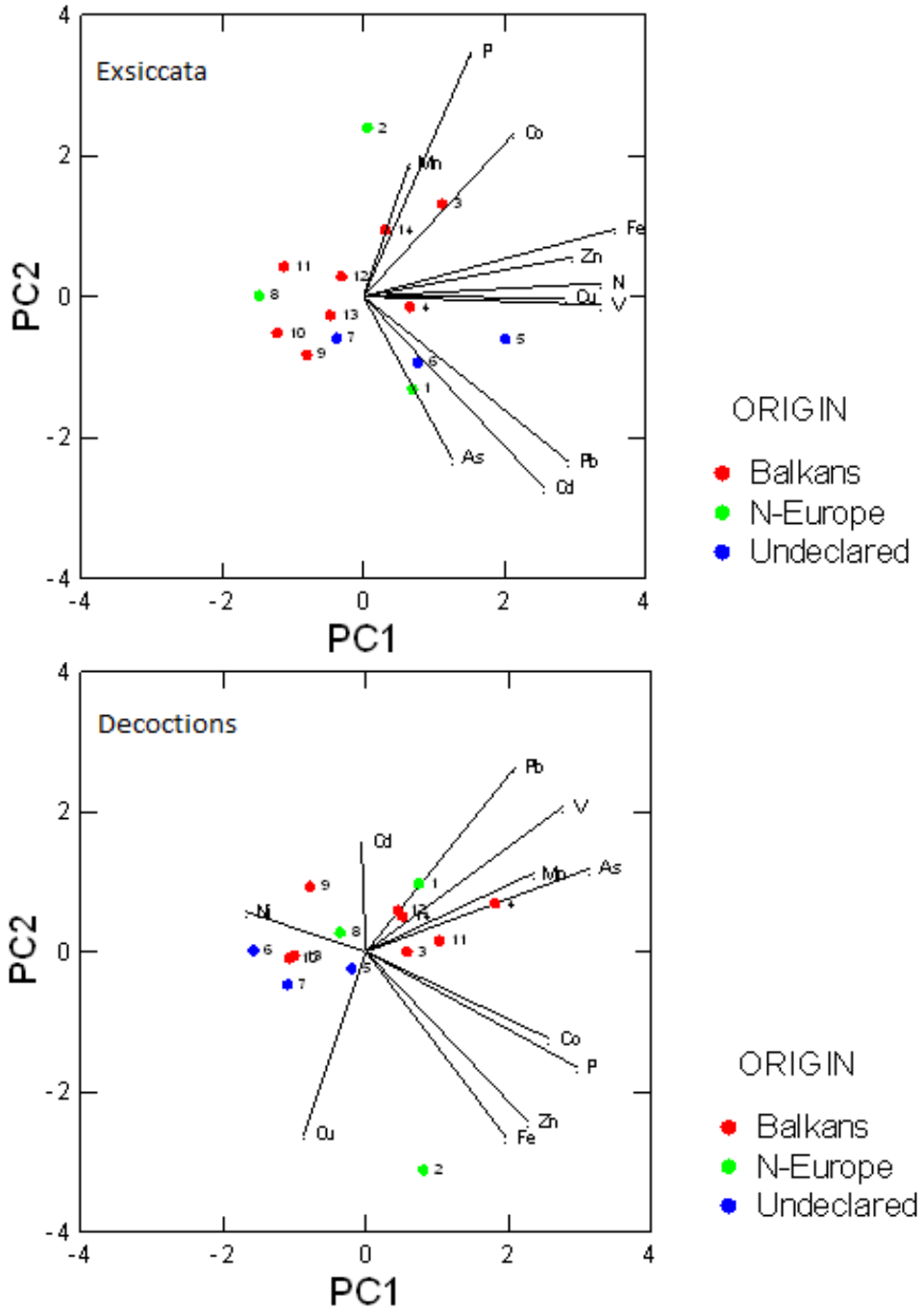

Fig. 5.1. Bi-plot of PCA applied to 14 samples of exsiccata (top) and to the corresponding decoctions (bottom) described by 11 variables (element concentrations). The different symbols represent different origins:
Green = Northern Europe; Red = Balkans; Blue = Undeclared.

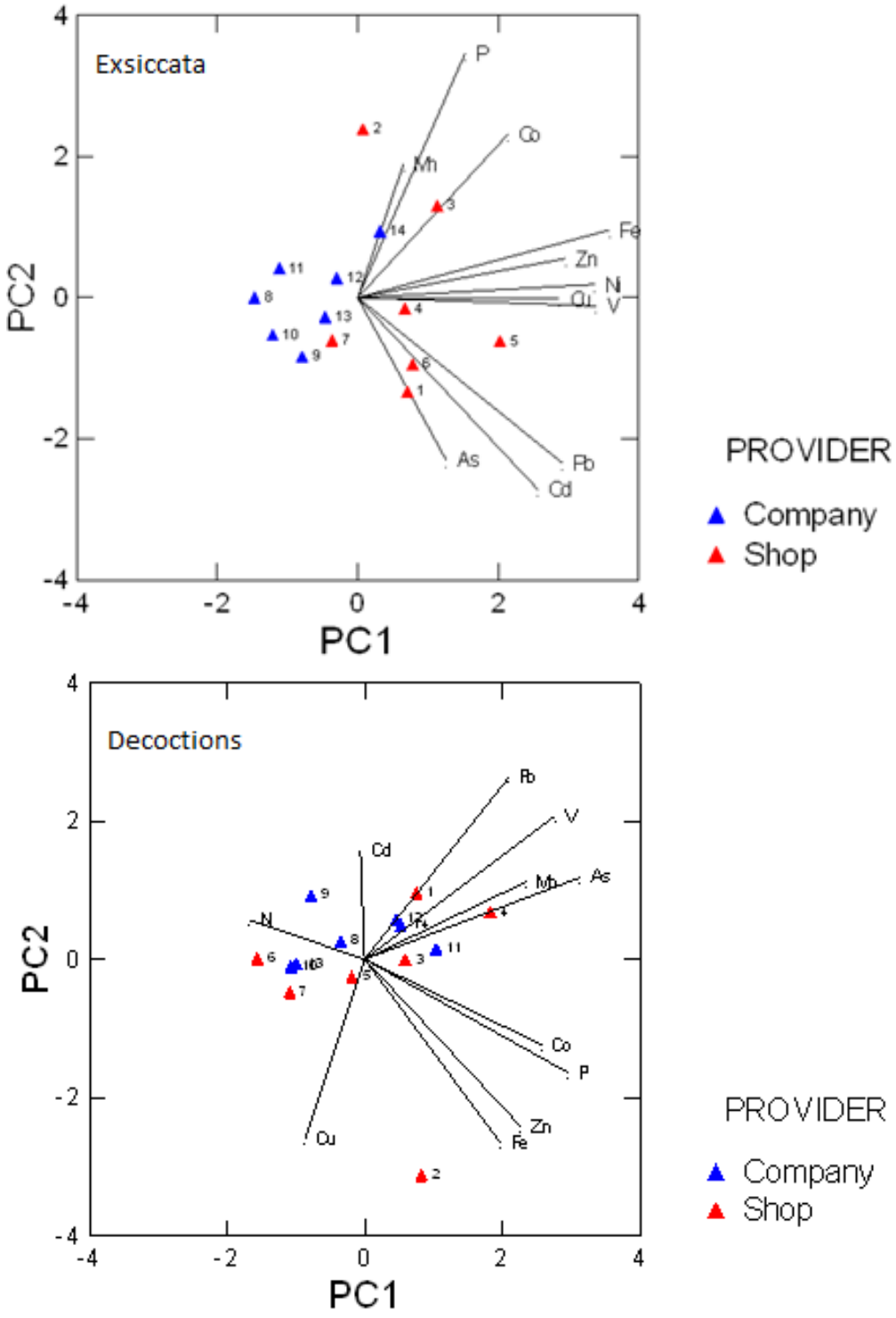

Fig. 5.2. Bi-plot of PCA applied to 14 samples of exsiccata (top) and to the corresponding decoctions (bottom) described by 11 variables (element concentrations). The different colors represent different providers:
Blue = Company; Red = Shop.

In addition, a transfer rate, for each element analyzed, was calculated as the percentage ratio of the concentration measured in the decoction and those measured in the exsiccata (based on the same amount of dried material as starting point). Figure 5.3 shows the graphic visualization of the transfer rate and it points out how that the transfer rate is element specific. Some elements had a low transfer percentage (Cd, Cu, Fe, Pb and V, less than 15%) while others reached over 70% of transfer rate (Mn, Ni, P and Zn).

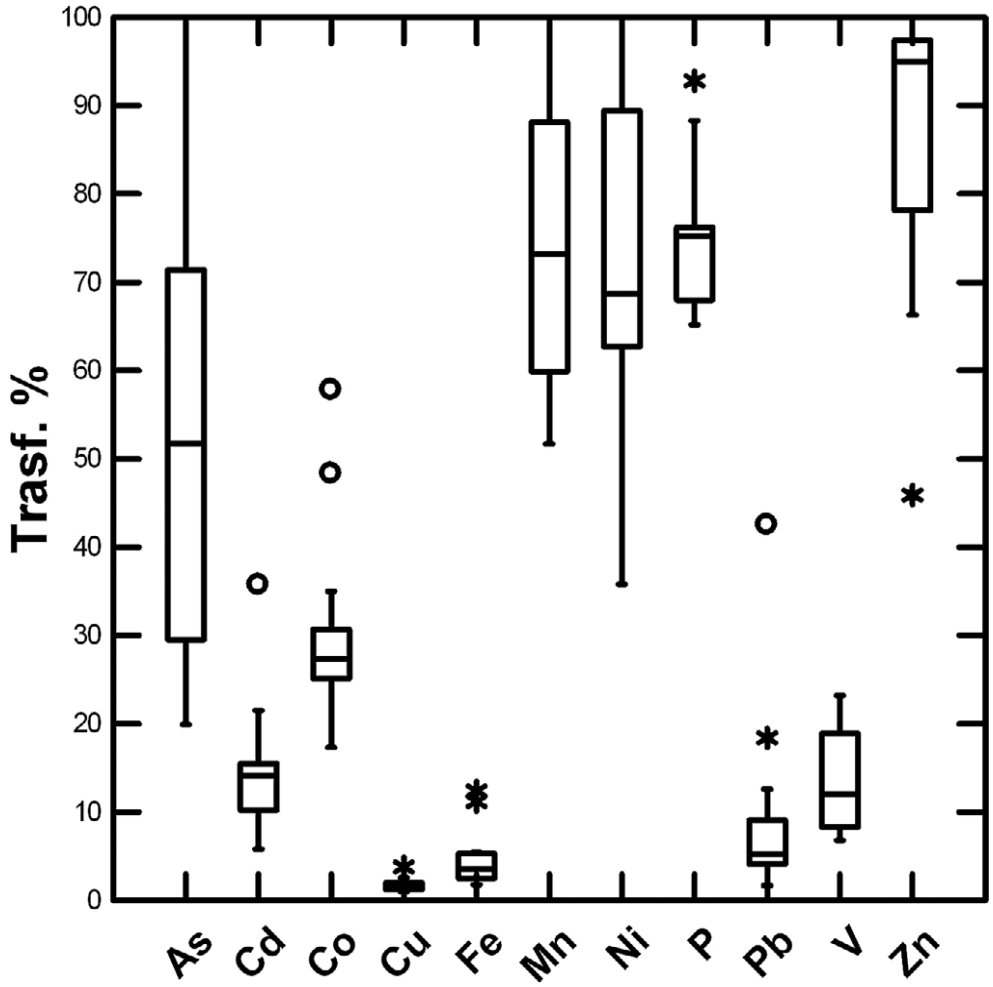

Fig. 5.3. Box-and-whisker plot showing the transfer rate (expressed as %) from exsiccata to decoctions for each element. In each box, the central line marks the median; the edges are at the first and third quartiles; the whiskers show the range of values falling within the interquartile range multiplied by 1.5; values falling outside that interval are plotted with asterisks; values falling outside the interquartile range multiplied by 3 are plotted by empty circles.

5.4. Conclusions

Trace element concentrations for lichen bioaccumulation in contaminated sites, as reported in literature, were higher than those obtained in this study with the analysis of raw material of *C. islandica*. In this study, the results obtained were in line with low contamination sites as reported also by Minganti et al. (2014).

In all the samples of exsiccata bought in shops, except the one from Iceland, the concentration of Pb was exceeding the European value of 3.0 mg kg^{-1} (European Community, 2008) ranging from 5.0 mg kg^{-1} to 8.3 mg kg^{-1}. Lead has been banned from automobile fuels since 2002 in the European Union and as an example, Minganti et al. (2014) reported that the decreasing trend in Pb concentration in herbarium samples for Italy reflected those of lead emission in the same country. However, some Eastern Europe countries used leaded fuels until few years ago, leading to higher concentration of Pb compared to the sample from Iceland. The concentrations of the other trace elements were in line with the recommended limits and comparable to those founded in other European countries (Cercasov et al., 2002).

5.4.1. The effect of geographic origin and providers

Manganese and P were the only two elements analyzed for which the effect of geographic origin was significant, while for the other elements it was negligible. No differences were observed between Northern and Eastern European countries, thus the metal contamination of the material bought from the European market could be considered homogenous. As for samples with unknown origin, the contamination related to the origin could be considered negligible for many trace elements. In this study, the concentrations of trace elements found in samples directly bought from the companies

were lower than those bought into shops. Thus, considering the possibly absence of correlation between geographical origin and elemental concentration, it is possible hypothesizing a contamination of shop samples during storage and transportation, which indeed are relevant steps in the distribution processes of herbal products.

5.4.2. Transfer rate of elements in the decoctions

Lichen raw materials showed a higher concentration of trace elements than those found in the corresponding decoctions, meaning that for these herbal preparations the health concern for the trace elements could be considered negligible. Furthermore, no differences among decoctions prepared from raw material provided by different suppliers were observed, in contrast with the differences detected among raw materials. The results in this study are in line with what found by Kim et al. (2015), pointing out that decoction can be used as a safe procedure for medicine preparation. The formation of insoluble compounds with tannin, alkaloids, proteins, etc., mainly located on the cell wall and inside the cell itself, is one of the main reasons for the concentration reduction of trace elements in decoctions, since they cannot be transferred from raw material to decoction after boiling (Purvis, 2014). The transfer rate was highly element-specific, with values ranging from an average of 2% for Cu to 95% for Zn.

Data reported in literature (Kim et al., 2015) for Pb and Cd transfer rates were lower than those founded in this study; in our data, the average transfer rates for Pb and Cd were respectively 9% and 14% against an average of less than 5% in literature data. Arsenic concentration levels in decoctions were higher (from two to seventy times) than those reported in literature for other herbal preparations such as *Angelicae tenuissimae radix*, *Carthami flos* and *Coptidis rhizoma* (Kim et al., 2015). The accumulation of

metals in lichens follows different main mechanisms such as cytoplasmic immobilization, detoxification of ions by chemical combination and/or transport in ionic state outside of the plasmalemma and even the cell wall (Purvis, 2014). Lichen secondary metabolites might also have an important role on the element-specificity transfer rate, since they can influence metal homeostasis of lichens. For example, in the lichen *Hypogymnia physodes* the secondary metabolite physodalic acid seems less efficient in regulating Fe^{2+} and Zn^{2+} uptake, while it is more efficient in reducing Cu^{2+} and Mn^{2+} uptake (Hauck, 2008). Thus, the localization of elements (e.g. extracellular localization in the mycobiont cell wall) and the stability of metal complexes and the chemical affinity in the cell wall are the main parameters, which can lead to different solubilization ability.

5.4.3. Implications for human health

Several trace elements play an essential role in human health, as integrated part of enzymatic systems or participating in structural and storage functions. Moreover, it has been observed that undesirable pathological conditions can be reversed or prevented with a suitable supplementation of trace elements (Fraga, 2005). However, the long-term assumption of trace elements can lead to higher concentrations and thus to potentially toxic effects. Moreover, the presence of toxic elements (i.e. Cd, Pb and Hg) in herbal preparations can lead to the outbreak of several adverse effects (Başgel and Erdemoğlu, 2006); the effects of these elements can be influenced by nutritionally essential metals. For example, Pb can compete with Ca in neurotransmitter release and in regulating cell metabolism (Goyer, 1995). In this sense, the ability of lichens of metal accumulation can generate potentially toxic effects for humans. For this reason, the European Food Safety

Authority (2012) has listed *C. islandica* among botanical species that can arise concern for human health. Given the lack of proper data, children, adolescents under 18 years of age, pregnant women and women during lactation should carefully consult warnings and precaution of use for this herbal preparation (Committee on Herbal Medicinal Products, 2014). People with verified nickel allergy are exposed to an enhanced risk of allergic events; in fact, Ni is among trace elements with higher transfer rate from exsiccata to decoctions. Moreover, considering an oral PDE (Permitted Daily Exposure) of 300 mg day^{-1} (6 mg Ni kg^{-1} day^{-1} in a 50 kg person) proposed by the European Medicines Agency, based on a NOEL (No-Observed Effect Level) of 5 mg kg^{-1} day^{-1}, and considering a safety factor of well over 800 (European Medicines Agency, 2007), the concentration of Ni in the Iceland moss decoctions (>0.5 mg kg^{-1}) could produce allergic reaction on people with Ni allergy.

6. General Conclusions

The different projects carried out during the three years of the PhD course highlighted different pollution patterns associated with the different sampling location; moreover, these studies contribute to strengthen the idea about how useful is the use of bio-monitors for assessing air pollution in urban areas.

The use of tree bark was tested in different urban environments across Europe (Genoa, N.W. Italy and Lisbon, Portugal) strengthening the effective applicability of techniques using such bioindicator. The possibility to work abroad increased the value of all the research performed, allowing to interact with new working environments and discover new ways to study the same problem. In such scenario, the use of magnetic analysis for air pollution monitoring, performed in Antwerp, Belgium, was efficiently correlated to the classic chemical analysis, providing a new and effective tool for a better understanding of the pollution problem in medium-big cities.

A new method for assessing air pollution was developed, involving the use of tree branch bark; such method was efficiently used for "*a posteriori*" analysis of time trend of pollutants; branch sections were easily associated to the corresponding year of growth and consequently the concentration of pollutants for each year was obtained. Time trends and time profiles of pollutants were the main output of this project giving the possibility to study past situations; this approach could be extended even in areas not covered by monitoring programmes.

In a parallel project, it was possible to test, using lichens for herbal preparation (infusions and decoctions), how safe these preparations are and how much of the trace

elements normally trapped by the lichen species considered was transferred to the solutions.

A new and intriguing project, involving tree branch bark and which is still on-going, aims to verify the correlation between the trace element concentrations, measured in bark samples and Particulate Matter data available from monitoring stations. The first preliminary results are really encouraging, showing a significant correlation for some of the main traffic related elements; these results should be considered as a positive starting point for future analyses to better define and confirm the results obtained.

Moreover, the good results obtained with the use of three branches should be used as a firm point from which continuing the use of such powerful natural archive.

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