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Monitoring environmental quality with trees - A multiscale approach towards the implementation of Nature-Based Solution in urban ecosystems

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Index

List of Figures and Tables

Chapter II.1: List of Figures

Chapter II.2: List of Tables

Chapter II.4: List of Tables

SHORT ABSTRACT

In Anthropocene, an era characterized by heavy human pressures on earth, biological assets are increasingly exposed to environmental changes. In particular, plants are required to detect and respond to environmental drivers, modify their functionality, and ultimately provide important goods and services. In this complex framework, there is a rising need towards the implementation of Nature-Based solutions, based on multidisciplinary and multiscale approaches, to effectively support decision-making processes. With this in mind, it was implemented a multi-scale experimental approach to study risks, opportunities and challenges related to the role of greenspaces in urban contexts, with the final aim to enhance human health and wellbeing. In this case, the multi-scale approach consists in studying the impact of environmental pollution through space and time from single-cell to landscape scale.

On one hand, the investigation in controlled conditions enables localization and quantification of particulate matter (PM) accumulation in plant tissues of target tree species, as well as highlighting related changes in physiological processes. On the other hand, the biomonitoring approach allows the spatial detection of pollutant signals and, combined with dendrochemistry, the retrospective analysis of the effect of pollutants in trees exposed to anthropogenic disturbances. The potentiality of plants to accumulate pollutants and the possibility to reconstruct pollution events provide support to assess the effectiveness of greenspaces in urban contexts. At the same time, the prompt assessment of the extension, structure and function of greenspaces at broad scale (e.g., national), through innovative approaches, further enhances the possibility of including these outcomes in real policy and planning processes.

Comprehensive studies offer an opportunity to include a deeper understanding of functional strategies and structural traits of trees within multi-scale planning processes, thus strengthening the implementation of Nature-Based Solutions towards more resilient and sustainable urban environments.

Keywords: *urban areas, environmental pollution, trees, biomonitoring, particulate matter, sustainable urban planning, NBS.*

EXTENDEND ABSTRACT

This PhD thesis deals with a multi-scale study approach that considers the role of trees in the urban context. The multi-scale approach consists in studying the impact of environmental pollution through space and time, from single-cell of plant to landscape scale, and implement this results in policy and planning processes towards the assessment of the extent and spatial distribution of trees and green areas at broad scale. The results provided useful information to support the implementation of Nature-Based Solution in the urban environment.

Monitoraggio della qualità ambientale attraverso gli alberi - Un approccio multi-scala attraverso l'implementazione di soluzioni Nature-Based in ambiente urbano.

Questa tesi di dottorato ha considerato un approccio sperimentale multi-scala per studiare il ruolo degli alberi nel contesto urbano. L'approccio multi-scala vuole studiare l'impatto degli inquinanti, nello spazio e nel tempo, dalla singola cellula a scala di paesaggio, e implementare questi risultati ottenuti con politiche di pianificazione attraverso la valutazione dell'estensione, struttura e funzione degli spazi verdi ad ampia scala. I risultati hanno fornito utili indicazioni per l'implementazione di soluzioni *Nature-Based* in ambiente urbano.

1.1 Introduction

In the last decades, the interest in the benefits delivered as Ecosystem Services by the forest and trees in and around the cities has been constantly increasing. Trees can detect signals of air pollution recorded in tree tissues (Nabais *et al.* 2001a; Robitaille, 1981; Rolfe, 1974), in order to obtain indication of environmental pollution in space and time (Cocozza *et al.* 2016; Danek *et al.* 2015; Odabasi *et al.* 2015). Trees can be considered a "natural capital" for human well-being due to their capacity to biomonitor and maintain the environmental quality. Therefore, the extent and spatial distribution of greenspaces facilitate the possibility to include trees into future decision on urban planning and management towards the implementation of Nature-Based Solutions (NBS) (Nesshöver *et al.* 2016).

In this work, the **first chapter** presents **a general framework** of the whole study.

The **chapter II.1** focuses on the detection of pollution signals in oak (*Quercus pubescens* Willd.), pine (*Pinus sylvestris* L.) and poplar (*Populus deltoides* Bartram) exposed to **nanoparticles (NP)** supplied in controlled conditions, **in** order to assess the potential of three different **woody species** to accumulate and remove NP from environment.

The **chapter II.2** emphasizes the detection of pollutants in tree growth rings, useful as bioindicators to provide **spatio-temporal information of environmental pollution** in an urban area near a large steel plant. The **chapter II.3** presents new aspects and **new possible researches approach** to the biomonitoring and pollutants detection in tree rings, applied for two different case studies.

The **chapter II.4** aims to develop and test an innovative and cost/time-saving approach to **achieve large-scale statistically-sound estimates of greenspaces** with particular regards to their structure (i.e., abundance, coverage, average size and tree cover density) and ecological functions (i.e., number of people living in the proximity and related ecosystem services provision).

Finally, this thesis reports the main results of the following three activities and includes **a general conclusion**, **chapter III,** on how the ecological studies can be effectively utilized in a multi-scale planning process.

1.2 Materials and Methods

The first study was carried out through an experiment approach, using plants grown in controlled conditions and envisaging the supply of nanoparticles in a greenhouse in Switzerland. The investigation was aimed to define particles accumulation in plants and to identify the effects of pollutants accumulation on physiological and biological processes.

The second study adopted a dendrochemical approach through sophisticated scientific analysis (e.g., combining laser ablation and synchrotron radiation). Tree cores of 32 downy oaks were sampled at different distances from several pollutant sources, including a large steel factory. Trace element concentrations in tree-ring wood were determined using high-resolution laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Then a synchrotron analysis was performed to visualize the trace elements in tree-rings in 3D.

The third study tested a methodology at national scale (Italy), based on the integration of inventory (the Italian Land Use Inventory- IUTI- Marchetti *et al.* 2012) and cartographic (the Italian Land Cover High Resolution Map, Congedo *et al.* 2016) approaches to detect urban forest and the contribution of very small greenspaces, dominated by trees or grasses.

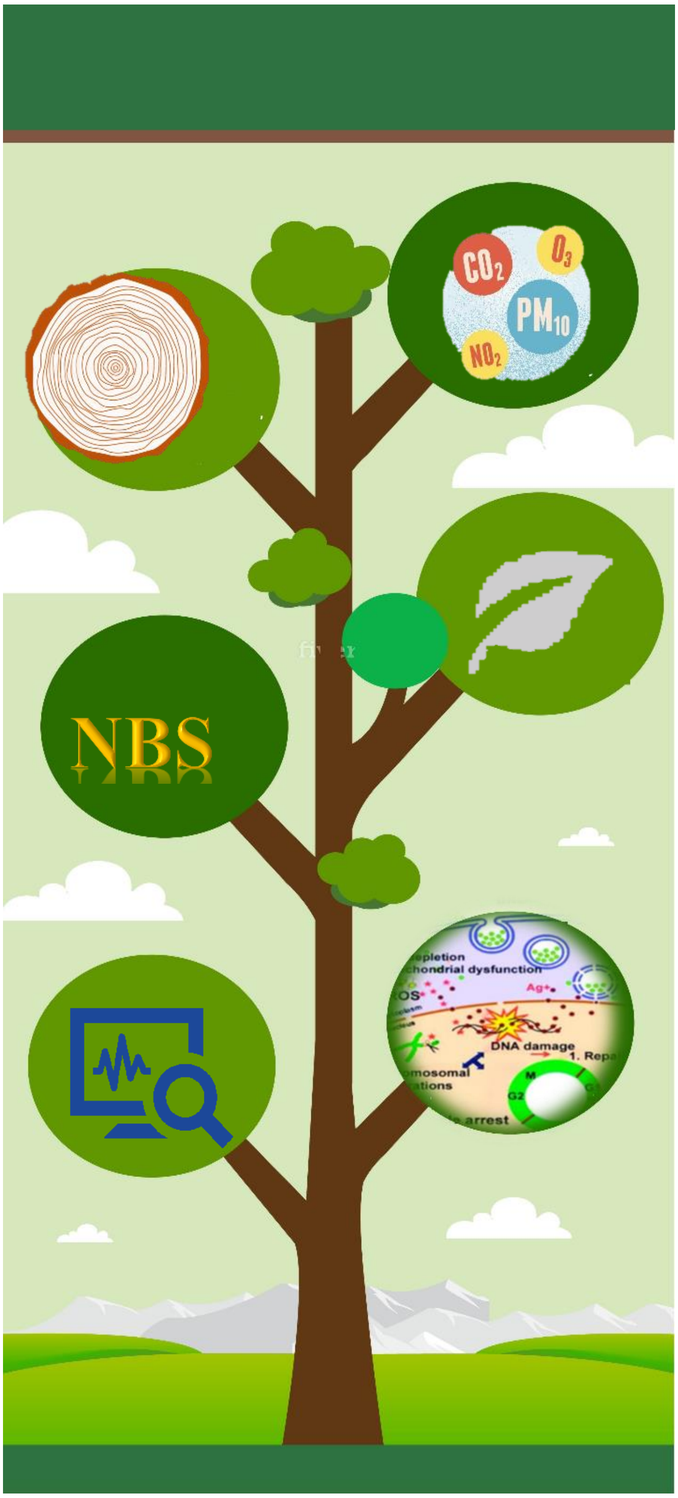
1.3 Results and discussion

The experimental approach (chapter II) evidenced that the pollutants accumulation depends on the species-specific affinity between nanoparticles and plant tissue, the exposure condition, the effective type of contamination and translocation, involving specific physiological and morphological plant modification. These results could be applied in environmental biomonitoring, especially in industrial or urban areas.

The second study (chapter III) revealed that the pollutants in the environment are detected in tree-rings providing information on the type of anthropogenic activities insisting in a defined area with annual resolution. Moreover, tree growth was negatively affected by the industrial plant defining a relevant role of tree monitoring in the assessment of environmental health in urban context.

The third study case (chapter IV) evidenced that in Italy a considerable portion of built-up areas (43.5%) is unsealed and filled by greenspaces (more than 18%), mainly dominated by urban forest. Results revealed that the number of forested greenspaces decreases with the increase of the population density, as well as their coverage and average patch size. The proposed approach provided reliable greenspaces estimates to better understand interactions between humans and nature along an urban-rural gradient, representing a valuable support towards the implementation of Nature-Based Solutions in urban planning.

I CHAPTER - GENERAL INTRODUCTION & STATE-OF-THE-ART



(I) 1. Air quality and pollution in urban environment

"Love is in the air, but air is highly polluted" (cit. environmental researcher Amit Abraham)

Industrialization provided humanity with materials and social benefits, though altering the environment and human behaviour (Sharma *et al.* 2016). Cities comprise less than 3% of the Earth's surface, where there is an extraordinary concentration of population, industry and energy use, leading to a massive local pollution and environmental degradation (Ksenija, 2016), that adversely affect ecological integrity and diversity and human health and well-being (EEA, 2017).

Air pollution is one of the most important problems related with industrialization and urbanization, and is of major concern to societies for its effects on the environment and human health. In the past two decades, the attention of science and society was particularly focused on the effects of air pollution particulate matter (PM, particles with aerodynamic diameter of less than 10 μm) and nanometer-sized particles or "nanoparticles" (NP; particulate matter with aerodynamic diameters $\leq$2.5 μm) (Wesselinova 2011).

These particles show a heavy toxicity, when accumulated in the environment and when are inhaled, causing diseases to humans and adverse physiological effects (Qian and Wang, 2013). The chemical composition of particles is defined by their composition, which is affected by the pollutant sources (Mazzei *et al.* 2008), and the size of particles. In the urban context, motor vehicles (emitting Pb and Cd) and industry (emitting Cd, Cr, Cu, Hg, Ni, V, Zn) exert the greatest influence on environmental health (Davydova, 2004). The composition, the size and the origin of atmospheric particles define the toxicity of a pollutant, as well as the processes of dispersion in the environment and the methods for the detection (Schauer *et al.* 1996).

One of the possible strategies for mitigating air pollution is the promotion of the remediation role of vegetation, that through the ad/absorption of pollutants and particulate matter (Varshney 1985) maintains the ecological balance in the urban environment.

(I) 1.1 Mechanisms of pollutants uptake by plants

"*A tree can save the world*" (cit. forester researcher Davis Nowak, 2014)

The biogeochemical behaviour of pollutants and particulate matter in environment-plant system is still not well investigated, although highly newsworthy (Shahid *et al.* 2017). **Plants can uptake and translocate contaminants through the root tissues and the aboveground organs** (e.g., cuticles, trichomes, stomata, stigma and hydathodes) (Wang *et al.* 2016) (Fig. 1). The interaction between plant and contaminants depends upon the intrinsic properties of contaminants and plant species (Máthé and Anton, 2002).

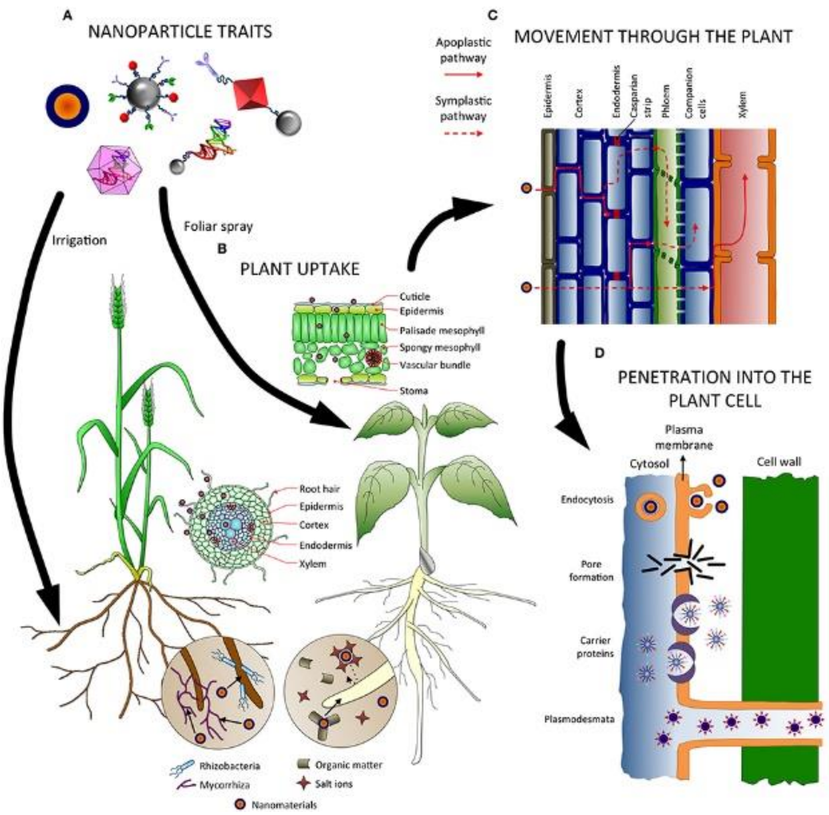


Figure 1: Different pathways of uptake and transport of nanoparticles in plants (Perez-de-Luque, 2017).

Several researches identified the root uptake of metals as the major pathway by which pollutants (heavy metals and particles) enter the plants, even if only few studies compared the influence of the two ways (roots and leaf) of pollutants uptake by plants (Schreck *et al.* 2013) and any of those give information on tree species. The role of foliar and root uptake of particles and heavy metals requires investigations for the understanding of speciation, toxicity, compartmentation and detoxification in plants (Schreck *et al.* 2014; Shahid *et al.* 2017). It is very difficult to differentiate that contaminants within plant tissues either is taken up by root from the soil or by leaf from atmosphere, because the two kinds of uptake pathways can occur simultaneously in urban and industrial areas. For root metal uptake, generally, the metal in the soil is adsorbed onto the root surfaces, then passively penetrates the roots and diffuses through translocating water streams (Seregin *et al.* 2001). In the case of foliar transfer, PM may be adsorbed on the leaf surface of plants due to wax cover and leaf hairs (Saebo *et al.* 2012), depending on their size (Schreck *et al.* 2012; Eichert *et al.* 2008). Based on the phobicity of pollutants, two pathways of foliar metal uptake have been hypothesized: the diffusion through the cuticle especially for lipophilic elements and the uptake via aqueous pores of the stomata and cuticle (Larue *et al.* 2014) (Fig. 1).

Even if remains still difficult to determine exactly the contribution of root uptake or leaf uptake during the contamination, plants can translocate contaminants into tree growth rings.

(I) 1.2 Tree-rings to biomonitor environmental pollution

Plants, as sink of pollutants, can be used to monitor environmental pollution (Lin, 2015). The responses of plants can be useful in order to detect or predict changes in the environment and to follow their evolution in space and time (Lin, 2015). These because plants give qualitative information on the status of environment, acting as bioindicator and provides quantitative information on the environment, acting as biomonitoring surces. Using plants is interesting in recent time as it is cost effective, sustainable and environmentally friendly compared to traditional physico-chemical methods. Some plant species are highly sensitive to particular pollutants and show specific responses to these elements by presenting specific effects and symptoms.

To study the role of plants as bioindicator and biomonitoring of environmental pollution it is possible to use tree-rings analysis. Annual growth rings of trees have the potential to store pollutants permitting to record of environmental pollution and analysis of wood chemistry of precisely dated tree-rings, a technique known as dendrochemistry (Watmough, 1999, McLaughlin *et al.* 2002). After entering the trees, heavy metals will deposit in the tree-rings during cambium development. The potential of tree-ring analysis in environmental pollution diagnosis is sill need to improve. The use of dendrochemical techniques to biomonitor environmental for a retrospective analysis of pollution is still on debate (Cheng *et al.* 2007; Garbe-Schonberg *et al.* 1997; Nabais et. al., 2001b, 1996). For example, the radial translocation of elements across tree-rings can compromise the temporal resolution of the technique (Cutter and Guyette, 1993). In fact a fundamental assumption of dendrochemical signals is that heavy metals are incorporated in wood of the recently developing, outermost annual growth increments, and then become immobile.

Even so, several investigation have shown successful ability of trees to store particles and heavy metals at the time of tree-ring formation (Watmough, 1999; de Vives *et al.* 2006; Odabasi *et al.* 2011), showing a correlation between environmental pollution and the concentration of heavy metals in the growth rings. Moreover, they may help also in reconstructing past pollution episodes and levels; however, in many situations this assumption appears tenuous at best (Kennedy, 1992, Poulson *et al.* 1995, Brabander *et al.* 1999). Tree-rings represent a widely available source of long-term data, and, therefore, offer considerable potential for identifying quality and changes in the environment (e.g., Cook, Innes 1989, Stolte *et al.* 1992).

(I) 2. Managing trees and green infrastructure in urban environment

"In this urban century when there are going to be an extra two billion people in cities, smart cities should be thinking about how nature and trees can be part of the solution to keep air healthy" (cit. Kinver from BBC, 2016).

Trees, with their capacity to biomonitor and maintain the environmental quality, can be considered a "*natural capital*" for human well-being. The term natural capital refers to elements of nature that, directly or indirectly, produce values for people (Bajer, 2017). Determining the location and quality of natural capital assets, is now underway especially across cities, as being the best tools for tackling and studying the human pressing problems (Bajer, 2017). Forest and green areas within urbanized landscapes were proved to be key components that significantly improve human health and wellbeing (Konijnendijk *et el.*, 2006) and thus support the city-nature bond (Beatley, 2009). Green in cities provide different ecosystem services: can take up CO_2, cause local cooling thereby ameliorating the urban heat island effect, and can reduce pollution.

To benefit functions of greenspaces, it is important to make sufficient provision of quality and quantity of these areas within urban context (Tang and Zang, 2017). Attributes associated to greenspaces, such as size, shape, accessibility, extend, spatial distribution within a city are directly related to their ecosystem services (Pietrzyk-

Kaszynska *et al.* 2017). **Integrating these indicators into a framework could also be used to monitor the effects or urbanization** and **to foster environmental benefits** in land and urban planning. Monitoring changes in extent and spatial distribution of green areas is important also to the implementation of "new greenspaces" or the modification of existing ones according to social and economic conditions (Lin *et al.* 2017, 2015). Nowadays, reliable large-scale information on this topic are usually limited and confused, depending on different terms used, that mean different things in different counties and socio-economic condition, and can be described and assessed in many different ways. For example the term "urban and peri-urban forest", as specified by many studies (e.g., Konijnendijk *et al.* 2000), usually refers to all trees within and close to urban areas, thus covering a quite generic and wide meaning (Konijnendijk *et al.* 2000).

The recently introduced concept of Nature-Based Solutions (NBS) (Nesshöver *et al.* 2016) has highlighted the general need of adopting clear, standardized and unambiguous definitions and methods to facilitate the effective implementation of a multidisciplinary and integrated approach in analyzing the hybrid and complex urban environment (Taylor and Hochuli, 2017).

(I) 2.1 Implementation of the ecological knowledge with NBS in urban planning

Nature-Based Solutions (NBS) aim to address a variety of environmental, social and economic challenges in a sustainable society (EU Green Infrastructure (GI) – Enhancing Europe's Natural Capital; COM (2013) 249 final). Several **actions are inspired by, supported or copied from nature**, using and enhancing existing solutions, as well as exploring more novel solutions, as green roofs, open green spaces, urban trees (European Commission, 2015). Nature-based solutions use the features and complex system processes of nature, such as its ability to store carbon and the ability of plants to reduce air pollution, **in order to achieve desired outcomes that improves human well-being through an inclusive green growth** (Nilsson *et al.* 2014). This implies that maintaining, assessing and enhancing natural capital (such as, green areas and urban forestry) are important as the basis for urban solutions. In addition, NBS safeguard urban biodiversity and increase the attractiveness and quality of life experienced in urban areas. Interpreting NBS is useful to define the resilience and adaptive processes that do not refer to stabilizing current conditions, but rather to the ability of a complex system to continuously adjust or adapt to changes in society, the economy and the environment (Nilsson *et al.* 2014).

(I) 3. Hypotheses and objectives

The development of a multi-scale investigation approach is essential to study the effects and the signals of pollutants in plant systems, allowing to evaluate risks, opportunities and challenges related to the role of trees in urban contexts and to enhance human health and wellbeing.

The main objectives of the thesis were to assess: a) whether signals recorded in trees, if any within the wood and other tissues, were caused by direct exposure to the high level of particles and heavy metals, if trees can be used as indicators of environmental pollution in space and time, to be useful to monitor environmental pollution; c) how environmental pollution influences tree-ring growth; d) if the biological and functional aspects of trees can be linked to the planning of sustainable urban forestry and e) in which way the monitoring and assessment of greenspaces in cities can be related to the ecological features.

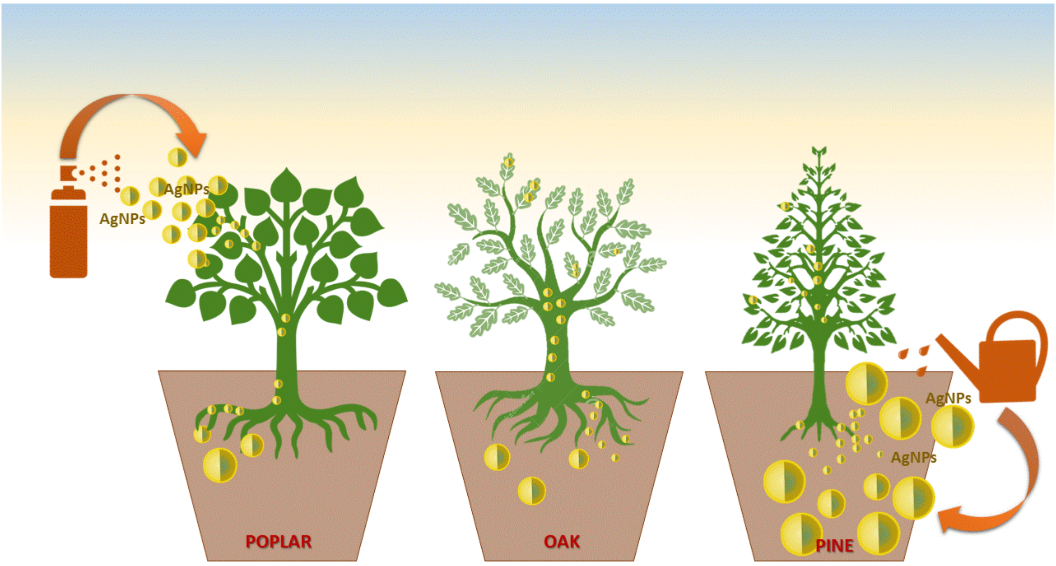
The study on the mechanisms of pollutants uptake by plants was carried out through an experiment, using plants grown in controlled conditions, and envisaging the supply of NP in a greenhouse in Switzerland. The investigation was aimed to define particles accumulation in plants and to identify the effects of pollutants accumulation on physiological and biological processes in different tree species.

The investigation on the capacity of plants to act as a biomonitoring tool and record environmental pollution was carried out through a dendrochemical approach. The study tested the capacity of trees grown in sites exposed to anthropic disturbance in Terni (Central Italy) to uptake target pollutants and translocate them into growth rings, where they can be stored.

The study on the structure of green areas derived from the development of a new methodology at national scale (Italy). The approach is based on the integration of inventory (the Italian Land Use Inventory- IUTI- Marchetti *et al.* 2012) and cartographic (the Italian Land Cover High Resolution Map, Congedo *et al.* 2016) dataset. The main aim was to develope spatial metrics to characterize greenspaces and to provide the need-to-know information to foster environmental benefits in urban planning and management.

II CHAPTER - MATERIALS and METHODS & RESULTS and DISCUSSION

II.1 Chapter - FOLLOWING SILVER NANOPARTICLES IN WOODY SPECIES THROUGH FOLIAR AND ROOT UPTAKE



Abstract

Particulate matter is one of the key global air pollutants. Nowadays the anthropogenic release of nanoparticles (NPs) in the environment poses a potential hazard to human, animal and plant health. NPs may enter in plants by active or passive uptake. Plants may transport and storage pollutants in tissues. An experiment was performed in greenhouse to assess uptake of NPs and to follow their transport in the leaves, stems and roots of three different tree species: oak (*Quercus pubescens* Willd.), pine (*Pinus sylvestris* L.) and poplar (*Populus nigra* L.). Soluble NPs of silver (Ag-NPs) were supplied to plants through leaf and soil application, separately, and they were not subjected in plants as reference treatment. The Ag uptake, transport and accumulation were investigated using a combination of spectrometry and microscopic techniques. The results show that Ag accumulation were strongly influenced by the type of exposure (root or leaf level) and the plant species. Leaf exposure to Ag-NPs evidenced through ESEM analysis the adsorption of NPs on leaf surface of three plant species. Leaf application of Ag-NPs showed effects on plant growth, aboveground biomass and stem length in poplar plants, whereas it did not affect leaf traits of oak and pine. The photosynthetic potential was not clearly affected by Ag-NPs, although species-specific changes in oxidative stress and antioxidant system were observed. Soil application of Ag-NPs showed a lower NPs transfer than leaf treatment. Moreover, Ag treatment induced some significant variations in the concentration of leaf and root cultivable bacteria and/or fungi. The results highlighted species-specific mechanisms of interaction, transport, allocation and storage of NPs in woody species.

Keywords*: Ag-NPs, pathway of uptake, Quercus pubescens* Willd., *Pinus sylvestris* L., *Populus deltoids* Bartram

(II.1) 1. Introduction

The plentiful release of particulate matter, namely nanoparticles (NPs), from natural and anthropogenic sources into the environment causes air pollution with heavy hazards to human, animal and plant health and life, particularly in urban areas (Dietz and Herth, 2011; Tripathi *et al.* 2017).

Plants can uptake and translocate NPs through the root tissues (Wang *et al.* 2016). After adsorption on root surface, pollutants passively penetrate the roots and diffuse through translocation water system (Shahid *et al.* 2014). Indeed, deep-rooted vegetation is a compelling hydraulic lift for the transport and storage pollutants in plant tissues (Lintern *et al.* 2013). However, the pollutants availability at root level identifies a minor pathway of particles transfer compared to foliar uptake (Schreck *et al.* 2014). Indeed, an important role in airborne NPs adsorption is assigned to the aboveground plant tissues (Schreck *et al.* 2014; Wang *et al.* 2016), where canopy represents a relevant NPs capture system (Birbaum *et al.* 2010; Nair *et al.* 2010; Turan *et al.* 2011; Larue *et al.* 2014; Sgrigna *et al.* 2015). Pollutant may be bound on the leaf surface through cuticular wax, as well as diffused through the lipophilic elements of cuticle and leaf hairs (Sæbø *et al.* 2012), and via aqueous pores of the stomata (Larue *et al.* 2014). Once inside the cells, NPs may be transported apoplastically or symplastically through plasmodesmata (Rico *et al.* 2011).

Foliar uptake of NPs is affected by several factors, as size (e.g., compatible to stomata aperture; Raliya *et al.* 2016) and form of nanoparticles (e.g., spheres, truncated cubes, rhombic dodecahedra, and rods; Raliya *et al.* 2016), environmental conditions (e.g., light, wind and moisture; Wang *et al.* 2013a), plant species (e.g., phloem translocation of NPs was not efficient in maize, Birbaum *et al.* (2010) in comparison to watermelon, Wang *et al.* 2013a), and mode of application (e.g., in powder or suspension treatment, Hong *et al.* 2014). The NPs-plant tissue affinity is species-specific, though the mechanisms of uptake, translocation, storage, and toxicity of NPs remain largely unknown (Máthé and Anton, 2002; Zhai *et al.* 2014). Indeed, NPs damage plant functionality and, consequently, plant growth (Tripathi *et al.* 2017).

Most of these studies were carried out on annual crops (e.g., Yang *et al.* 2006; Lin and Xing 2007), whereas only few researches were focussed on woody plants (e.g., Nowack and Bucheli, 2007; Navarro *et al.* 2008; Zhai *et al.* 2014). Although NPs may accumulate in woody tissues once transported through the vascular system (Seeger *et al.* 2009; Schreck *et al.* 2014; Shahid *et al.* 2017), the role of foliar and/or root uptake in element speciation, toxicity, compartmentation and detoxification in woody plants has been rarely explored (Schwab *et al.* 2016). In the present study, a new research framework is presented in order to explore the complex process of NPs fate in woody plants, mainly considering the plant exposure to NPs (leaf and root exposure) and the species-specific tissue affinity to NPs. The research was performed supplying silver NPs (Ag-NPs) at leaf and root level, separately, in three different woody species, common in urban and peri-urban environment: downy oak, black poplar and Scots pine, showing different growth habits, leaf traits and stress tolerance. Silver is rarely represented in the Earth's crust, is predominantly concentrated in basalt (0.1 mg/kg) and igneous rocks (0.07 mg/kg), and it tends to be naturally concentrated in crude oil and in water from hot springs and steam wells (Howe and Dobson 2002). Whereas, anthropogenic sources of silver are smelting, hazardous waste sites, cloud seeding with silver iodide, metal mining, sewage outfalls, and especially the photoprocessing industry (Eisler, 1997). The industrial revolution provides numerous applications of silver, such as medicine, consumer products (cosmetics and sporting equipment), environmental remediation and information technology (Maillard and Hartemann 2012). In this context, the study is aimed to identify risks for the environment, in terms of silver NPs uptake by trees, that can be assessed to: a) quantify Ag within leaves, stem and roots, considering the NPs supply at top-down (leaf spray) and bottom-up (pot watering) level, in order to define the Ag-NPs translocation pathway and its efficiency in different woody species; b) localize NPs in plant tissues using microscopic analysis, in order to detect apoplastic or symplastic NPs transport pathway; c) assess the effects of NPs on plant traits, growth and functionality.

(II.1) 2. Materials and methods

(II.1) 2.1 Plant material and experimental setup

A pot experiment was performed supplying NPs at leaf and root level of *Quercus pubescens* Willd. (downy oak), *Populus nigra* L. (black poplar) and *Pinus sylvestris* L. (Scots pine). The experiment was carried out in controlled conditions of the greenhouse of the Swiss Federal Institute of Forest, Snow and Landscape Research (WSL) at Birmensdorf, in Switzerland. In winter 2015, 24 three-year-old trees of downy oak, Scots pine and woody cuttings of black poplar were planted into 15L plastic pots filled with soil fertilizer mixture and grown in the greenhouse under well-watered conditions. After 4 months of growth, in May 2016, plants of similar height and one main branch were selected for the experiment. Temperature and humidity were kept constant (20 °C at night and 25 °C during day-time with 40–60% relative humidity). The experimental layout was a randomized block design with eight replicates per species and treatment.

The experimental treatment comprised the supply of NPs using nanopowder, amorphous-carbon-coated with Ag nanoparticles (Ag-NPs) (Novacentrix, Austin, TX). The Ag-NPs were 98% pure, with an average particle size of 25 nm, specific surface area of 23 m^2/gm. The Ag-NPs were directly dispersed in water through surfactant substance to reduce the surface tension of water.

Three different treatments (8 trees for each treatment) were set up: “control”, plants were pot irrigated with tap water; “leaf supply” of Ag-NPs, plants were treated with leaf spray solution of 1 mg Ag-NPs L^{-1} of water. A plastic membrane was placed on the top of the pot to avoid NPs availability in the soil. The leaf spray treatment was performed in a separate room to avoid contamination of other plants. “Root supply” of Ag-NPs, plants were pot watered with a solution of 1 mg Ag-NPs L^{-1}. The NPs concentration was chosen according to literature (Wang *et al.* 2013b; Antisari *et al.* 2014). Fertilizer was not used during the experiment. The Ag-NPs treatments were performed in 10 weeks from May 19 to July 28 (4 weeks with single supply followed by 6 weeks with twice supply), in 16 events of Ag-NPs supply to simulate a chronic exposure to NPs. The final exposure of plants to Ag-NPs was 16 mg L^{-1} in both leaf and root treatments.

(II.1) 2.2 Determination of Ag

(II.1) 2.2.1 Inductively Coupled Plasma - Mass Spectroscopy (ICP-MS)

Root, leaf, stem and soil (n = 5) were sampled at the end of experiment and dried at 80 °C until constant weight, then ground in a metal-free mill (Retsch GmbH, Haan, Germany). Leaf tissues of “leaf supply” plants were washed in distilled water (according to Ugolini *et al.* 2013), for the detection of Ag adsorbed on tissue surface and absorbed in tissue. To determine the concentration of NPs in samples, 0.5 g was digested in aqua regia (Nitric acid: hydrochloric acid, 3: 1). Digested samples were diluted with 4.5 mL 1% nitric acid. Silver concentration (μg kg^{-1}), in each sample was measured with ICP-MS (PerkinElmer, Inc., USA). Silver content (μg) in tissue was calculated as:

Ag content = [Ag] x M;

where [Ag] is the amount of Ag per unit of weight (μg kg^{-1}), and M is the dry plant weight (kg).

(II.1) 2.2.2 Environment scanning electron microscopy (ESEM)

The leaves were analysed using FEI Quanta 200 environment scanning electron microscope (ESEM, FEI Corporation, The Netherlands), operating in low-vacuum mode (the chamber pressure was kept at 1 Torr) at 25 kV, without preparation of the samples. The chemical composition of the tissue was determined by energy-dispersive X-ray spectroscopy (EDX) determined by back scattered electron detection. Each leaf area analysed by EDX in our experimental conditions corresponded to a spot of approximately 50 nm in diameter. The ESEM images (300x magnification) of upper and lower surface leaves were analyzed using the ImageJ analysis software. Stomatal size and density (n°/mm^2), trichrome typology, density and length per unit area (μm^2) were measured.

(II.1) 2.2.3 Transmission electron microscopy (TEM)

Electron microscopy was performed on the middle sections of leaves. The samples were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 1 h and then washed twice with the same buffer, prior to a post-fixing procedure with 2% osmium tetroxide in phosphate buffer (pH 7.2). The specimens were dehydrated in a graded ethanol series (25, 50, 75, 90 and 100%); finally, the ethanol was replaced by propylene oxide. The samples were gradually embedded in Spurr resin (Spurr, 1969) and polymerised at 70 °C for 24 h. Ultrathin sections (70-90 nm in thickness) were obtained using an LKB IV ultramicrotome (Rankin Biomedical, USA), collected on Formvar-coated copper grids, stained with uranyl acetate and lead citrate and finally examined with a Philips CM12 transmission electron microscope (Philips, The Netherlands) operating at 80 kV.

Leaf and root tissues were analysed by TEM (PHILIPS CM 12, FEI, Eindhoven, The Netherlands) equipped with an EDS-X-ray microanalysis system (EDAX, software EDAX Genesis, AMETEK, Mahwah, NJ, USA) for the detection of Ag-NPs. The images were recorded by a Megaview G2 CCD camera (software iTEM FEI, AnalySIS Image Processing, Olympus, Shinjuku-ku, Japan).

(II.1) 2.3 Plant traits analysis

(II.1) 2.3.1 Plant growth

Stem and root length was measured at the end of experiment. Biomass allocation in the different organs (roots, leaves, and stems) of control, leaf- and root-treated plants were determined as dry weight (DW) after 24 h of drying in the oven at 80 °C. The ratio between the biomass of non-photosynthetic and photosynthetic organs ("C/F") was calculated by dividing the sum of stem and root DW and leaf DW (Monsi and Saeki, 2005).

(II.1) 2.3.2 Photosynthetic response curves

Photosynthetic response curves (A/Ci curves), derived from the response of net photosynthesis (A) to intercellular CO_2 concentration (Ci), were measured on LPI 5 leaves of three plants of each species × treatment after 4 weeks of single Ag-NPs supply (June 13) and at the end of treatment (July 28) with a portable photosynthesis system (LI-6400, Lincoln, NE, USA). The leaf area clipped by the chamber was 6 cm^2. Leaf chamber temperature and humidity were adjusted to maintain a leaf-to-air vapor pressure difference of 1.1 kPa. The CO_2 response curves were obtained by changing the [CO_2] entering the cuvette from 50 to 800 μmol mol^{-1} with an external CO_2 cartridge mounted on the LI-6400 console and automatically controlled by a CO_2 injector. The CO_2 assimilation rate was first measured by setting the reference [CO_2] near ambient (400 μmol mol^{-1}) and then at 300, 200, 100, 50, 400, 400, 600 and 800 μmol mol^{-1}. Gas exchange was determined at each step after exposure of the leaf to the new [CO_2] for 60–120 s; saturating light (Q) was maintained at 1500 μmol m^{-2} s^{-1}, 28°C block temperature, and relative humidity of 60%.

The maximum rate of ribulose-1,5-bisphosphate carboxylation (Vc_{max}), the maximum rate of electron transport used in the regeneration of RuBP (J_{max}), triose phosphate use (TPU), mesophyll conductance (g_m) and day respiration (R_{day}) were computed from the A/Ci curves using the A/Ci curve fitting model developed by Sharkey *et al.* (2008). TPU was determined as the highest A, regardless of whether symptoms of TPU were present, which makes TPU similar to the parameter A_{max} reported by other investigators. Gas-exchange in Scots pine is reported on a unit surface area basis calculated using a geometric approximation of the needle surface.

(II.1) 2.3.3 Determination of ROS and antioxidant compounds

Hydrogen peroxide (H_2O_2) was measured spectrophotometrically after reaction with KI, in according to a modified method of Alexieva *et al.* (2001). A sample (0.25g of fresh leaves) was homogenised with liquid nitrogen and then transferred in 13 ml tube with 2ml of 0.1% tricholoacetic acid (TCA); the extract was centrifuged at 10000 rpm for 10 minutes at 4°C. The reaction consisted 0.5ml 0.1% TCA leaf extract supernatant, 0.2 ml of 100 mM K-phosphate buffer pH 7.8 and 2ml 1M KI. The blank probe consisted of 0.1% TCA in absence of leave extract. The reaction was developed for 1h in darkness and absorbance measured at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared with known concentrations of hydrogen peroxide. The results were expressed as µg/g leaf FW.

Superoxide anion (O_2^-) was measured as described by Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of O_2^-, with some modifications (Jiang and Zhang 2001). Frozen sample (1g of leaf) was homogenised with liquid nitrogen and then put in 13 ml tube with 3 ml of 65 mM potassium phosphate buffer, pH 7.8, and centrifuged at 10000 rpm for 10 minutes at 4 °C. The incubation mixture contained 0.9 ml of 65 mM potassium phosphate buffer, 0.1 ml of 10 mM hydroxylamine hydrochloride, and 1 ml of supernatant. After incubation at room temperature for 1 hour, 17 mM sulphanilamide and 7 mM α-naphthylamine were added to the incubation mixture. After 5 minutes 10000 rpm centrifuged and 15 minutes at room temperature. The sample is ready to be read at 530nm. A calibration curve was established using sodium nitrite. The results were expressed as μg/g leaf FW.

Ascorbic acid (AsA) was determined through the modified method of the Okamura (1980) by Law *et al.* (1983). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA in acidic solution. The Fe^{2+} then forms complexes with bipyridyl, giving a pink colour that absorbs at 525 nm. Total ascorbate (AsA + DAsA) is determined through a reduction of DAsA to AsA by dithiothreitol. For the concentration quoted, half sample was assayed for total ascorbate content, and the other half was assayed for AsA only. DAsA concentration was then deduced from the difference.

A sample (0.5 g of fresh leaves) was homogenised with liquid nitrogen and then transferred in 13 ml tube with 2 ml of 10% tricholoacetid acid (TCA), the extract was centrifuged at 12000 rpm for 20 minutes; after that supernatant was allowed to stand in ice for 5 minutes. NaOH 5M (10 μl) was added, mixed and centrifuged for 2 minutes. To a sample (200 μl) was added 200 μl of 150 mM NaH_2PO_4 buffer, pH 7.4, and 200μl of water. To another 200 μl of sample was added 200 μl of buffer, 100 μl of 10 mM dithiothreitol and, after through mixing and being left room temperature for 15 minutes, 100 μl of 0.5% (w/v) N-ethylmaleimide. Both samples were vortex-mixed and incubated at room temperature for > 30s. To each was then added 400 μl of 10% (w/v) trochloroacetic acid, 400 μl of 44% (v/v) H_3PO_4, 400 μl of 4% (w/v) bipyridyl in 70% (v/v) ethanol and 200 μl of 3% (w/v) $FeCl_3$. After vortex-mixing, samples were incubated at 37 °C for 60 minutes and the A_{525} was recorded. A standard curve of ascorbate (AsA) was used for calibration. The results were expressed as μg/g leaf FW.

Glutathione (GSH) was determined using the modification of the Sedlak and Lindsay (1968) method. A sample (0.5 g of fresh leaves) was homogenised with liquid nitrogen and then transferred in 13 ml tube with 1.5 ml of 5% tricholoacetid acid (TCA), the extract was centrifuged at 12000 rpm for 20 minutes; after that 0.5 ml of supernatant was mixed in 13 ml tube with 1.5 ml of 0.2 M tris buffer, pH 8.2, and 0.1 ml of 0.01M DTNB [$5,5^{l}$-dithiobis-(2-nitrobenzoic acid)] or Ellman's reagent, and then centrifuged at

3000 rpm for 15 minutes. The absorbance was read at 412 nm; standard curve was made with glutathione. The results were expressed as μg/g leaf FW.

(II.1) 2.3.4. Determination of cultivable bacteria and fungi

Cultivable bacteria and fungi concentration was determined in leaves and roots of oak, poplar and pine trees. Leaves and roots were collected from the plants of each plot, packed into plastic bags and immediately brought to the lab to perform microbiological analyses. Roots were vigorously shaken to eliminate the coarse soil particles. Five grams of leaves (or capillary roots) were suspended in 90 ml of saline buffer (0,8 % NaCl) and shaken for 1 hour at room temperature. Suspensions were serially diluted and aliquots of 100 μl of each dilution were spread on agar media specific for the two different microbial groups: tryptic soy agar (TSA) medium amended with 200 ppm cycloheximide for bacteria; potato dextrose agar (PDA), acidified with a solution of 25% lactic acid (200μl/lt) and amended with 100 ppm streptomycin for fungi (Schaad *et al.* 2001). Plates were incubated at 27°C and monitored for one week. Colonies were counted and the number of colony forming units per gram of leaves or roots was calculated (cfu gr^{-1}).

(II.1) 2.4 Statistical measurement

All data were presented as mean ± SE (standard error), based on at least five replicates per treatment. Data were analysed by one-way ANOVA to assess the difference between treatments for the measured parameters. A post hoc comparison of means was performed using the least significant difference (Tukey-HSD) test at the 0.05 significance level. Statistical analysis was performed with the OriginPro 9.1 (OriginLab), scientific data analysis and graphing software.

(II.1) 3. Results

(II.1) 3.1 Ag content in plants

Silver concentration (μg kg^{-1}) (Fig. 1A) and content (μg) (Fig. 1B) varied among plant tissues (root, stem and leaf) and treatments ("control", "leaf supply" and "root supply") within each species. The Ag concentration (μg kg^{-1}) in "leaf supply" treated plants was higher in stem than root tissues; whereas, the difference between leaf concentrations between treatments was not detectable because below detection threshold for Ag in leaf of “root supply” treated plants (Fig. 1A). “Leaf supply” treatment in poplar showed the highest allocation in stem, with 21808.99 (± 1714.72) µg kg^{-1}, which represents 84.3% of the total Ag concentration detected, then leaf (15.5%) and finally roots (0.1%). Pine showed Ag distribution of 73.8%, 25.8% and 0.23% in stem, leaf and root, respectively. In oak, Ag distribution was of 68.7%, 30.8% and 0.4% in stem, leaf and roots, respectively.

The Ag concentration in soil samples of Ag-NPs “root supply” was 7.47 ± 0.1 µg kg^{-1}, 25.0 ± 0.3 µg kg^{-1}, 12.99 ± 0.2 µg kg^{-1}, in oak, pine and poplar, respectively; whereas, in Ag-NPs “leaf supply”, it was 2.88 ± 0.1 µg kg^{-1}, 7.24 ± 0.2 µg kg^{-1}, 1.01 ± 0.1 µg kg^{-1}, in oak, pine and poplar, respectively (data not shown).

High values of Ag content were found in Ag-NPs “leaf supply” than “root supply” plants; whereas, Ag was not found in “control” plants (Fig. 1B). Higher values of Ag content were found in pine than oak and poplar. In Ag-NPs “leaf supply” plants, the Ag content was higher in stem than leaf and root tissues in the three species (Fig. 1B). In detail, in Ag-NPs “leaf supply” treatment, in pine 70.7% of the Ag content was allocated in the stem, 28.9% in leaf and 0.4% in root tissues. In oak, Ag was higher in stem (255 ± 93.23 µg) than leaf (22.94 ± 8.59 µg); the same Ag distribution was detected in poplar with higher content in stem (94.50 ± 7.14 µg) than leaf (25.82 ± 4.51 µg), whereas it was not detected in root. Ag was not found in leaf of NPs “root supply” plants in the three species (Fig. 1A, 1B). In root, Ag was preferentially accumulated in “root supply” in comparison with “leaf supply”; whereas, higher Ag content was found in the stem of “leaf supply” than “root supply”, in the three species.

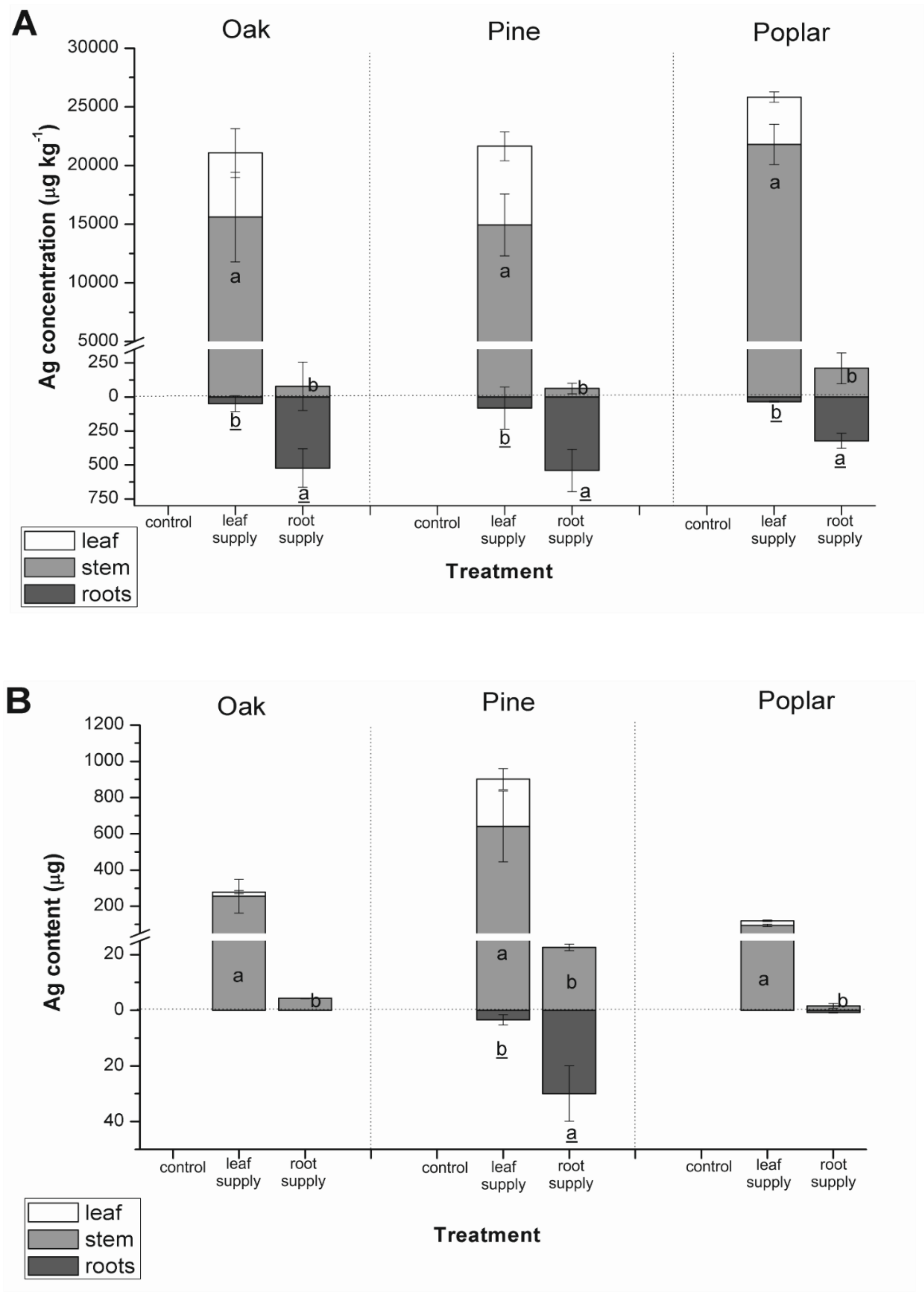


Figure 3: (A) Silver concentration (µg/ kg) and (B) content (µg) in root, stem and leaf of oak, pine and poplar plants differently treated: "control", "leaf supply" and "root supply" with Ag-NPs for 11 weeks. The values are mean ± SE (n = 5) for each species and each treatment. Different letters correspond to statistical difference between treatments within tissues (lowercase letter for stem; underlined letter for root) (Tukey-HSD, p < 0.05 level).

Leaf analysis at ESEM detected NPs on the leaf surface of "leaf supply" plants, chemically validated by EDX; whereas, Ag was not detected on leaf surface of "root supply" and "control" plants (Fig. 2).

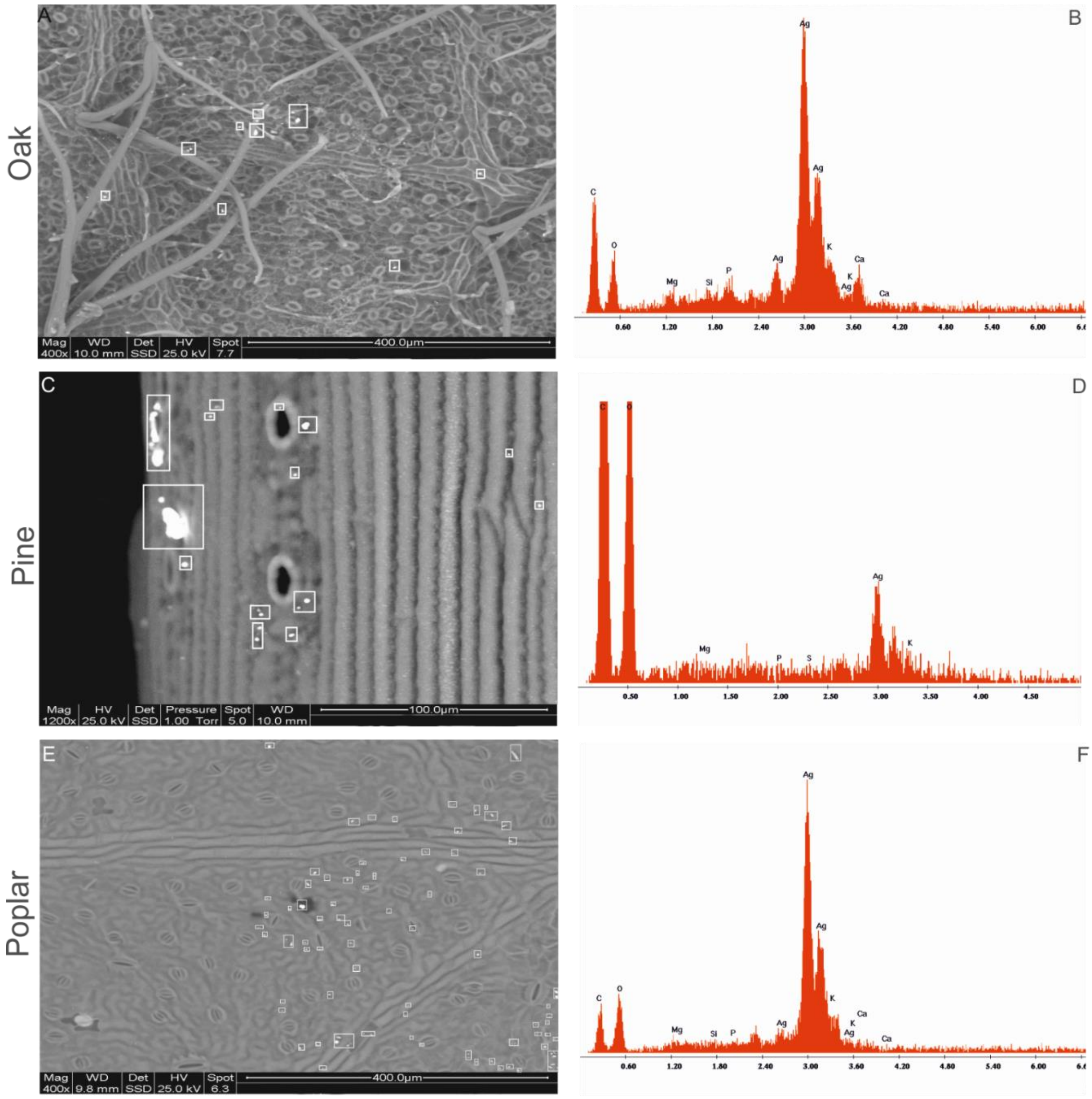


Figure 4: Environment scanning electron micrographs of oak (A), pine (C) and poplar (E) leaves, and the corresponding energy-dispersive X-ray spectroscopy in oak (B), pine (D) and poplar (F). In squares are indicated the particles detected by back scattered electrons on leaf surface.

Analysis at TEM did not detected nanoparticles in the leaf structure of "leaf supply" plants, though EDS-X-ray did not reveal Ag-NPs.

(II.1) <u>3.2 Plant traits</u>

Biometric traits showed significant differences between treatments in poplar ($p < 0.05$), which were more marked than in oak and pine (Fig. 3A, 3B). Poplar showed a significant reduction in biomass of leaf and stem tissues when exposed to Ag-NPs “leaf supply”; whereas, no significant differences in oak and pine biomass were found in relation to Ag-NPs supply (Fig. 3A). The stem length was reduced by Ag-NPs “leaf supply” in poplar; whereas, Ag-NPs supply did not affect stem and root length in pine and oak (Fig. 3B).

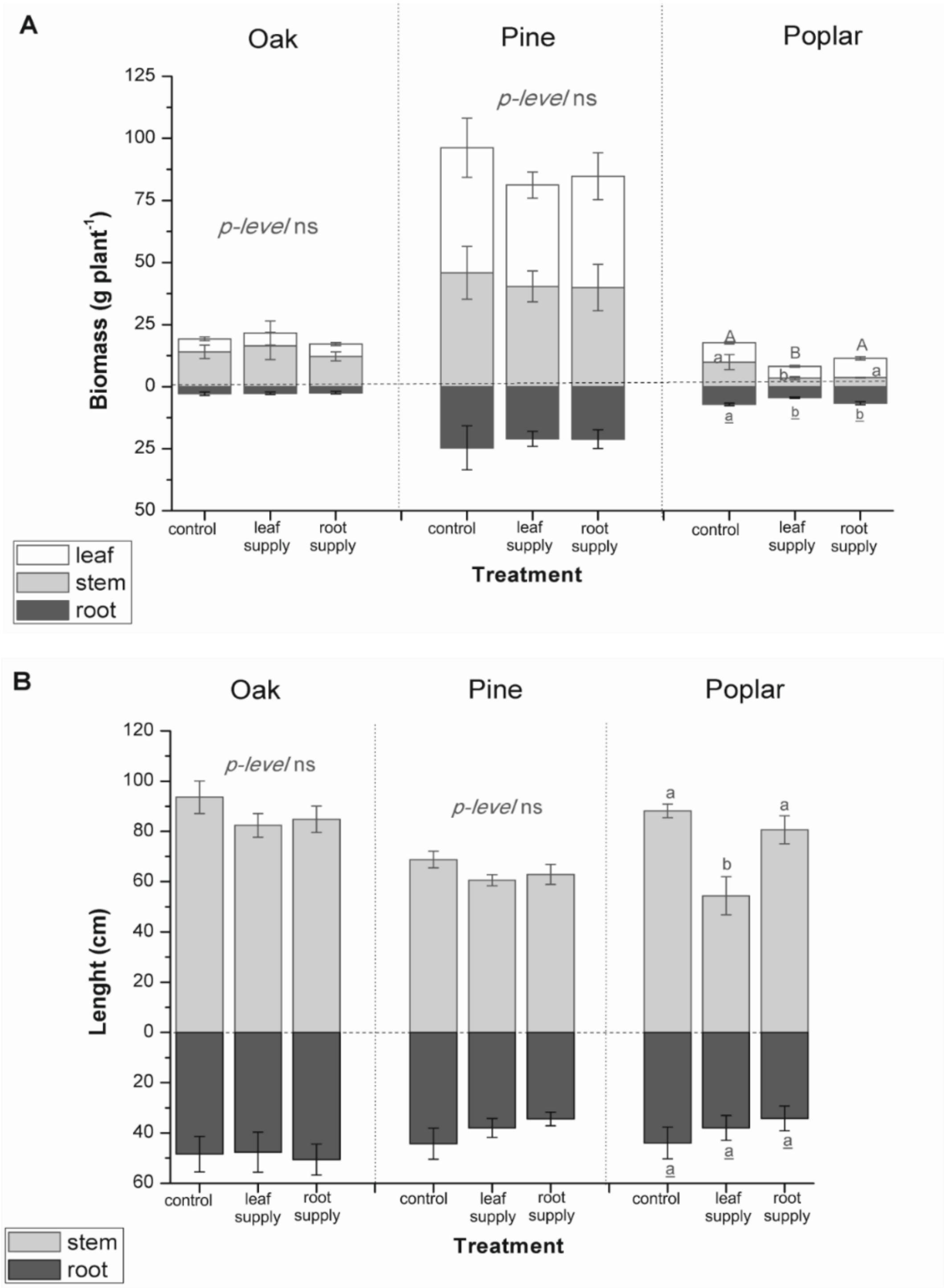


Figure 3: Leaf, stem and root biomass (dry weight, g) (A) and stem and root length (cm) (B) of oak, pine and poplar plants exposed to "control", Ag-NPs "leaf supply" and "root supply". The values are mean ± SE (n = 5). Different letters represent statistical differences between treatment within tissues (capital letter for leaf; lowercase letter for stem; underlined letter for root) (Tukey-HSD multiple comparisons at p < 0.05 level).

The "C/F" was significant ($p < 0.05$) in poplar, decreasing when exposed to Ag-NPs supply, "control" > "leaf supply" > "root supply", while pine and oak did not show significant differences (Table 1).

Table 1: Ratio of dry weight ("C/F") of non-photosynthetic (the sum of stem and root) and photosynthetic organs (leaf) in oak, pine and poplar plants exposed to "control", "leaf supply" and "root supply". The values are mean ± SE (n = 5). One-way ANOVA was applied to measure significant differences between treatments within each species (p-level values are given; ns: not significant).

Species	Treament	C/F ($g\ g^{-1}$)		p-level
Oak	control	1.40	± 0.08	ns
	leaf supply	1.50	± 0.05	
	root supply	1.36	± 0.07	
Pine	control	3.07	± 0.08	ns
	leaf supply	3.65	± 0.05	
	root supply	2.96	± 0.07	
Poplar	control	2.24*	± 0.41	0.05
	leaf supply	1.66*	± 0.14	
	root supply	1.32*	± 0.05	

Values of photosynthetic capacity (Vc_{max}, TPU, R_{day}) and internal CO_2 diffusion (g_m) did not change between treatments. J_{max} significantly changed in the three species, resulting lower in "root supply" in pine (13 June) and oak (18 July), in "leaf supply" in pine (18 July), whereas, it was higher in "leaf supply" in poplar (13 June); Vc_{max} was significantly lower in "leaf supply" (13 June) in poplar than in the other two species (Table 2).

*Table 2: Maximum carboxylation rate (Vcmax), electron transport rate (Jmax), triose phosphate use (TPU), day respiration (Rday) and mesophyll conductance (gm) of oak, pine and poplar plants differently treated with Ag-NPs for 11 weeks. The values are mean ± SD (n = 3) of each species within each treatment. The values are mean ± SE (n = 5). One-way ANOVA was applied to measure significant differences between treatments within each species (p-level values are given; ***, p < 0.001; ns, not significant).*

	species	treatment	Vc_{max} ($\mu mol\ m^{-2}\ s^{-1}$)			J_{max} ($\mu mol\ m^{-2}\ s^{-1}$)		TPU ($\mu mol\ m^{-2}\ s^{-1}$)		R_{day} ($\mu mol\ m^{-2}\ s^{-1}$)			gm ($mmol\ m^{-2}\ s^{-1}$)		
13 June			mean		st.er	mean	st.er	mean	st.er	mean		st.er	mean		st.er
	oak	control	35.4	±	2.9	411.1	± 18.0	6.1	± 0.6	1.6	±	0.2	25.4	±	0.6
		leaf supply	34.7	±	0.9	430.9	± 2.0	6.5	± 0.3	2.6	±	1.6	23.4	±	2.5
		root supply	37.8	±	1.9	430.9	± 0.1	7.0	± 0.3	2.2	±	1.0	26.2	±	0.6
			ns			ns		ns		ns			ns		
	pine	control	21.2	±	1.9	406.4	± 19.8	5.7	± 0.9	2.3	±	0.5	26.2	±	0.2
		leaf supply	24.2	±	1.9	401.3	± 30.0	5.8	± 0.8	1.6	±	0.2	26.4	±	0.3
		root supply	28.8	±	1.5	69.7	± 7.1	6.6	± 0.5	1.6	±	0.1	26.2	±	0.6
			ns			***		ns		ns			ns		

	poplar	control	155.1 ± 6.8	83.4 ± 10.6	6.5 ± 0.9	2.3 ± 0.9	22.0 ± 2.5
		leaf supply	38.5 ± 6.5	429.0 ± 0.9	5.9 ± 0.4	1.5 ± 0.2	26.5 ± 1.3
		root supply	148.6 ± 13.1	84.9 ± 2.5	6.8 ± 0.4	2.0 ± 0.7	20.7 ± 3.0
			***	***	ns	ns	ns
18 July							
	oak	control	37.8 ± 9.4	428.0 ± 1.8	7.0 ± 1.2	1.6 ± 0.4	26.0 ± 0.9
		leaf supply	29.5 ± 14.0	411.7 ± 15.1	4.3 ± 2.2	1.0 ± 0.7	27.6 ± 1.3
		root supply	60.9 ± 5.8	298.2 ± 5.4	9.3 ± 0.7	1.9 ± 0.7	22.8 ± 4.1
			ns	***	ns	ns	ns
	pine	control	25.7 ± 12.7	412.9 ± 8.2	5.9 ± 1.8	4.8 ± 3.2	26.5 ± 1.7
		leaf supply	36.1 ± 0.6	111.1 ± 48.7	7.4 ± 0.5	3.3 ± 2.1	25.8 ± 1.0
		root supply	25.2 ± 16.9	412.9 ± 6.1	5.9 ± 2.1	3.0 ± 1.8	23.5 ± 3.8
			ns	***	ns	ns	ns
	poplar	control	75.0 ± 5.3	411.1 ± 29.1	7.2 ± 0.3	2.8 ± 1.0	24.6 ± 1.2
		leaf supply	51.6 ± 1.4	433.2 ± 1.8	7.5 ± 0.7	3.0 ± 0.6	27.3 ± 0.8
		root supply	42.8 ± 13.3	434.3 ± 1.9	7.1 ± 1.2	2.0 ± 0.6	27.1 ± 0.8
			ns	ns	ns	ns	ns

The leaf morphological characterization showed significant differences in stomatal density in poplar; whereas, no differences between treatments were found in stomatal density in oak and pine (Fig. 4A). Stomatal density of poplar was different between treatments in the abaxial leaf surface, showing higher values in "leaf supply" plants (205.2 ± 17.1 stomata mm^{-2}) than "root supply" and "control" plants; no differences in stomatal density were found in the adaxial leaf surface (Fig. 4A).

Stomatal length was affected by Ag-NPs treatment in the three species (Fig. 4B). Stomatal length was higher in "leaf supply" and "root supply" plants in oak and pine in comparison with "control" plants; whereas, it was higher in "control" than Ag-NPs treated poplar (Fig. 4B). In poplar, stomatal length was significantly different between treatments in both leaf surfaces, abaxial and adaxial (Fig. 4B).

The trichome structure in leaves of oak was affected by Ag-NPs treatment (Fig. 4C). Trichome density was significantly lower in the abaxial and adaxial leaf surfaces in "leaf supply" plants and in the adaxial leaf surface of "control" and "leaf supply" plants, in comparison with "root supply" plants. Trichomes length were significantly different in "leaf supply" plants than in other treatments (Fig. 4C).

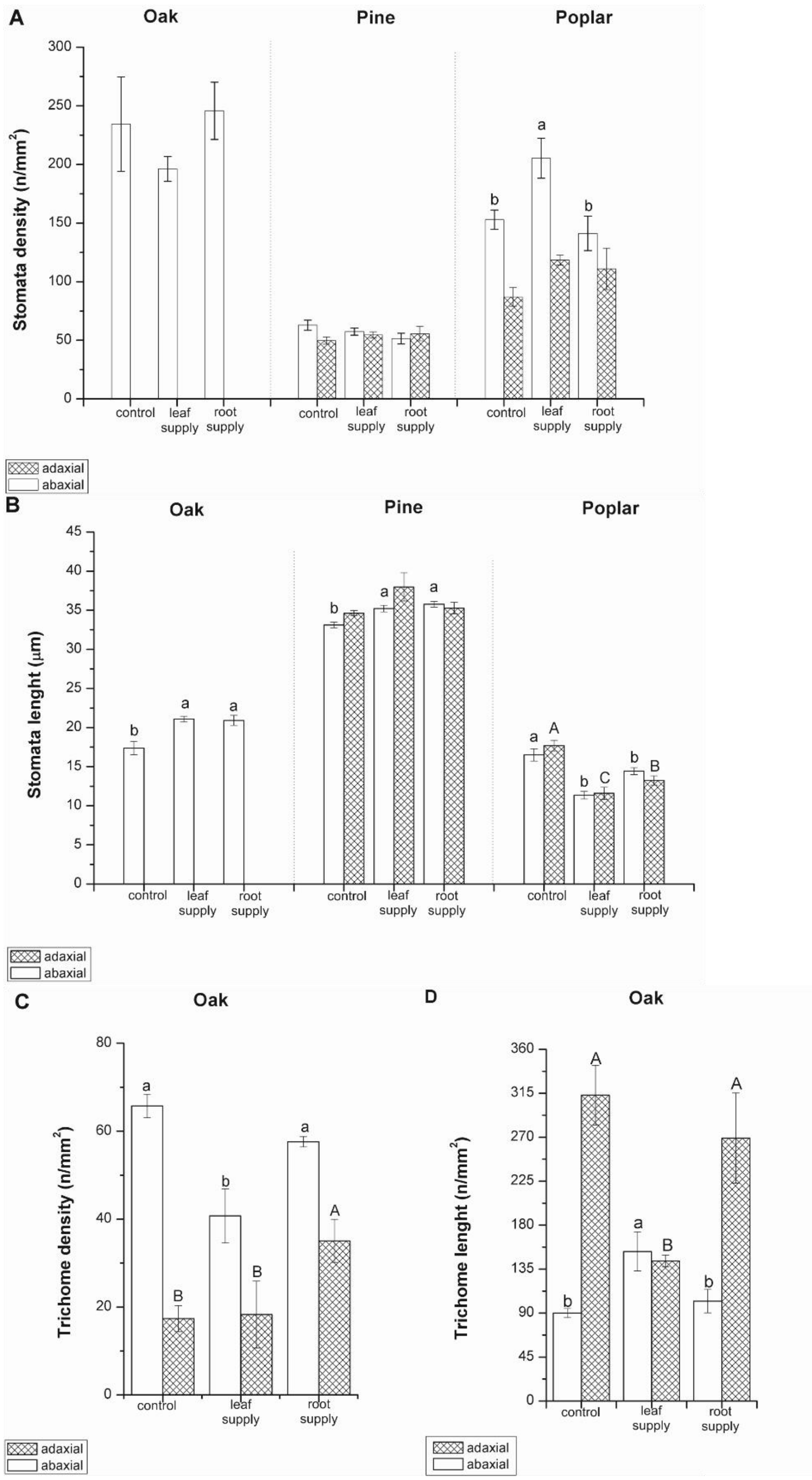


Figure 4: Stomatal density (A) and length (B) of adaxial and abaxial leaf surface of oak, pine and poplar; trichrome density (C) and length (D) in leaf surface of oak plants exposed to "control", "leaf supply" and "root supply". The values are mean ± SE (n = 5). Different letters represent statistical differences between treatment within tissue (lowercase letter for abaxial leaf surface; capital letter for adaxial leaf surface) (Tukey-HSD multiple comparisons at p < 0.05 level).

Determination of reactive oxygen species (ROS), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) was performed to detect oxidative stress. Ag-NPs “leaf supply” treated plants showed a significant accumulation of H_2O_2 in oak plants; in poplar, higher values of O_2^- were found in leaves of Ag-NPs “leaf supply” and “root supply” plants in comparison with “control” plants; whereas, in pine, ROS were not found (Fig. 5). The concentration of antioxidant compounds (AOX), ascorbic acid (AsA) and glutathione (GSH), was performed to detect the biochemical antioxidant system. Ascorbic acid was significantly higher in “leaf supply” plants of oak and in “root supply” plants of pine in comparison with “control” plants; whereas, it was not different between treatments in poplar (Fig. 5). In pine and poplar, GSH concentration was higher in “root supply” plants than “leaf supply” and “control” plants (Fig. 5).

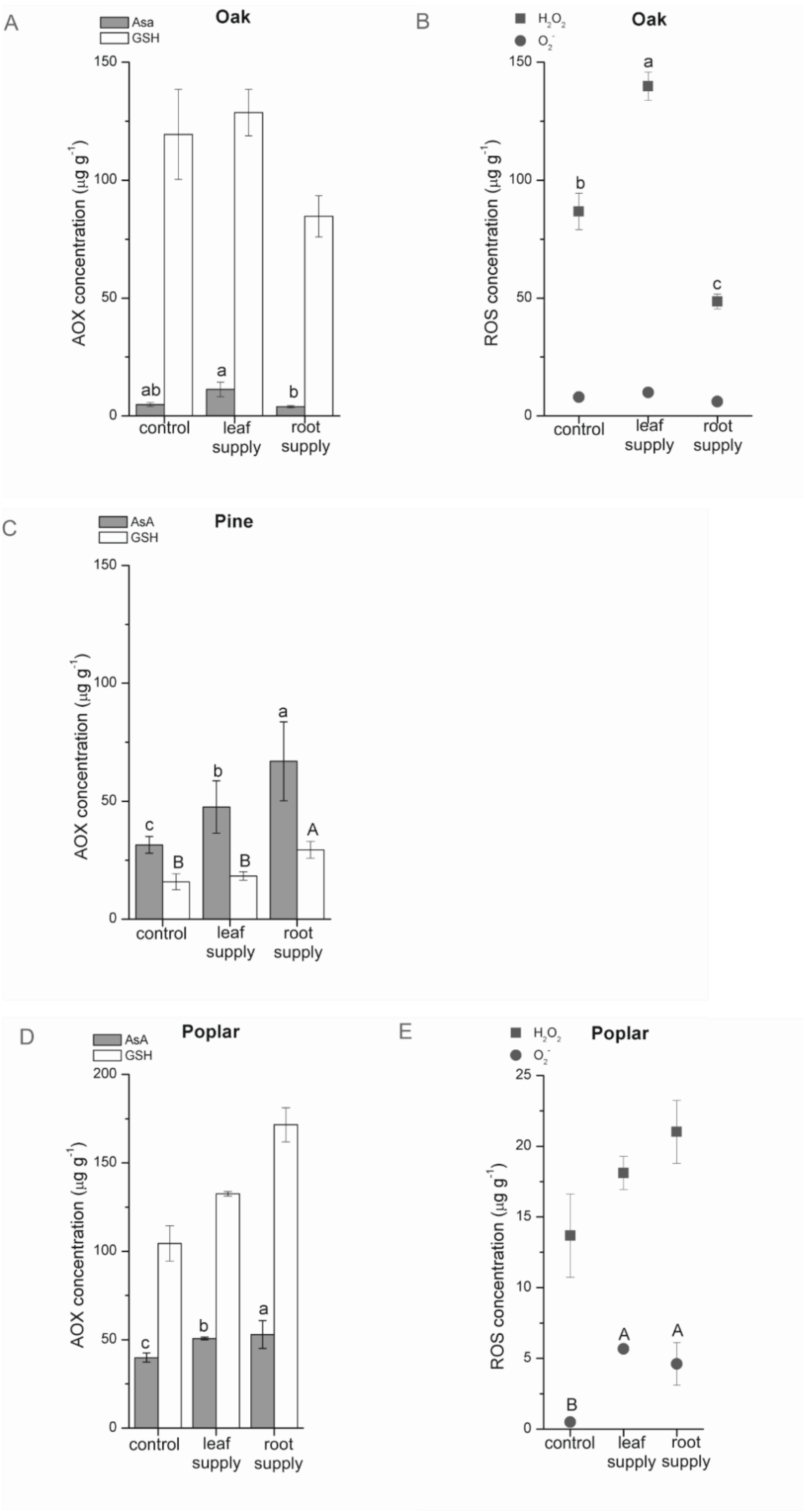


Figure 5: Antioxidant compounds (AOX: ascorbic acid, AsA; glutathione, GSH) and reactive oxygen species (ROS: hydrogen peroxide, H_2O_2; superoxide anion, O_2^-) in leaves of oak, (A, B) pine (C) and poplar (D, E) plants, exposed to "control", "leaf supply" and "root supply". The values are mean ± SE (n = 5). Different letters represent statistical differences between treatments per compound (lowercase letter for AsA and H_2O_2; capital letter for GSH and O_2^-) (Tukey-HSD multiple comparisons at $p < 0.05$ level).

Ag-NPs supply affected bacterial and fungal populations in roots and leaf tissue of plants exposed to “control”, “leaf treatment” and “root treatment”. Ag-NPs “leaf supply” treatment significantly reduced the leaf bacterial population in oak and poplar and fungi population of pine (Table 3). “Root supply” did not affect microbial population of oak and pine roots, whereas the bacterial and fungal population of roots were reduced by Ag-NPs treatment in poplar (Table 3).

*Table 3: Total fungal and bacterial population in leaves and roots of oak, pine and poplar plants exposed to related Ag-NPs supply; leaf of “leaf supply” and root of “root supply” treatments were considered. Data are reported in logarithmic scale. Significant differences between treatments (ANOVA test) are indicated: *, P<0.05; **, P<0.01.*

		OAK (cfu/ml)		**PINE (cfu/ml)**		**POPLAR (cfu/ml)**	
Treatment	Tissues	*Fungal*	*Bacterial*	*Fungal*	*Bacterial*	*Fungal*	*Bacterial*
control	leaf	$7.7x10^{3}$	$7.7x10^{4*}$	$1.1x10^{4***}$	$5.9\ x10^{4}$	$5.4x10^{3}$	$2.8x10^{5*}$
leaf supply	leaf	$7.7x10^{3}$	$7.7x10^{3*}$	$1.6x10^{3***}$	$5.9\ x10^{4}$	$2.3x10^{3}$	$2.8x10^{3*}$
control	root	$1.3x10^{5}$	$3.0x10^{8}$	$4.8x10^{5}$	$4.3x10^{8}$	$2.3x10^{5**}$	$1.8x10^{8*}$
root supply	root	$1.4x10^{5}$	$2.3x10^{8}$	$5.5x10^{5}$	$7.3x10^{9}$	$1.3x10^{6**}$	$8.2x10^{7*}$

(II.1) 4. Discussion

The importance of woody species and NPs in urban ecosystem is relevant, considering the continuous high anthropogenic impact and the potential of trees to improve environmental quality. To simulate the effects of nanoparticles under natural-like conditions, we supplied Ag-NPs through suspension spray on leaf and watering of root in greenhouse conditions. Although the potential of poplar to adsorb NPs has been documented (Zhai *et al.* 2014; Wang *et al.* 2013b), the uptake capacity of oak and pine remains to be explored. Several studies were carried out in hydroponic system (Ma *et al.* 2010; Shahid *et al.* 2014), focusing on soil-root transfer and removing the effect of soil in the interaction between plant and NP. We focused on the attitude of downy oak, black poplar and Scots pine to interact with available Ag-NPs in the surrounding environment. The concentration of Ag-NPs in the environment is low, e.g., it ranges between 0.1 ng/L in the USA and 0.8 ng/L in Europe in surface waters (Gottschalk *et al.* 2009).The photosynthetic potential of trees was variably altered by the treatment with Ag-NPs, which was accompanied by random changes in oxidative stress and antioxidant system parameters, as well as leaf structural traits. This was reflected in poor changes in biomass of different plant compartments, although significant in poplar (“leaf supply”), which points to minor toxicity of Ag-NPs at present concentrations. Zhai *et al.* (2014) observed that Au-NPs did not elicit adverse effects on poplar physiology and biomass, while Cao *et al.* (2017) found that the effects of cerium oxide NPs on physiology and biomass of soybean depended on the concentration and surface coating properties of the NPs.

(II.1) 4.1 Silver in plant tissues

Oak, pine and poplar showed evident differences of Ag allocation between tissues in relation to Ag-NP supply at tissue level, highlighting the variable availability of elements in suspension in the air (leaf supply) and in storage in the soil (root supply). “Leaf supply” showed high Ag concentrations (i.e., the “effective” amount of Ag per unit of weight) in aboveground tissues of oak, pine and poplar, where more than 50% of the total Ag stored was in stem tissue. Meantime, “root supply” did not exhibit Ag translocation to leaves, although a low Ag concentration was detected in stems. As a consequence of the different biomass of the three species, species-specific Ag contents were measured, resulting in higher values in pine for the copious needle foliage in comparison with oak and poplar. Although the comparison between tree species can be inappropriate, poplar showed shorter stems, leading to high Ag concentration in plant tissue in “leaf supply” treatment. Silver concentration was allocated into the leaf tissue to a lesser extent in poplar than in the other species (15.5%, oak 25.8%, pine 30.8%; Fig. 2). The reason can be attributed to the higher capacity of poplar to store NPs in the stem compared to oak and pine. The accumulation of NPs in plants strictly depends by the properties of NPs as well as by plant species-specific traits (Ma *et al.* 2010).

The localization of Ag in leaf tissues was performed through image analysis, adopting ESEM technique, which allows to detect nanoparticles without pre-treatment of samples (Xiong *et al.* 2014). Leaves showed a spread and random localization of Ag-NPs on their surface, which was confirmed by chemical composition. Stomatal length ranged between 15 and 40 μm, which makes stomata a potential way for Ag uptake from the air boundary layer, considering the size of Ag-NPs (mean 25 nm, namely 0.025 μm). Foliar uptake of NPs can be driven by two main processes: the adsorption and internalization via the cuticle and the penetration via stomatal pores (Schreck *et al.* 2012b). Foliar uptake through the stomatal pathway may easily occur through stomatal cells, taking advantages by the diffusion of water vapour (Roth-Nebelsick, 2007). Indeed, Ag-NPs have a strong tendency to agglomerate, negatively influencing the uptake capacity through the cuticle pores (Fernandez and Eichart, 2009; Geisler-Lee *et al.* 2013). Extracellular matrix polymers of leaf, polysaccharides, cuticular lipids and waxes might be responsible of holding the leaf surface with Ag-NPs, as observed though ESEM imaging, which may then diffuse in the leaf tissue (after dissolution or translocation through the cuticle) (Schreck *et al.* 2012b). Physical (stomatal pore) and chemical (agglomeration) limitations to the uptake of NPs include species-specific resistances (Schreck *et al.* 2012a, b). An important role in the adsorption of airborne NPs is defined by traits of aboveground plant tissues (e.g., cuticles, trichomes, stomata, stigma and hydathodes) (Wang *et al.* 2016). Poplar can retain high amounts of particles due to the broad leaf surface area even if it has a low cuticle thickness (Hull *et al.* 1975). Pine is characterized by hydrophobic epicuticular wax (Percy *et al.* 2013), resulting in a low contact surface between cuticle and contaminants (Schreck *et al.* 2012a, b). Trichomes of oak leaf can trap NPs on the leaf surface, interfering with their translocation (Dietz and Herth, 2011; Schwabe *et al.* 2016). In our study, trichome density was reduced by Ag-NPs in abaxial leaf surface of "leaf supply" oak. Although we did not characterize the chemical composition of organic compounds of leaf tissues, the organic composition of leaf structures can electrostatically bind the NPs (Hong *et al.* 2014), which, in turn, can be internalized into the leaf tissues and translocated within the plant tissues (Keller *et al.* 2010).

We used TEM in order to provide an unequivocal demonstration to locate NPs in plant cells but, Unfortunately Ag-NPs were not found in the observed samples. However, the analysed sections, through numerous, referred to specific observation points, sections with a very limited area of about 0.3-0.4 mm^2. For this reason and for the low concentration of Ag particles in plant tissue, NPs were not found, but it does not mean that they are not present in the plants tissues. However, the process of metal translocation within plants through leaf uptake remains to be investigated, considering that we found Ag allocated in stem tissues in "leaf supply" treatment. Additional experiments with emerging visualization techniques, having spectral, and/or three-dimensional resolution (i.e., synchrotron) can be useful to investigate NPs in plant cells to accurately describe their faith in translocation processes. In poplar, Au-NPs were found to be transported from one cell to another through plasmodesmata (Zhai *et al.* 2014); at high concentrations NPs may interfere with nutrient translocation.

(II.1) <u>4.2 Ag-NP effects on plant traits</u>

Foliar application of Ag-NPs resulted in evident effects on plant growth, aboveground biomass and stem length in poplar; whereas, this was not the case in oak and pine (Fig. 3). Lower biomass and stem length in “leaf supply” treated poplar plants in comparison with “root supply” treated and control ones corresponded to high value of Ag concentration in stem tissue. The effect of Ag on poplar can be related to fast growth and high transpiration, which favour translocation efficiency in this genus (Robinson *et al.* 2000). In oak and pine, the concentration of NPs supplied in this experiment was less than that determining a phytotoxicity threshold, e.g. 350 and 790 mg kg^{-1} as found by Sweet and Singleton (2015) for the Bishop pine and a value higher than 50 ppm as resulted after Ag-NPs applied to foliage in English oak (Olchowik *et al.* 2017). The permeability of tissues of these species may also play a role (Dudonné *et al.* 2009). This latter factor can be particularly relevant for poplar plants, considering the structural traits of their leaves in comparison with oak and pine (Bondada *et al.* 2004). Moreover, poplar responded to NP supplied at leaf level by decreasing biomass with an increase of the proportional investment in photoassimilating organs (“C/F”) in comparison with control plants, supporting the ‘optimal partitioning theory’ that higher biomass is allocated to leaves if environmental disturbances come from the above ground, and *viceversa* (Gedroc *et al.* 1996). The supply of Ag to leaves, indeed, may bring to modification of leaf structures (Schreiber, 2010).

The supply of Ag-NPs induced significant changes in poplar leaf traits, increasing stomatal density in “leaf supply” and decreasing stomatal length in “leaf supply” and “root supply” (Fig. 4). In oak, stomata were longer in treated than control plants; whereas, trichome density in abaxial and length in adaxial surface were negatively affected by Ag-NPs “leaf supply”. These changes represent compensatory mechanisms to balance gas exchange or adaptive strategy to increase assimilation surface in response to stress conditions (Marchi *et al.* 2008). For instance, trichome density is considered a key factor in the interception of NPs, as well as cuticular waxes (Rao and Dubey, 1992; Uzu *et al.* 2010; Dietz and Herth, 2011). Phenotypic plasticity of leaf structures may change in response to environmental conditions, following defensive adaptation strategies (Holesky, 2007), such as the storage of NPs to detox plant tissues (Cifuentes *et al.* 2010). Indeed, leaf features were found sensitive to metal availability in different ways, according to the species and morphological traits (Tognetti *et al.* 2004; Abbruzzese *et al.* 2009; Di Baccio *et al.* 2009), as well as an increasing of the stomata density in poplar and stomata length in pine.

The photosynthetic potential was not clearly affected by Ag-NPs. Although phytotoxic effects of Ag were previously observed by Kumari *et al.* (2009), Berahmand *et al.* (2012), Sweet and Singleton (2015), the concentration of Ag-NPs used here was probably nontoxic to plants. Alternatively, the young and efficient photosynthetic tissues (Marcelis, 1996), as well as the plastic structural (leaf structures and "C/F") and biochemical (antioxidant system) traits, may favour plant adjustments (Peng and Jong, 2014; Regier *et al.* 2014; Xiong *et al.* 2017). The supply of NPs affected progressively J_{max} in oak "root supply" and in pine "leaf supply", pointing to stress conditions (Sperlich *et al.* 2016). An investment in the electron transport system resulting in a relatively low J_{max} can be supported by a relatively high maximum rate of carboxylation (Vc_{max}). Poplar was relatively able to optimize resource allocation to preserve a balance between enzymatic (Rubisco) and light-harvesting (chlorophyll) capabilities across treatments (Tognetti *et al.* 2004), by the end of experiment. The sensitivity of simulated carboxylation rates to variation in Vc_{max} and J_{max} suggests a response to Ag-NPs (Xiong *et al.* 2016), although photosynthetic gain and costs of energy dissipation were not impaired. Dynamic responses of Vc_{max} and J_{max} define the plant ability to adjust optimally to environmental variability and resource supply providing estimates of carbon and water fluxes in the soil-plant-atmosphere continuum (Quebbeman and Ramirez, 2016).

Oak and poplar produced ROS when supplied with NPs, which may stimulate the scavenging system (Di Baccio *et al.* 2009). In particular, oak accumulated H_2O_2 in "leaf supply", whereas poplar O_2^- in both "leaf" and "root supply"; whereas pine did not produce ROS. Abiotic stress may lead to the overproduction of ROS in plants, inducing oxidative stress with consequent damages to proteins, lipids, and carbohydrates, and to photosynthetic rate and biomass production (Nel *et al.* 2006; Yin *et al.* 2012). In this study, the production of ROS was accompanied by the synthesis of AsA in oak and GSH in poplar in plants supplied with Ag-NPs, activating antioxidant mechanisms involved in the protection of plant tissues from oxidative stress, maintaining vital physiological activities. Moreover, although ROS were not detected in pine, antioxidant compounds were produced, suggesting that ROS can be quickly detoxified. Otherwise, the antioxidant activity in pine needle may be attributed to the high content of aromatic and phenolic compounds, which act for prevention of chain initiation, decomposition of peroxides, free radical scavenging (Jia e al., 2010; Leopoldini *et al.* 2011; Zeng *et al.* 2012).

A decrease in microbial populations was observed in "leaf supply" plants of the three species, highlighting a direct effect of Ag-NPs (e.g., Cao *et al.* 2016). It is known that the antimicrobial role of Ag-NPs induces damages to cell walls and cell division (Cox *et al.* 2017), inducing the activation of oxidative stress (Wang *et al.* 2009) and, in turn, a reduction in plant biomass (Colman *et al.* 2013). Significant changes in fungal and bacteria populations occurred in poplar tissues, representing the sensitivity and plasticity of this genus to environmental changes (Tognetti *et al.* 2013). Microbial communities influence the dissolution in the substrate and the availability of metals or metalloids in plants (Bravin *et al.* 2012; Rajkumar *et al.* 2012), through excretion of various inorganic and organic compounds (Lindow and Brandl, 2003) and/or acidification of environment (Ma *et al.* 2011). The assessment of microbial colonization in plant-polluted system, therefore, may provide useful information on the tolerance mechanisms of plant-microbe systems to pollutants, as well as on the choice of suitable phytotechnologies (Cocozza *et al.* 2014, 2015).

(II.1) 5. Conclusions

The present study is the first exploring the whole transfer pathway of Ag-NPs at different plant tissue scales, comparing three tree species and combining various investigation techniques. The Ag-NP content was significantly higher in plants contaminated by leaf treatment, compared to root treatment; suggesting a strong dependence of Ag uptake and localization to exposure conditions (NPs supplied at leaf and root levels). A specific affinity plant species-Ag-NP was observed influencing the plant morphology and physiology (plant biomass, stomata density and lenght). A challenge of this study was that woody plants present more complex morphology and physiology than herbaceous species. Nevertheless, we provided a synergistic combination of ecophysiological aspects for improving the knowledge of the mechanisms involved in the uptake of NPs by woody plants.

II.2 Chapter - OAK TREE-RINGS RECORD SPATIAL-TEMPORAL POLLUTION TRENDS FROM DIFFERENT SOURCES IN TERNI (CENTRAL ITALY)

Abstract

Monitoring atmospheric pollution in industrial areas near urban center is essential to infer past levels of contamination and to evaluate the impact for environmental health and safety. The main aim of this study was to understand if the chemical composition of tree-ring wood can be used for monitoring spatial-temporal variability of pollutants in Terni, Central Italy, one of the most polluted towns in Italy. Tree cores were taken from 32 downy oaks (*Quercus pubescens*) located at different distances from several pollutant sources, including a large steel factory. Trace element (Cr, Co, Cu, Pb, Hg, Mo, Ni, Tl, W, U, V, and Zn) index in tree-ring wood was determined using high-resolution laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). We hypothesized that the presence of contaminants detected in tree-rings reflected industrial activities over time. The accumulation of contaminants in tree-rings was affected by anthropogenic activities in the period 1958-2009, though signals varied in intensity with the distance of trees from the industrial plant. A stronger limitation of tree growth was observed in the proximity of the industrial plant in comparison with other pollutant sources. Levels of Cr, Ni, Mo, V, U and W increased in tree-ring profiles of trees close to the steel factory, especially during the

80's and 90's, in correspondence to a peak of pollution in this period, as recorded by air quality monitoring stations. Uranium contents in our tree-rings were difficult to explain, while the higher contents of Cu, Hg, Pb, and Tl could be related to the contaminants released from an incinerator located close to the industrial plant. The accumulation of contaminants in tree-rings reflected the historical variation of environmental pollution in the considered urban context.

Keywords*: Laser ablation, tree-rings, trace elements, urban trees, steel factory.*

(II.2) 1. Introduction

Monitoring atmospheric pollution in the proximity of industrial plants and urban areas is essential to infer past levels of contamination and to evaluate the impact of environmental regulations. Unfortunately, stations monitoring air pollutants have been installed during the 1980's and, therefore, only short time series are available. However, trees may help in reconstructing past pollution episodes and levels. In fact, pollutants deposited over aerial plant surfaces (Schreck *et al.* 2012) can, eventually, be transported via phloem to cambium zone at different tree heights (Lepp, 1975), and assimilated by roots (Watmough *et al.* 2004). A number of studies has shown the ability of trees to take up and incorporate pollutants into their annual growth rings (Nabais *et al.* 2001a; Robitaille, 1981; Rolfe, 1974), so that the accumulation of pollutants in tree-rings may reflect to some degree the variation of pollutant concentrations in the environment at the time of tree-ring formation (Watmough, 1999). The possibility of using element profiles in tree-ring series represents a powerful approach to biomonitor retrospectively pollution events and trends (Jensen *et al.* 2014). Indeed, tree-rings have been used to provide annual records of pollution over decades, tracing pollutants on a spatial and temporal scale in relation to their sources (Cocozza *et al.* 2016; Danek *et al.* 2015; Odabasi *et al.* 2015).

High coherence between the chemical composition of tree-rings and the chemistry of the surrounding environment, in space and time, was previously found in *Quercus* spp. (Cutter and Guyette, 1993; Eklund, 1995; Jonnson *et al.* 1997), showing that oak trees are suitable indicators of sources of metal contamination. This is based on the permeability of ring porous wood of these species, as well as their low number of rings in the sapwood, ecological amplitude, longevity, and wide geographical distribution. Downy oak (*Quercus pubescens* Willd.) seedlings were found to absorb Cd in roots, leaves and stems, with negative impact on photosynthetic capacity varying progressively with increasing Cd concentration in the soil (Cocozza *et al.* 2012). Thus, accurate dendrochemical indicators may integrate information collected from traditional passive and active sampling devices (Lin, 2015), determining large scale patterns of pollutant distribution over time and providing a tool to forecast the impacts of pollution on green infrastructures in relation to human activities (Haase *et al.* 2014; Watmough, 1999).

Nevertheless, the use of dendrochronological techniques for a retrospective biomonitoring of pollutants have been questioned (Cheng *et al.* 2007; Garbe-Schonberg *et al.* 1997; Nabais *et al.* 2001b, 1996), and the debate on whether elemental concentration changes in tree-rings are reliable indicators of environmental alteration through time is still open and topical (Baes and Mclaughlin, 1984; Bindler *et al.* 2004; Pearson *et al.* 2005). Several studies found no correlation between element concentrations in tree-rings and changes of their amount in the air, possibly due to the translocation of elements across tree-ring boundaries (at least) in the sapwood (Kennedy, 1992) and their accumulation in the outermost rings (Poulson *et al.* 1995), as well as the lack of confidence on rough analytical approaches (Brabander *et al.* 1999). This controversy illustrates the need to carefully choose appropriate sampling design, tree species and analytical methods (Cutter and Guyette, 1993). Furthermore, analytical methods were recently improved, enabling the determination of very low concentration (ppm to ppb levels) and reducing the quantity of material needed for the analysis, avoiding the necessity of using more than one growth ring and thus enhancing analytical resolution.

The most powerful analytical methods in dendrochemistry are the GC/MS (Gas Chromatography/Mass Spectrometry), GC/FID (Gas Chromatography/Flame Ionization Detection), XRF (X-Ray Fluorescence), and LA-ICP-MS (Laser Ablation Inductively Coupled Plasma Mass Spectrometry) (Hoffmann *et al.* 1994; MacDonald *et al.* 2011). LA-ICP-MS is a sophisticated analytical technique with high level of accuracy that precisely allows the description of the elemental composition of solid samples, including biological tissues (Limbeck *et al.* 2015). Such as, LA-ICP-MS is potentially suited to investigate contaminants that generally are present at very low concentrations in plant material. In particular, LA-ICP-MS is promising for the determination of trace elements in individual tree-rings and in their portions (Witte *et al.* 2004), allowing high-resolution analysis of element distribution in early and late wood (Monticelli *et al.* 2009).

We hypothesized that trace elements taken up by trees are fixed in the growth ring produced in a particular year, providing a spatial-temporal pollution record. A strongly polluted town in central Italy was selected as study area. The main aim of the study was to demonstrate the feasibility to detect changes in environmental contamination across space and time by measuring pollutant levels in tree-rings, corresponding to a period for which monitoring data were not available, using updated LA-ICP-MS method. Downy oak trees, growing in the proximity of a steel factory and at distal sites in the town of Terni, were sampled and elements in tree cores were measured using LA-ICP-MS. High levels of metals were emitted into the environment by the industrial plant through time, specifically particulate matter (Sgrigna *et al.* 2016, 2015). In particular, we verified whether: 1) these oak trees took up and stored pollutants in the annual tree-rings; 2) element levels in tree-rings indicated spatial-temporal distribution of pollutants; and 3) climate and pollution had interactive effects on tree growth.

(II.2) 2. Materials and methods

(II.2) 2.1 Site description

The study area is located in the town of Terni in central Italy (42° 34’ N; 12° 39’ E, elevation 130 m a.s.l., 112’000 inhabitants). The area is within a valley, surrounded by three main mountain chains, Sabina mountain (NS direction), Martana chain (ESE-WNW direction) and the Narnese-Amerina mountain (NNW-SSE direction) (Cattuto *et al.* 2002) (Fig. 1), and winds are mainly blowing from N-NE (Sgrigna *et al.* 2015). The morphology of the town leads to the persistence of atmospheric pollutants and the area is one the most polluted in Italy, especially for particulate matter (PM), in winter and summer months (Sgrigna *et al.* 2016, 2015) (www.arpa.umbria.it). There is a huge industrial pole mostly located in the town center (Capelli *et al.* 2011) characterized by one of the largest stainless steel production site in Europe (Moroni *et al.* 2013) (around 150 ha), established at the end of 19^{th} century. The industrial plant produces 1 million tons/year of manufacted steel (communication of factory). The emission is daily monitored by environmental stations around the steel factory, which detect particulate matter and heavy metals (Pb, Cd, As, Ni, Cr). At around 7 km from the steel factory, there is an area contaminated and identified as a remediation site by a project aimed to implement environmental restoration and monitoring activities, which started in 2003 - area of national interest (SNI): “Terni-Papigno” (DM 468/2001 and DMA 08/07/02). “Terni-Papigno” includes a treatment plant of wastewaters of the steel factory and two storage sites: one for waste and special waste, and another for dangerous waste have been active since 1982 and 2006, respectively. Close to “Terni-Papigno” and near the d8 site, there is another waste disposal. Three incinerators of solid waste are also located in the area of “Maratta” (Fig. 1). In 2008, an incinerator was closed due to environmental laws related to suspected harmful and radioactive substances in waste (Mosca, 2008). In 2007, during the excavations for the construction of a tunnel, a dense underground lake of hexavalent Cr (VI) was found below the landfill. Moreover, a large hospital with nuclear medicine department and other industries are also located near the city center (Fig. 1), contributing to very complex environmental conditions.

Eight sites in urban and peri-urban area were selected: four sites (p1, p2, p3, p4) at a distance of 0.5 km from the steel factory, indicated as proximal sites (P-chronology), and four sites (d5, d6, d7, d8) at 1 km from the plant, indicated as distal sites (D-chronology) (Fig. 1).

Air quality of the area is monitored through environmental stations that supply a real-time air quality (PM level, dioxins, ozone, sulphur and nitrogen dioxide and heavy metals in urban airborne). The PM levels were detected by 6 different monitoring stations located in the urban and peri-urban area (Fig. 1). PM_{10} levels of 40±1.60 μg/m^3 in station 1, 28±1.35 μg/m^3 in station 2, 34±1.64 μg/m^3 in station 3, 30±1.68 μg/m^3 in station 4, 32±1.62 μg/m^3 in station 5, and 33±2.23 μg/m^3 in station 6 were calculated as annual mean values in the period 2004-2011 (www.arpa.umbria.it). The highest PM level was detected by the monitoring station number 1 (N 42° 34’ 20.78”, E 12° 40’ 32.68”), located in p2, the area closest to the steel factory. According to the WHO (2016), 20 μg/m^3 annual was chosen as mean annual level of PM in the long-term guideline value to avoid health problem. This value represents the minimum of the range over which significant effects on survival rate were observed, causing air pollution-related deaths by around 15% (WHO, 2016). This issue had attracted media and legal attention for the "real dangers to the citizen health, linked to the spread of pollutants in water and air” (Moroni *et al.* 2013).

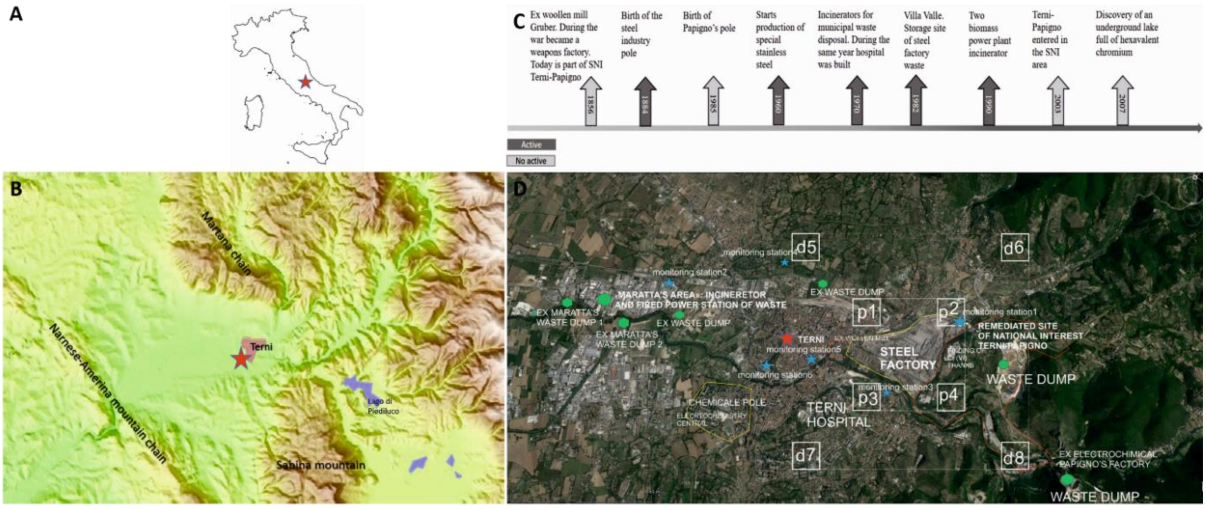


Figure 5: A) Location map of the study area in Central Italy. B) Orographic map of the Terni area. C) Reconstruction of the temporal trends of industrial activity. The arrows indicate the start of specific activities in the area. D) Location map of sampling site

(II.2) 2.2 Tree-rings core collaction and sampling procedure

Tree cores of downy oak, the most common tree species in the study area, were sampled. Downy oak was previously used for dendrochemical studies with positive results, as correlation between pollution level of the environment and observed metal content in tree-ring profile was observed (Cocozza *et al.* 2016). To avoid the effect of any wood alteration, only trees without abrasion scars or other visible evidence of injury were selected. Four healthy downy oak trees were randomly identified in each site, at both proximal and distal sites. Trees were chosen with diameter at breast height (DBH) ranging from 30 to 80 cm and height around 10 to 15 m. Two cores for each tree were sampled using an incremental borer of 50 cm length and an inner diameter of 5.15 mm (Haglof Sweden AB). Coring was performed at breast height (1.30 m).

Tree cores were collected in winter (December, 2015), in dormancy season when no significant mobility of the elements should occur once stored (Hagemeyer and Schiifer, 1995). Later, the samples were prepared for LA-ICP-MS analysis. The cores were fixed on hemicilindrical-grooved plastic supports with wood vessels perpendicularly oriented with respect to the support surface. The cores were sectioned with a microtome, and mounted for insertion into the LA-ICP-MS instrument. The core sections were reduced to half of their original diameter, thus exposing the inner part.

(II.2) 2.3 Ring width measurement

Tree-rings were dated and then measured with a resolution of 0.01 mm, using the LINTAB-measurement equipment (Rinntech, Heidelberg, Germany), coupled with a Leica MS5 stereoscope (Leica Microsystems, Germany), and TSAP Win 0.55 software (Rinn, 1996). Cross-dating is one of the fundamental principles of dendrochronology. Each ring-width chronology should be cross-dated within and between trees and among all trees within the same site. Raw ring-width chronologies should be first cross-dated visually and then statistically (Cherubini *et al.* 2002). Usually the visual cross-dating is validated statistically using Student t-test, Pearson correlations and more dendrochronologically suited statistical tests, i.e., the Glk (the *Gleichläufigkeit*), to determine the significance of the correlation between the curves (Cherubini *et al.* 2002). Statistical crossdating was performed through the software COFECHA to assess the quality and accuracy of tree-ring series measurement (Holmes, 1983). The ARSTAN program was used to remove variability due to age, size and other non-climatic factors affecting tree growth, and to standardize ring width data into final chronologies producing tree-growth indices (Cook *et al.* 1990). To preserve the long-term fluctuations in the series, a conservative method of detrending was used. Only the negative exponential curves and/or linear regressions with negative or zero slopes were used and a 20-year smoothing spline.

The standardization produced two-chronologies, one for proximal sites and one for distal sites. Standardized tree-ring index series are combined into a mean value of all series to obtain a standardized chronology (STD). Means of each year are computed as either the bi-weight robust estimate or the arithmetic mean (Cook, 1985). The bi-weight mean is an integral part of the ARSTAN methodology and strongly recommended to remove effects of endogenous stand disturbances and to enhance the common signal contained in the data. Standardized tree-ring chronologies were statistically characterized by: raw mean ring width, calculated as the arithmetic average of the raw data of each elementary series; mean ring width of STD chronology; mean sensitivity (MS); standard deviation (SD) to assess the high-frequency variations (Fritts, 1976); and expressed population signal (EPS) as threshold indicating an acceptable level of coherence in dendrochronology (Wigley *et al.* 1984).

The ring widths of P- and D-chronologies were converted in tree growth index (TGI). This index is a ratio calculated as follows:

$$TGI = 3 \text{ consecutive rings width data} / (\text{means of a series}^{\wedge 1/n}) - 1$$

for P- (TGI P) and D-chronologies (TGI D).

Each point represents the annual average growth rate, based on the procedure of Robitaille (1981), calculated as the ratio between the mean of the values measured on 3 consecutive annual rings and the mean of the series for a specific interval time (n). Higher or lower values for a given year represent proportionally higher or lower tree growth for that year. The index is useful to interpret the growth variations in terms of climate or other environmental factors.

(II.2) 2.4 Climatic data

The role of climatic variables in influencing tree growth was analysed with the R Statistical Software version 3.0.2 (R Development Core Team, 2010), package “bootRes”, using moving correlation function (MCF) for detecting stationarity and consistency of correlation functions over time. The MCF considers air temperature and precipitation, the main parameters to assess the climatic long-term effect on radial growth (Zywiec *et al.* 2017). Moving correlation function analysis is a valuable statistical method for the assessment of the influence of climate on tree growth (e.g., Carrer *et al.* 2007, Reynolds-Henne *et al.* 2007, Palombo *et al.* 2013. The calculation is performed year by year. Each square represents a climatic response (correlation coefficients) in 20-yr time windows on the basis of a linear regression analysis of year by year data set. Correlations were calculated separately for each month, allowing the observation of seasonal sensitivity of correlations, for the period from March of the year prior to ring formation to October of the year of growth (according to Zang and Biondi, 2015). This technique is useful to summarize large amount of data and the colour scale corresponds to both the sign and strength of the correlations. The standardized tree-ring chronologies were used to correlate tree growth with mean temperature and total precipitation. The climatic data used for the correlations were downloaded from ftp://palantir.boku.ac.at/Public/ (Moreno and Hasenauer, 2016). The resolution of climatic data is 0.0083° (about 1×1 km grid) obtained combining the gridded climate data set of E-OBS (European Observations) version 8.0 at 0.25° resolution (approximately 30 km) and WorldClim climate surfaces. Mean temperature and total precipitation, with monthly detail in the period from 1951 to 2012, were considered. The coordinates of the center of the steel plant (42°46’39’’ N; 12°22’31’’ E) were used to extract climatic data from the grid.

(II.2) 2.5 Trace elements in tree: LA-ICP MS determination

Trace elements were detected in tree cores using LA-ICP-MS at the laboratory of the Institute of Geochemistry and Petrology (ETH, Zürich, Switzerland). Standard operating conditions of LA-ICP-MS were set up (Table 1). LA-ICP-MS analysis was carried out on 2 cores of 3 trees in each site. The analysis was performed along the tree cores.

Table 1: Laser Ablation ICP-MS parameters.

Instrumental parameters	Dendrochemical analysis
Instrument Host	LA-ICP-MS ETH Zurich
Type	RESOLUTION 155S (asi)
ICP-MS	Element XR (Thermo Fischer)
Laser Type	193 nm excimer
Setting	10 Hz, 3.5 J/cm2 single hole, 400 pulses
Spot size	257 micron diameter
Normalized	^{13}C intensity

Helium carrier gas transported the ablated material to the ICP-MS at a flow rate of 0.7 L/min. Argon makeup gas was mixed within the funnel of the 2-volume ablation cell at a flow of 1-1.1 L/min. To optimize the ICP-MS system and ensure high sensitivity and stability, the glass standard reference material (SRM) NIST 612 was ablated using single line patterns (laser settings: energy 3.5 J/cm^2; spot size 43 µm; scan speed 3 µm/s; shot frequency 10 Hz). One large spot (257 µm crater) was ablated in each tree-ring. The spot dimension was similar to that used by Marten *et al.* (2015) (200 µm spot dimension). The spot dimension, the method of spot ablation, and the type of wood (e.g., wood density and pore dimension) depend on tree species. The spot ablation technique has the advantage to associate the result to a specific tree ring (age assignment of ablation results). All oak wood samples were ablated orthogonally to the tree-rings in the drilling direction. The analysis was done in late wood, because during dormancy no significant mobility of the elements should occur once stored (Hagemeyer and Schiifer, 1995). Moreover, in ring porous species (as in the case of oak), late wood has smaller vessels and a denser and more uniform structure than early wood (Danek *et al.* 2015). This ensured that the spot size chosen was quantitatively sufficient for the combustion of the ablated particulate. For data reduction, Sills software (Guillong *et al.* 2008) was used to select integration intervals, remove spikes and calculate net count rates (cps) for all measured elements. For better comparability, all count rates were normalized to ^{13}C to correct for differences in ablation yield, beside the fact that carbon is difficult to accurately measure (Frick and Günther, 2012). Due to the lack of a suitable reference material, absolute concentration was not calculated and the element/^{13}C ratios were taken as proxy for the element level. Each analysis consisted of about 30 seconds of gas blank data; used for background correction and 40 seconds of sample ablation. The following isotopes were measured to obtain the elemental contents in the tree-rings: Cobalt (^{59}Co), Copper (^{65}Cu), Chromium (^{53}Cr), Lead (^{208}Pb), Mercury (^{202}Hg), Molybdenum (^{95}Mo), Nickel (^{62}Ni), Tungsten (^{182}W), Thallium (^{205}Tl), Uranium (^{238}U), Vanadium (^{51}V), and Zinc (^{66}Zn).

(II.2) 2.6 Elements level

Data of three trees were averaged to obtain a single time series for each site and for each element.

The level of each element was determined by normalization procedures (Bukata and Kyser, 2007). The normalization defined common temporal levels of element in tree-rings of different sites. An index value was assigned for year (I_x), using the following equation:

$$(I_x) = (level_x - level_{lowest}) / (level_{highest} - level_{lowest})$$

where, $level_x$ refers to the level of a specific year, $level_{lowest}$ and $level_{highest}$ refer to the lowest and the highest levels, respectively, measured in tree-rings of each site.

Finally, normalized levels of each element in tree-rings of the sites near the steel factory (p1, p2, p3, p4) and far from that (d5, d6, d7, d8) were averaged for the characterization of element levels, and the two series in P- and D-chronologies were plotted.

(II.2) 2.7 Statistical analysis

The trend of Cr, Cu, Hg, Mo, Ni, Pb, Tl, U, V, W, and Zn contents in P- and D-chronologies was assessed using the non-parametric Man-Kendall test (MK test) (Mcleod, 2005; Tognetti *et al.* 2014). According to Man-Kendall test, the null hypothesis H0 assumes that there is no trend (the data are independent and randomly ordered) and this is tested against the alternative hypothesis H1, which assumes that there is a trend (Önöz and Bayazit, 2003). Each element was compared between P- and D-chronologies. The initial value of the Mann-Kendall statistic, S (Mann-Kendall score), is assumed to be zero (i.e., no trend; null hypothesis). If a data value in a later time period is higher than a data value in an earlier time period (i.e., a trend is detected; alternative hypothesis), S is higher than one. On the other hand, if the data value in a later time period is lower than a data value in an earlier time period, S is lower than one. The incremental and decremental values define the final value of S. This test allows to investigate long-term trends of data without assuming any particular distribution. Then, the rank correlation coefficient, Kendall's tau, was calculated to compare the strength of the correlation between two data series; tau ranges between −1 and 1, measuring the degree of similarity between ranks of pairs of chronologies. The tau coefficient corresponds to: value 1, when the agreement between the two rankings is perfect (i.e., the two rankings are the same); value -1, when the anticorrelation between the two rankings is perfect (i.e., one ranking is the reverse of the other); value 0, when the rankings are completely independent. The resultant MK test statistic was performed using XLSTAT-Time statistical analysis software.

The trends in P- and D-chronologies were tested using the non-parametric Levene's t-test. This test evaluates the significant difference between the means of two independent samples in order to define the relationship between two variables and the trends of the relationship. The Levene's t-test correlation coefficients were determined by linear regression. Differences between groups were considered to be significant when *p-value* ≤ 0.05. The Levene's test verifies two hypotheses. The null hypothesis indicates that the variance between series is equal. The null hypothesis is rejected when the p-value of Levene's test is less than 0.05, assuming the hypothesis that series are different. The analysis was performed using the SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA).

(II.2) 3. Results

(II.2) <u>3.1 Ring width chronologies</u>

The longest chronologies of downy oak for the investigation were 68 years old (1947-2015) in P-chronology and 122 years old (1893-2015) in D-chronology (Fig. 2A).

The raw mean ring width showed higher growth rates in P- than D-chronologies. Values of STD, MS, SD, and EPS did not show differences between P- and D-chronologies (Table 2). The low MS indicated that downy oak is relatively complacent at this site (Douglass, 1919; Fritts, 1976). The high STD expressed consistency between the values of the time series analysed. The EPS value showed a good level of coherence of the chronologies, indicating consistency of the studied chronologies (Wigley *et al.* 1984).

Table 2: Descriptive statistics of two chronologies (P- and D-chronologies): mean tree-ring width calculated on the raw chronology and the STD chronology; tree-ring standard deviation (SD), which estimates the variability of measurements for the whole series; mean sensitivity (MS), which is an indicator of the mean relative change between consecutive ring widths; and EPS, the expressed population signal to indicate the level of coherence of the constructed chronology (ARSTAN analysis).

Statistical value	P-chronology	D-chronology
Measured years (n)	69	123
Raw mean ring width (mm) of chronology	4.05	2.46
Mean ring width of STD chronology	0.995	0.992
Mean sensitivity (MS)	0.117	0.121
Standard deviation (SD)	0.14	0.17
Expressed Population Signal (EPS)	0.81	0.81

The tree-ring widths were converted into a growth index and plotted (Fig. 2B). The chronologies revealed a growth decrease of 0.04 unit/year in P-chronology, whereas, a growth decrease of 0.02 unit/year was observed in D-chronology, in the period 1985-2015. Overall, after 1985, tree growth exhibited a 58% decrease in D-chronology, and a 158% drop in P-chronology.

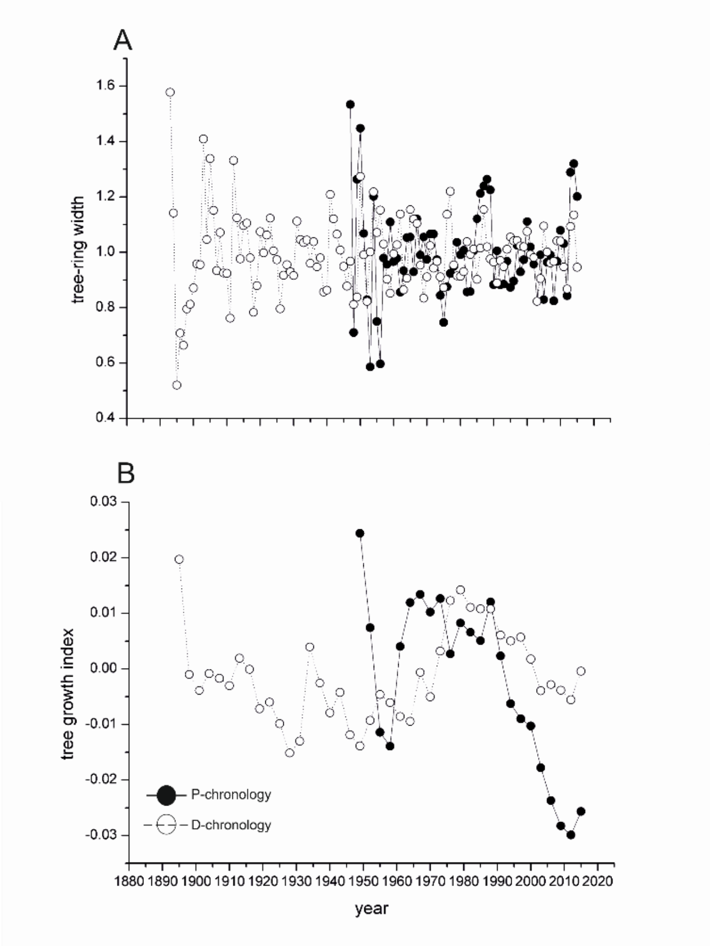


Figure 6: A) Tree-ring width of P-chronology (black circles) and D-chronology (white circles). The tree-ring index is produced through the ARSTAN programme applying a conservative method of detrending with negative exponential curves and/or linear regression with negative or zero slopes; B) Tree growth indices (TGI) of Q. pubescens in D and P-chronologies were calculated as the ration between the mean of the values measures on 3 consecutive annual rings and the mean of the series for a specific interval time (n). The index is useful to interpret the growth variation in terms of climate or other environmental factors.

(II.2) 3.2 Growth climate relationships

Tree growth in P- and D-chronologies correlated weakly with climatic factors. Correlations between tree growth and climatic variables (temperature and precipitation) in P- and D-chronologies were generally weak (strength of correlation), although resulting in statistically significant correlations (Fig. 3). The bootstrap graph showed positive correlation between radial growth and precipitation in November of the previous year in P-chronology (Fig. 3). Whereas, correlations between plant growth and precipitation were negative in October of the previous year and January (1968-1987), and October of the current year (1988-2007) in P-chronology (Fig. 3). In summer, negative correlations between radial growth and precipitation occurred in July (1993-2012) and in August from 1973 to 2002 in P-chronology (Fig. 3). In D-chronology, growth-climate analysis revealed positive significant correlations of precipitation, in October of the previous year (1958-1977), in March (1963-1982), in April and September of the previous year (1993-2012), in June (1993-2012) and September (1988-2007) of the current year. Whereas, negative correlations between growth and precipitation in D-chronology were found in July of the previous year (1973-1992) and in August (1988-2007) of the current year of analysis (Fig. 3).

In P-chronologies, the relationships between air temperature and radial growth were significantly positive in April and May of the previous year (1978-1997), in November of the previous year (1993-2012), in August (1993-2012), and in October (1963-1982; 1973-1992) of the current year. Whereas, negative correlations between growth and air temperature in P-chronology were found in April (1993-2012) and December (1963-1982) of the previous year, April (1973-1992), June (1978-1997) and October (1958-1977) of the current year (Fig. 3).

Significantly positive correlations between tree-growth and temperature in D-chronology were observed in March (from 1973 to 2007), June and October of the previous year (1973-1992), January (1958-1997), February (1993-2012), June (1978-1997) and October (1958-1977) of the current year (Fig. 3). Negative correlation was found between radial growth and air temperature in January of the previous year (1978-1997) (Fig. 3).

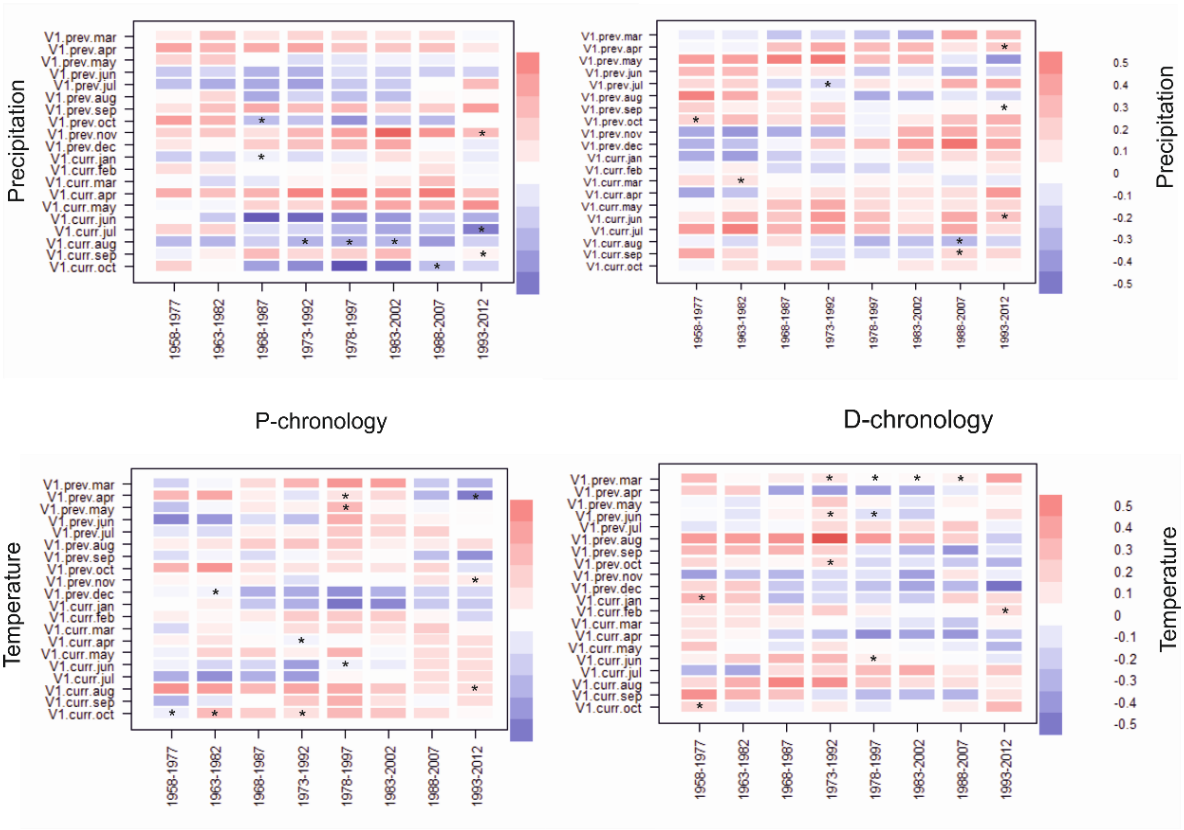


Figure 7: Running means of bootstrapped statistically significant correlation coefficients between climate data and tree-ring indexed chronologies (MCF) in P- and D-chronologies. Correlations were calculated separately for each month for the period from March of previous year to October of the current year. Each rectangle represents a correlation calculated over a 20-yr period plotted in the last year of each 20-yr period. Colour scale correspond to the sign and strength of the correlations, asterisks refer to significant correlation.

(II.2) 3.3 Spatial and temporal variation patterns of elements level and sources

Higher levels of Co, Cr, Mo, Ni, U, V, W, and Zn were detected in P- than D-chronologies, within the analysed period 1958-2009 (Fig. 4). Data in the period 1967-1974 are missing due to unclear signal of the ablation analysis in these years.

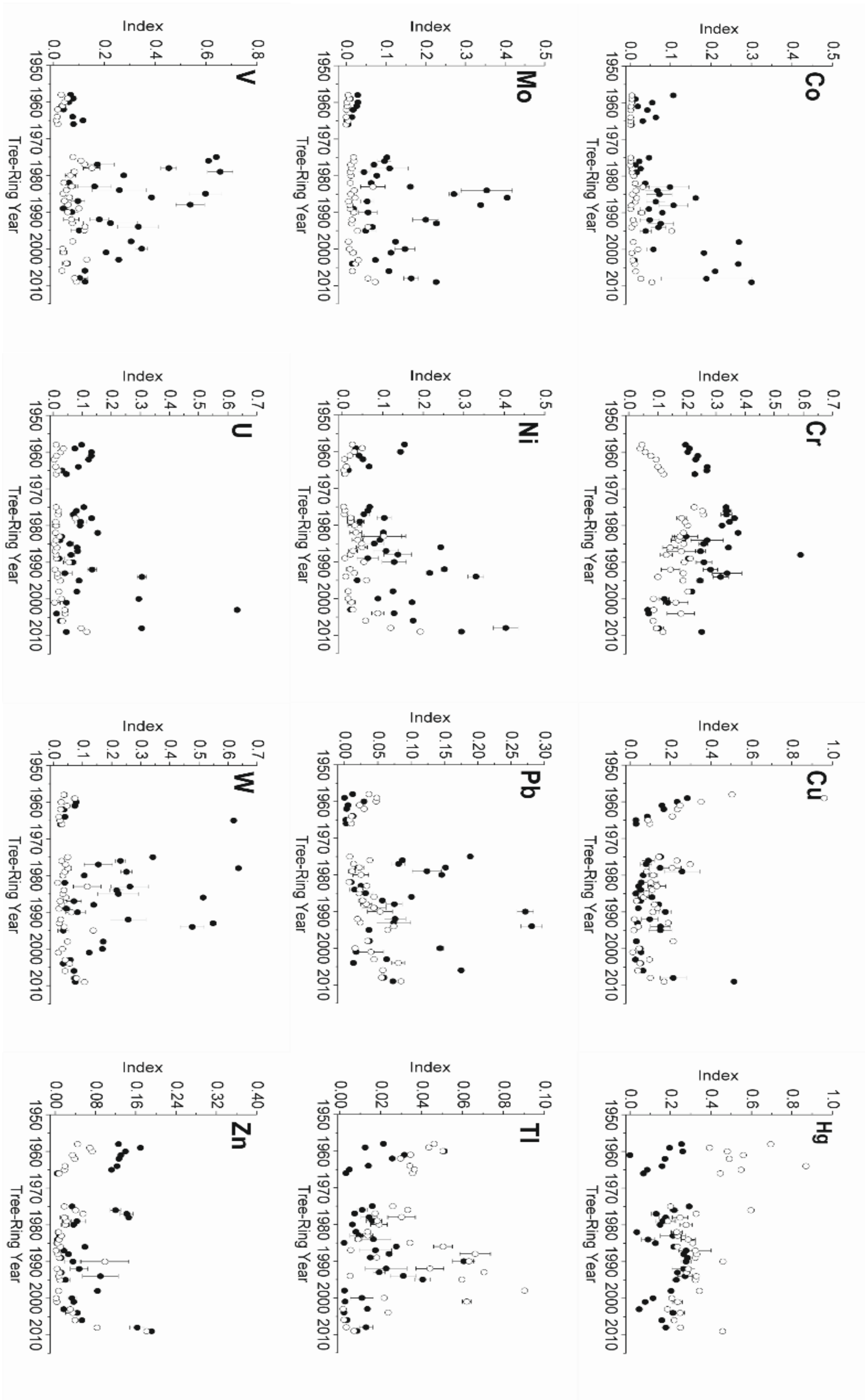


Figure 8: Tendency of elements distribution (mean normalized data) in tree-rings of P-chronology (black circles) and D-chronology (white circles).

The levels of Co, Mo, Ni, Pb, and U over time (*p-value* ≤ 0.05) (MK test rejected the null hypothesis, $H0 \leq 0.01$) increased significantly in both P- and D-chronologies. Vanadium and Cu levels showed an increasing trend only in D-chronology. Chromium, Tl, Zn and W levels did not show any temporal pattern (*p-value* ≥ 0.05, accepting the null hypothesis H0) (Table 3), whereas, Hg and Pb levels showed a decreasing pattern over time (Table 3).

Table 3: Mann–Kendall rank correlation test of trace elements in P- and D-chronologies. Values of Tau of Kendall, S (Kendall score, expressed as negative or positive value indicating decreasing or increasing trend, respectively), p-value and trend function, defined by arrows, are reported. Significant elements were marked in grey.

		P-chronology pattern		***D-chronology pattern***
Co	Tau di Kendall	0.398	Tau di Kendall	0.425
	S	237	S	253
	p-value (bilateral)	≤0.01	p-value (bilateral)	≤0.01
	trend	↗		↗
Cr	Tau di Kendall	-0.173	Tau di Kendall	-0.045
	S	-103	S	-27
	p-value (bilateral)	-	p-value (bilateral)	-
	trend	no		no
Cu	Tau di Kendall	-0.2	Tau di Kendall	-0.466
	S	-119	S	-277
	p-value (bilateral)	-	p-value (bilateral)	≤0.01
	trend	no		↗
Hg	Tau di Kendall	-0.324	Tau di Kendall	-0.237
	S	-193	S	-141
	p-value (bilateral)	≤0.01	p-value (bilateral)	≤0.05
	trend	↘		↘
Mo	Tau di Kendall	0.331	Tau di Kendall	0.435
	S	197	S	259
	p-value (bilateral)	≤0.01	p-value (bilateral)	≤0.01
	trend	↗		↗
Ni	Tau di Kendall	0.371	Tau di Kendall	0.314
	S	221	S	187
	p-value (bilateral)	≤0.01	p-value (bilateral)	≤0.01
	trend	↗		↗
Pb	Tau di Kendall	-0.096	Tau di Kendall	-0.24
	S	-57	S	-143
	p-value (bilateral)	-	p-value (bilateral)	≤0.05
	trend	no		↘
Tl	Tau di Kendall	-0.119	Tau di Kendall	-0.2
	S	-71	S	-119
	p-value (bilateral)	-	p-value (bilateral)	-
	trend	no		no

U	Tau di Kendall	0.257	Tau di Kendall	0.361
	S	153	S	215
	p-value (bilateral)	≤0.05	p-value (bilateral)	≤0.01
	trend	↗		↗
V	Tau di Kendall	0.113	Tau di Kendall	0.227
	S	67	S	135
	p-value (bilateral)	-	p-value (bilateral)	≤0.05
	trend	no		↗
W	Tau di Kendall	-0.018	Tau di Kendall	0.143
	S	-11	S	85
	p-value (bilateral)	-	p-value (bilateral)	-
	trend	no		no
Zn	Tau di Kendall	-0.129	Tau di Kendall	-0.197
	S	-77	S	-117
	p-value (bilateral)	-	p-value (bilateral)	-
	trend	no		no

Moreover, Cu, Hg and Tl were unevenly distributed in tree-rings in P- and D-chronologies. The normalized levels of these elements (I_x) varied in D-chronology in the period 1958-2009: Cu in d7 > d5 > d8 > d6 (0.3 ± 0.039, 0.3 ± 0.010, 0.11 ± 0.034, 0.1 ± 0.014, respectively); Hg in d5 and d7 > d6 and d8 (0.4 ± 0.016, 0.3 ± 0.014, respectively); Pb in d7 and d8 > d5 and d6 (0.04 ± 0.001, 0.03 ± 0.003, respectively); Tl in d5 > d8 > d6 and d7 (0.06 ± 0.002, 0.05 ± 0.001, 0.04 ± 0.003, respectively).

Significant differences in temporal trends of each element were found between P- and D-chronologies (*p-value* ≤ 0.01), except for Cu levels (Table 4).

Table 4: Levene's t-test of element patterns in P- and D-chronologies. The test evaluates the significant differences between the means for each element index in P- and D-chronologies (p-value ≤ 0.05). Significant elements were marked in grey.

		F	*p-value*
Co	Equal variances assumed		
	Equal variances not assumed	29.466	0.000
Cr	Equal variances assumed		

	Equal variances not assumed	3.670	0.060
Cu	Equal variances assumed	0.717	0.400
	Equal variances not assumed		
Hg	Equal variances not assumed		
	Equal variances assumed	6.648	0.012
Mo	Equal variances not assumed		
	Equal variances assumed	34.890	0.000
Ni	Equal variances assumed		
	Equal variances not assumed	15.516	0.000
Pb	Equal variances not assumed		
	Equal variances assumed	21.573	0.000
Tl	Equal variances not assumed		
	Equal variances assumed	8.658	0.004
U	Equal variances assumed		
	Equal variances not assumed	11.032	0.001
V	Equal variances assumed		
	Equal variances not assumed	44.811	0.000
W	Equal variances not assumed		
	Equal variances assumed	34.890	0.000
Zn	Equal variances assumed		
	Equal variances assumed	18.849	0.000

(II.2) 4. Discussion

(II.2) 4.1 Impact of urban environment on tree growth

Growth-climate analysis (weak relationships) highlighted that climatic effects do not completely explain the reduction of tree growth in plants grown at increasing proximity to the steel factory (P-sites). Whereas, pollution probably played a progressively negative effect on plant growth, as shown by the tree growth index (Fig. 2), in the 1950s, from 1973 and from 1990, in correspondence with the start of steel production, waste incinerator and biomass incinerator, respectively (Fig. 1). The periods of lowest precipitation in Terni were those from 1967 to 1975 and from 1988 to 2002, with cumulative annual precipitation ranging from 570 to 800 mm (Meloni and Carpine, 2004). This precipitation pattern did not fully explain the observed reduction of tree growth in the '80s.

Precipitation somewhat affected tree growth in D- and P-chronologies differently. Site-specific environmental conditions were probably the cause of a different microclimate between P- and D-chronologies, though at a short distance to each other. P-chronology is in the urban context with compacted soils and more heat (urban heat island), while D-chronology refers to the peri-urban area with less drastic transformations affecting soil conditions and moisture availability. The intensified warming caused by altered land surface properties and the lowered evapotranspiration-induced cooling due to sealed surfaces added to detrimental pollution levels, posing challenges to the growth of urban trees (e.g., Gregg *et al.* 2003; Robitaille, 1981; Svensson and Eliasson, 2002).

Although a stronger limitation to tree growth was observed in the proximity of the industrial plant, the impact exerted by other pollution sources could not be ruled out. The timing of the reduction of tree growth coincide with the development of a landfill in the same area during the end of '80s. Results obtained here pointed to higher levels of pollutants in P- compared to D-chronology. A marked reduction in tree growth due to the interactive effects of climatic conditions and industrial activities (paper manufacturing, aluminium-refining and phosphate fertilizer factories) was also observed in *Picea abies* Karst (Tomakomai forest), near a major industrial district in Hokkaido, Japan (Kobayashi *et al.* 1997). However, in the present case, the unfavourable urban context probably enhanced the environmental pollution effect in limiting tree growth more in P- than D-chronology (Barniak and Krąpiec, 2016; Fischer *et al.* 1994; Robitaille, 1981). More thorough examinations of elemental index profiles in stem wood and tree ecophysiological responses to stress factors are, therefore, needed before drawing unambiguous conclusions about the impact of environmental conditions on downy oak or other tree species as derived from dendrochemical analyses.

(II.2) 4.2 Trace elements in tree-rings

Different trace element profiles in tree-rings were detected with annual resolution in trees grown at different distance from the potential pollutant source, in the period 1958-

2009, reflecting industrial activities in the urban area. Levels of Co, Cr, Mo, Ni, V, W, and Zn were higher in annual rings of trees closest to the steel factory (Fig. 4). Moreover, the levels of Cr, Mo, Ni, V, and W in growth rings increased during the ‘80s and ‘90s, in agreement with a high peak of pollution history in Terni. During those pollution peaks, a waste disposal of the steel factory was implemented in “Terni-Papigno” (Arpa Umbria, 2010), causing an increase in particulate matter index. These findings were detected by monitoring stations. Yet, high depositions of particulate matter on leaves of downy oaks were observed in the same area (Sgrigna *et al.* 2016). The use of Cr, Mo, Ni, V, Zn, and W is common during the productive processes of steel manufacture (Santonen *et al.* 2010), for the corrosion resistance of flat-rolled stainless steel, and W, in the welding procedure (Santonen *et al.* 2010). These elements are, consequently, potential tracers of the industrial activity in the studied area, their elemental index varying with the level of pollution. Distinctively, concentrations of Cl and Cu were consistent in the area particularly exposed to the vehicular traffic.

Although U is a naturally occurring element found in low levels within rock, soil and water (UNSCEAR, 1993), it was difficult to explain the presence of U in our tree-rings, exceeding the background noise in P-chronology. The occurrence, source, distribution, level, mobility, and speciation of U in the soil environment might depend on both natural and anthropogenic factors. A potential pollution source of U accumulation in tree-rings could be found in the radioactive waste of the hospital, where the depleted U and radioactive isotopes are used, for example, for sterilization of medical equipment and for cancer therapy (Davidovits, 1994). The multiplicity and variability of possible pollution sources (in space, time, type, and level) added to the complexity of the pollution issue caused by the steel factory.

The uneven distribution of Cu, Hg, Pb, and Tl found in tree rings of P- and D-chronologies, with higher contents in the latter (Figure 4), may be explained by the accumulation of pollutants emitted by the incinerator in the peri-urban area of “Maratta” and the waste disposal areas around the D-chronology (Figure 1). According to recent researches, Hg, Pb and Tl (Crowley *et al.* 2003; Ruck *et al.* 1989) are trace metals commonly used to fingerprint emissions from waste incinerators (Font *et al.* 2015) and waste disposal (National Research Council, 2000). The area of Terni is interested by different pollution sources, such as incinerator and waste dump. A report of Regione Umbria (2008) identified, in the area of Terni, activities of burning or dumping of urban waste, hospital waste and electronic materials, which contain toxic metals, such as Hg, Pb and Tl. In fact, D-chronology showed higher values of Hg and Tl indexes compared to P-chronology. It is worth noting that trees grown in the plots closer to the incinerator in "Maratta" (d7 and d5) and waste disposal (d8) showed higher accumulation of elements differently distributed in the external plot, within the period 1958-2009.

Lead and Cu showed an erratic and unclear spatial and temporal distribution, probably due to other unspecific and/or multiple urban sources of pollution, such as local vehicular traffic and other industrial processes (Cansaran Duman *et al.* 2014; Harrison *et al.* 1994). However, Cu naturally occurs in the earth's crust, in rocks, soils, and waters (Manahan, 2010). It is interesting to note also that the decreasing patterns of Pb and Hg could be due to Pb replacement policies, and the replacement of Hg with non-toxic liquid metal alloy (NewMerc, e.g., gallium and indium), also in medical devices, (Rice *et al.* 2014). Copper did not show a specific temporal pattern in P- and D-chronologies, probably due to the varying sources of contamination (industrial and waste pollution), or the common background presence in the soil.

Although some difficulties in identifying the exact source were encountered, the investigation of tree-ring elemental index variability over time allowed the assessment of temporal patterns of pollutant accumulation and the comparison of differences among different sites. The increasing pattern of Co, Cr, Mo, Ni, U, V, W, and Zn levels in tree-rings within the monitored time span suggests a continuous contamination, in the town of Terni (Corona and Serneri, 2007; Terni, 2004).

Elements can be translocated through the xylem, phloem or ray cells, or can be mobile across tree-ring boundaries, radially or vertically translocated in the stem (Hagemeyer and Schiifer, 1995), which may complicate their analytical detection. Some elements are more mobile, and actively translocated within the plants through metabolic processes (e.g., Cl, K, Mg, P, S, etc.), than others (e.g., B, Ba, Ca, Cu, Fe, Li, Mn, Mo, Sr, and Zn, either immobile or restricted in their mobility within the phloem; Bukovac and Wittwer, 1957), which may provide controversial results. Instead, As, Cd, Cr, Cu, Fe, Hg, Mo, Ni, Sb, Sn, V, and Zn are not highly mobile within the stem (Odabasi *et al.* 2015). Therefore, a complication of the analytical detection can be determined by the migration of elements between individual tree-rings, or by the different mobility of elements that determine the elemental index in tree-ring series. The interpretation of temporal patterns is difficult and the possibility to use dendrochemistry for reflecting historical changes in the environment limited (Garbe-Schonberg *et al.* 1997).

(II.2) 5. Conclusion

The ring width growth decreased in trees grown in the proximity of sites impacted by pollutant sources, whereas the accumulation of trace elements increased. Although there were no simple correlations between the contents of trace elements in tree-rings and the index of pollutants in the surrounding environment, the LA-ICP-MS spot analysis was successful in describing historical variation in this highly polluted urban context. Determining the variability of trace element levels in tree-rings with annual resolution is of crucial importance when assessing contamination episodes and trends to identify the legacy of environmental contamination associated with industrial activities and subtle pollutants. The use of LA-ICP-MS spot analysis proved to be useful in providing systemic information regarding changes in historical pollutant loading, making the reconstruction of temporal trends of environmental contamination possible. However, a clear understanding of how elements become physiologically incorporated into tree-rings is still needed to have a precise interpretation of temporal trends in tree-ring elemental contents.

II.3 Chapter - NEW APPROACHES TO ENVIRONMENTAL RESEARCH

(II.3) a - Particulate matter in tree-rings of *Quercus pubescens* (Willd): micro-tomography synchrothrone analysis

Introduction: Currently, many studies use the bioindicators to qualitatively and/or quantitatively measure pollution. Tree growth rings represent a relevant bioindicator that records changes of the environment in the wood with an annual resolution. In detail, tree-rings are bioindicator of pollutants input from anthropogenic emissions (Temmerman *et al.* 2004), showing interesting indication of the effects of environmental conditions on tree growth (Lepp, 1975). The analysis of tree-rings may provide a signature of the temporal evolution of environmental stress, in order to identify heavy metal pollution and to distinguish various types of environmental contamination (Watmough, 1999). In the present study, the same tree-rings cores of *Quercus pubescens* (Willd), sampled in the city of Terni (Central Italy) and previously analysed using standard dendrochronological and LA-ICP-MS method (Perone *et al.* submitted, 2017), were examined with the application of synchrotron analysis. Synchrotron radiation X-ray tomographic microscopy analysis allows to characterize and quantify the three-dimensional (3D) network formed by vessels inside single growth rings and consequently detect the nanoparticles pollutants that tree transported into the stem (chemically identified with LA-ICP MS analysis). In the present study, we hypothesize that nanoparticles are stored in woody tissue and these produced changes in the structure of tree-ring wood related to changes in air quality and air pollution episodes and trends. The goal of this research activity was to detect, localize and identify nanoparticles in tree-rings differently exposed to pollution sources and pollution events.

Materials and methods: *A) tree cores collection*: four cores of *Q. pubescens* trees in each plot will be collected for the analysis according to the protocol described by (Perone *et al.* 2017), to identify the effects of the anthropic activities on tree growth and on the elements accumulation in the time. Samples were prepared to synchrothrone analysis.

B)Experimental procedure: the measurements were performed at the TOMCAT beamline of the Swiss Light Source, Paul Scherrer Institute (PSI), Villigen, Switzerland.7 The configuration used leads to a field-of-view of 720 x 720 µm2 and a nominal pixel size of 0.35 um/pixel. In order to ensure the high quality of our images and a faster acquisition, we employed a 2 x binning factor, resulting in 1024 x 1024 pixels radiographic images of 0.70 um/pixel. The detector, located at a distance of 30 mm from the sample, acquired 1001 (plus 2 dark fields and 2 flat fields) radiographic projections at equiangular positions over a total rotation angle range of 180" with an exposure time of 150 ms each, resulting in a total scanning time of approximately four minutes. After reconstruction, a tomographic dataset consists of 1024 radial-tangential cross section images (or slices) stacked at one pixel interval along the longitudinal direction. Each cross-section image has 1024 x 1024 pixels. *C) Tomographic data analysis:* the images were analyzed developing a specific algorithms and implementing a software which use specific morphological (spherical shape), colorimetric (grey scale), dimensional (≤ 5µm) and positional (border) parameter to identify particles in woody tissue. These automatize analysis were validated by visual analysis by an expert.

Preliminary results and discussion: The developed software, allowed on the one side to speed up the particle identification process (PM) and on the other side to identify particles that were initially ignored during a first visual analysis (Fig.1).

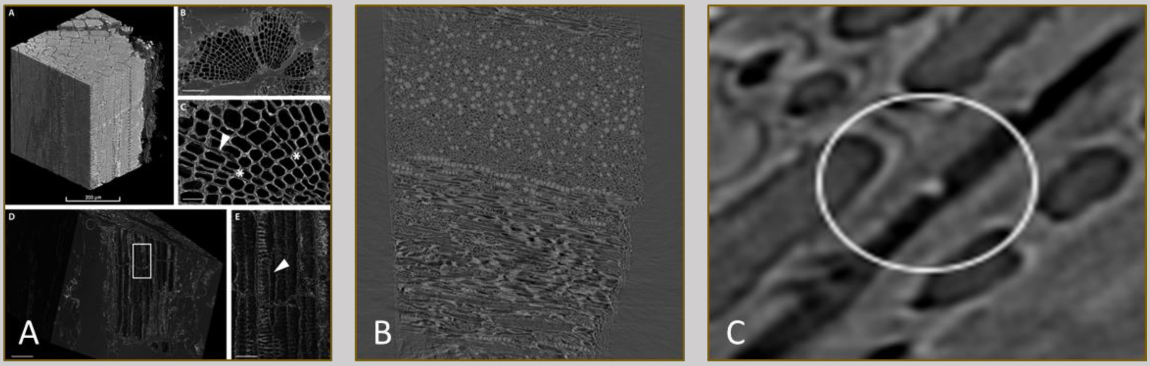


Figure 1: A) Tomographic images, B) analysis by a specific algorithms, C) localization of particles in woody tissue.

The research has a strong impact on the development of new strategies and approaches using trees to biomonitor emerging pollutants in urban ecosystems.

(II.3) b - Comparing pollutants in epiphytic lichens and tree-rings to detect pollution in urban forestry

Introduction: The use of organisms to assess pollution, has developed notably during the last few decades (Temmerman *et al.* 2004). Such organisms assume environmental contaminants and may be used as indicators of bioavailability of a given contaminant over time, allowing, in certain cases, comparison between contamination levels in geographically areas. Lichens have been widely used as bioaccumulators of trace elements, since their metabolism is very dependent on pollutant atmospheric exchanges and they are metal tolerant (Bargagli and Nimis, 2002). Lichens are able to accumulate trace elements in very high levels, and the concentrations of the trace elements inside the thalli of several lichen species seem to be directly correlated with the environmental levels of these elements (Paoli *et al.* 2013). In the case of trees, they may accumulate anthropogenic pollutants either from the atmosphere, by deposition onto foliage and/or bark, and/or following deposition on the soil and subsequent uptake by roots. These pollutants induced datable signals in tree-rings that can be useful to reconstruct environmental pollution over time. While several studies have focused on both lichens and mosses as bioaccumulators, which take up and retain metals differently (e.g., Szczepaniak and Biziuk, 2003; Giordano *et al.* 2013), few have used a multi-source approach. This research, focused on the integration of tree-rings of downy oak and lichens to biomonitor Terni, one of the most polluted towns in Italy. The area of Terni is characterized by several industrial plants and the particulate matter (PM) pollution is a serious problem for the city. Downy oak (*Quercus pubescens*) tree-rings and transplants lichens (*Evernia prunastri*) were sampled in eight plots of the urban area and analyzed for the trace elements. The study was aimed to characterize the heavy metal composition of tree-rings and lichen thalli in order to compare the pollution signals recorded by these organisms, which can be implemented in monitoring activities.

Materials and methods: Tree-ring cores of 32 oaks (*Quercus pubescens*) were sampled in 8 plots (Perone *et al.* 2017) (Fig. 1), where lichens (*Evernia prunastri*) were transplanted from an unpolluted area on the sampling trees for 3 months. Trace elements signals in tree-ring cores were determined using high-resolution laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), while element concentrations in lichen thalli were determined by ICP-MS.

Figure 1: Location map of sampling site

Preliminary results and discussion: The following trace elements, Cr, Co, Cu, Pb, Mo, Ni, Tl, W, U, V, and Zn, were detected, indicating the sensitivity of both sample types for the same anthropogenic sources. Results highlighted that high pollutant accumulation occurred in the same plots for both lichens and tree-rings (Fig. 2).

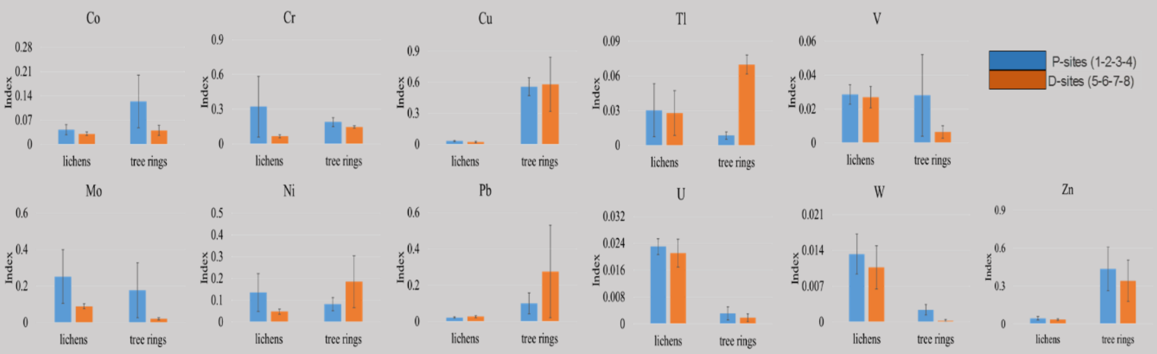
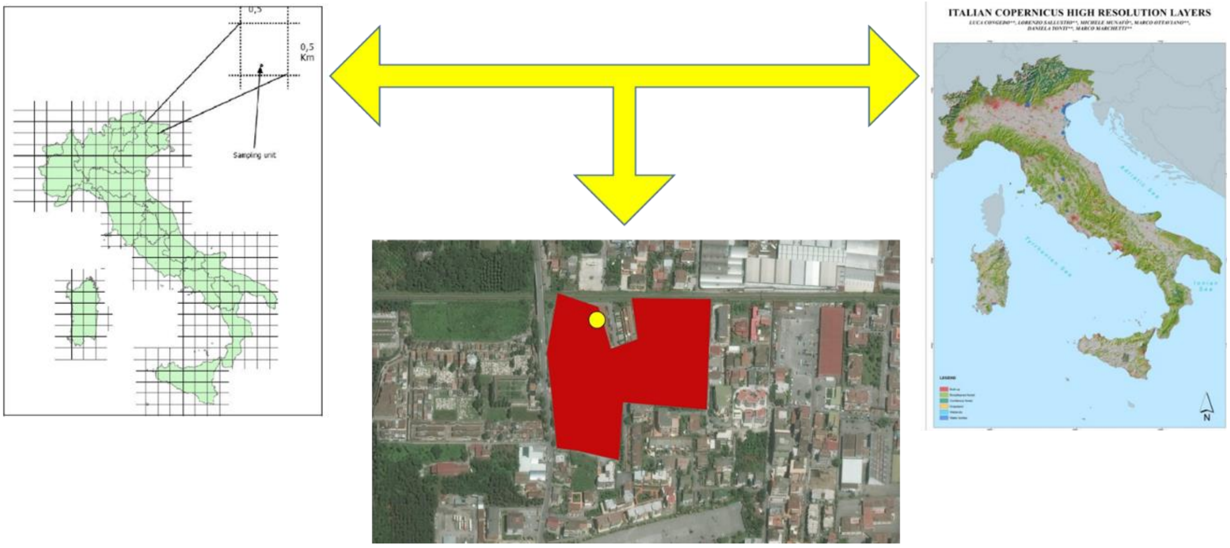


Figure 2: Comparison between accumulation pattern of elements in tree-rings and lichens in Terni (Central Italy)

However, the accumulation pattern of elements was species-specific and related to environmental factors, such as the dominant wind direction. The comparison of pollution signals in different organisms and tissues is important to define pollution thresholds for sensitive environments.

II.4 Chapter- THE GREEN SIDE OF THE GREY: ASSESSING GREENSPACES IN BUILT-UP AREAS OF ITALY



Abstract

Although greenspaces in built-up areas (GSB) heavily contribute to human health and wellbeing in anthropized environments, large-scale data and information on GSB are still lacking. This study aims to estimate abundance, coverage and average size of GSB in Italy through the integration of inventory and cartographic data, even considering GSB patches smaller than 0.5 ha. GSB were classified according to their type (forested and non-forested), canopy cover and location with respect to population density (from thinly to densely populated areas). We refer to built-up areas instead urban, thus including, for example, road infrastructures, buildings and sparse settlements in rural areas, otherwise neglected.

Results show that 43.5% of built-up areas in Italy is unsealed, of which more than 18% is covered by GSB, mainly dominated by forested ones. Results also reveal that the number of forested GSB, their coverage and average patch size decrease with the increase of the population density, while their canopy cover follows the opposite trend. Main findings demonstrate that the proposed approach provides reliable GSB estimates to better understand interactions between humans and nature along an urban-rural gradient, thus

representing a valuable support towards the implementation of Nature-Based Solutions in urban planning.

Keywords: *land inventory, statistical sampling, green infrastructures, Nature-Based Solutions, urban planning*

(II.4) 1. Introduction

We are living in the Anthropocene, an era characterized by heavy human modifications of the planet (Steffen *et al.* 2015). Considering that more than half of human population lives within cities, and 66% of world's population is projected to be urban by 2050, some authors even refer to this period as "Urban Age" (e.g., Derickson, 2015). Since the Industrial Revolution, the fast economic and social changes have brought massive expansion, redevelopment, and restructuring of built-up areas (Jim and Chen, 2006). These have led to a permanent conversion of 'greenspaces' into 'grey' impermeable surfaces, with several negative impacts on the environment, such as rising concentrations of pollutants, climate alteration, modification of hydrologic and biogeochemical cycles, and habitats fragmentation (Kong and Nakagoshi, 2006; Uy and Nakagoshi, 2007). However, these changes offer the opportunity to recognize the complementarity between built and natural forms (Jim and Chen, 2006).

Greenspaces can be distinguished from their 'grey' counterparts considering their spatial connectivity and multi-functionality (Lafortezza *et al.* 2013). These forested and green areas within urbanized landscapes were proved to be key components that significantly improve human health and wellbeing (Panno *et al.* 2017), and thus support the city-nature bond (i.e., *biophilic urbanism* (Beatley, 2009). In addition, greenspaces provide environmental benefits (Gómez-Baggethun and Barton, 2013), including carbon sequestration (Livesley *et al.* 2016) and particulate matter removal (Grote *et al.* 2016). These benefits depend on different features, among which the typology, extension and management of greenspaces (Rall *et al.* 2015, Kim, 2016). As human-induced disturbances are becoming increasingly frequent and relevant, the current land use planning system appears inadequate to tackle the vulnerabilities of urbanized landscapes. This aspect emphasizes the importance of monitoring greenspaces, also as isolated forest patches, not only within cities but also in peripheral contexts (Salvati *et al.* 2016).

The recently introduced concept of Nature-Based Solutions (NBS) (Nesshöver *et al.* 2016) has highlighted the general need of adopting clear, standardized and unambiguous definitions to facilitate the effective implementation of a multidisciplinary and integrated approach in analysing the hybrid and complex urban environment (Taylor and Hochuli, 2017). For example, Taylor and Hochuli (2017) found that there are different uses and definitions of the term greenspace that limit *i*) the availability of Country-scale information, *ii*) the possibility for comparative studies, and iii) the capacity to effectively support decision making processes. Particularly, the term greenspace is often followed by secondary terms or adjectives to better describe the location or objective of the study, such as "urban", "open", "public" etc., which in turn define various subsets of analysis. Similarly, there is a lack of a standardized description of what is urban and what is not, depending on urban growth patterns and specific socio-economic contexts for which these definitions are conceived (MacGregor-Fors, 2011). For example, the term "urban and peri-urban forest", as specified by many studies (e.g., Konijnendijk *et al.* 2000), usually refers to all trees within and close to urban areas, thus covering a quite generic and wide meaning (Konijnendijk *et al.* 2000). Although urban areas usually refer to definitions based on both biophysical and population density characteristics (e.g., MacGregor-Fors, 2011), the term "built-up areas" only refers to biophysical ones, thus including, for example, road infrastructures and sparse settlements in rural areas, otherwise neglected. Therefore, adopting the concept of built-up areas instead of urban ones is unambiguous and appropriate. Accordingly, in the present study we consider Urban Green Spaces (UGS) as a subset of Green Spaces in Built-up areas (GSB), being the former only related to urban areas and the latter to all the built-up areas, even where they are represented by road networks or sparse settlements in rural contexts.

The availability of data on UGS and GSB strictly depends by the different classification systems that can be based on land use or land cover aspects (Sallustio *et al.* 2016). Particularly in the EU context, another limitation is attributable to a lack of available homogenized large-scale forest data (e.g., extension of isolated forest patches, tree density and distance from non-forest and forest areas to the nearest forest in the urbanized landscape, etc.) (Gómez-Baggethun and Barton, 2013), which has so far hampered the comparative assessment of urban and peri-urban forests. In addition, GSB usually consist in small, fragmented and isolated patches, thus increasing the difficulties related to their large-scale detection and monitoring. Moreover, technical constraints related to the minimum mapping unit of the most widely-used land use and land cover maps often hamper the detection of small GSB patches. For example, considering the minimum mapping unit of 25 ha (Di Gregorio and Jensen, 2005), the adoption of the Corine Land Cover map hinders the detection of patches under this threshold. On the other hand, the use of more accurate data, such as those provided by Urban Atlas (EEA, 2011), is currently restricted to only the biggest EU cities (i.e. those with more than 100,000 inhabitants) (Salvati *et al.* 2017), thus strongly limiting a comprehensive evaluation of GSB.

The monitoring of changes in extent and spatial distribution of GSB is important to foster environmental benefits in land and urban planning through the implementation of "new greenspaces" or the modification of existing ones according to social and economic conditions (Lin *et al.* 2017, 2015). Nowadays, reliable large-scale information are usually limited to UGS instead GSB, and to those provided by forestry-related surveys like the National Forest Inventories (NFI) (Corona, 2016; Corona *et al.* 2002; Schnell, 2015). Corona *et al.* (2012a) applied a methodology to estimate the abundance, coverage and average size of urban forests in Italy from NFI data. Indeed, in Italy, available data are currently limited to urban forests while neglecting other greenspaces in built-up areas (e.g., lawns, gardens, cemeteries, uncultivated lands).

The aim of the present study is to assess the abundance, coverage and average size of GSB in Italy, including small-size patches, lawns, gardens and other greenspaces dominated by grasslands instead of only urban forests as defined by the FAO classification's system. Accordingly, we improved the large scale monitoring of GSB through the implementation of an inventory approach able to provide statistically-sound estimates. GSB were classified according to *i*) their type (forested and non-forested GSB), and *ii*) their location with respect to the population density following a rural-urban gradient (from thinly to densely populated contexts), which has a fundamental role affecting the provisioning of environmental services (Gómez-Baggethun and Barton, 2013), as well as their beneficiaries (i.e., "serviceshed"; Tallis *et al.* 2015). The methodology is based on GIS and statistical analysis to integrate the inventory data provided by the Italian Land Use Inventory (Pagliarella *et al.* 2016) with those of the Italian High-Resolution Land Cover Map (Congedo *et al.* 2016). Others ancillary data were then added to this framework to characterize the spatial distribution of GSB.

(II.4) 2. Materials and methods

(II.4) 2.1 Study area

The investigated area covers the whole Italian territory of about 302,070 km^2, which is mainly dominated by croplands (33.4% of the national territory) and forests (32%) (Pagliarella *et al.* 2016). Built-up area covers about 7.1% of the national territory, one of the highest relative coverages among EU countries (Corona *et al.* 2012b), and has increased during last decades in spite of the generalized negative national demographic trend (ISPRA, 2016). The national land take pattern is affected by the distribution of mountains, as well as by the weak implementation of land use plans, resulting in a highly scattered and fragmented urban mosaic (Romano *et al.* 2017). Due to spatial configuration, mapping and assessment of built-up areas as well as of the related greenspaces are rather difficult. Fragmentation processes lead to the availability of unsealed spaces within and surrounding built-up contexts, as generally demonstrated by the most recent data on soil consumption at national scale (ISPRA, 2017) and by the detailed study on the highly industrialized Lombardy region, northern Italy (Sanesi *et al.* 2016). More in depth, for example, Sallustio *et al.* (2016) have recently estimated that almost 32% of the built-up area in the Molise region (central Italy) is unsealed.

(II.4) 2.2 Available data and classification systems

We integrated inventory data and cartographic information in order to discriminate the portion of built-up areas that is unsealed from those covered by greenspaces. In particular, built-up areas were classified through an inventory approach, and their characterization related to the biophysical coverage was possible thanks to the integration with very high-resolution land cover layers. The overall approach is outlined in Figure 1: starting from the assessment of the built-up areas and following a hierarchical classification system, the GSB class was derived and in turn classified according to *i)* the presence of trees (forested and non-forested GSB); *ii)* the patch size (forests in GSB and small woods); *iii)* the crown coverage, represented by the level of the canopy cover (high, medium and low coverage).

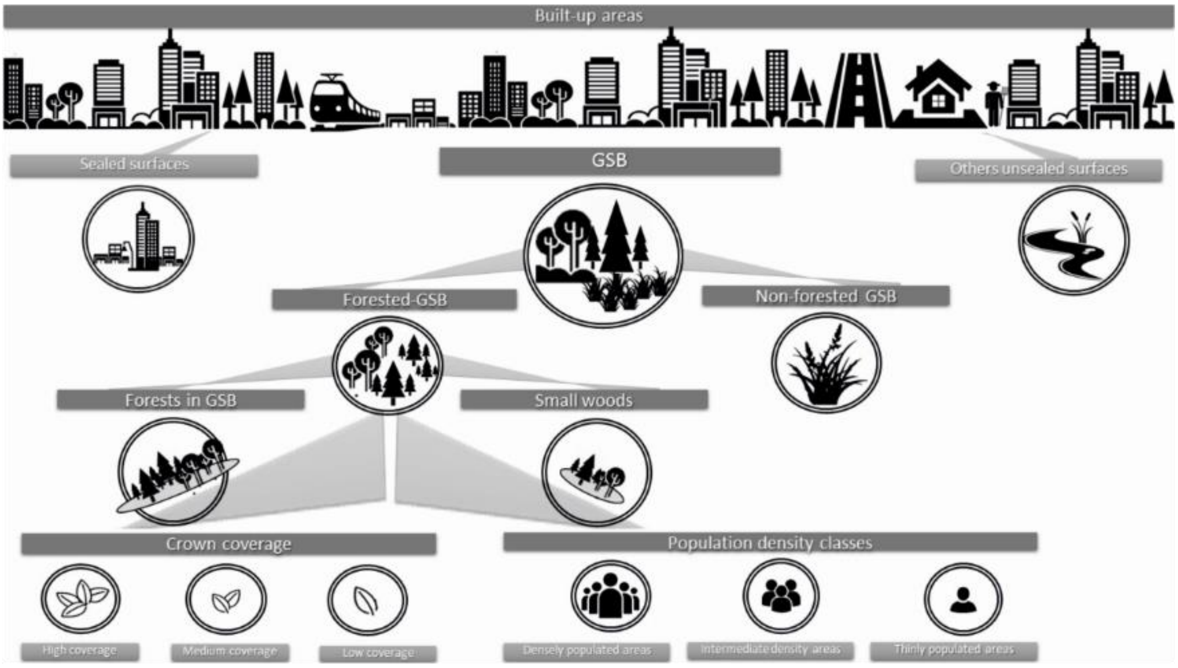


Figure 9: The hierarchical approach used to discriminate and characterize GSB

Furthermore, the forested-GSB class was refined and characterized according to the population density (densely populated areas, intermediate density areas and thinly populated areas). Details on the classification system adopted are reported in Table 1.

Table 5: Classification system adopted.

Class	***Definition/Description***
Built-up areas	Area dominated by settlements (continuous and discontinuous) larger than 0.5 ha and larger than 20 m, including roads and railways network, ancillary works, industrial and commercial areas, ports, dumps, cemeteries, urban parks and garden (Marchetti *et al.* 2012)
Greenspaces in built-up areas (GSB)	Lands covered by natural or man-made vegetation, enclosed in built-up areas (modified from Kong and Nakagoshi, 2006; Uy and Nakagoshi, 2007)
Forested-GSB	GSB dominated by trees
Forests in GSB (F-GSB)	Forested-GSB larger than 0.5 ha (modified from Konijnendijk *et al.* 2006)
Small woods (SW)	Forested-GSB smaller than 0.5 ha (modified from de Foresta *et al.* 2013)
Non-forested GSB	GSB dominated by bushes, herbs, lawns and gardens

(II.4) 2.2.1 The Italian Land Use Inventory

The Italian Land Use Inventory (*Inventario dell'Uso delle Terre d'Italia* – IUTI) was used to assess built-up areas at national scale. IUTI was implemented by the Italian Ministry of Environment, Land and Sea as an instrument of the National Registry for forest carbon sinks for the accounting of GHG emissions (Corona *et al.* 2012b). IUTI served to estimate land use changes in Italy from 1990 to 2008, according to the Good Practices Guidance for Land Use, Land Use Change and Forestry classification system (IPCC, 2003). IUTI is based on a tessellation stratified sampling scheme, where the Italian territory was covered by a network of 1,217,032 quadrats of 25 ha, and a random point was then located in each of them. Each sample point was photo-interpreted and classified, according to standard size of references: a) extension greater or equal to 0.5 ha; and b) width greater or equal to 20 m. Due to its large sample size and the design-based approach, the uncertainty values of IUTI estimates have been precisely assessed, with relative standard errors lower than 1% for all the land use classes at national scale (i.e., 0.3% for urban areas). Largely due to its simplicity, IUTI can rapidly be updated, if needed (Pagliarella *et al.* 2016).

(II.4) 2.2.2 The Italian High-Resolution Land Cover Map

We used the Italian High-Resolution Land Cover Map at 2012 to investigate the land cover within built-up areas. It covers the entire national territory with the aim of monitoring soil imperviousness (sealed surfaces) and natural (unsealed, permeable) covers, namely forests, grasslands, wetlands, and water surfaces, with a high spatial resolution of 20 m (Congedo *et al.* 2016). Afterwards, we used the grasslands and the forests high-resolution layers to discriminate respectively non-forested and forested GSB, among the unsealed built-up areas. The high-resolution raster layers were converted into polygons to discriminate unique and continuous patches of forests or grasslands. An overlap analysis was then performed in a GIS environment to identify patches containing at least one IUTI point classified as "built-up" at 2008. According to the classification system in Table 1, the IUTI points classified as built-up falling within grasslands patches were classified as non-forested GSB, while those falling within forests patches were classified as forested GSB. The latter, according to the patch size, were then classified as forests in GSB (F-GSB) or small woods (SW), if falling within forested GSB patches, respectively, larger or smaller than 0.5 ha. Moreover, the high resolution layer of forests contains information on the crown coverage expressed as percentage per pixel. A mean crown coverage value was calculated for each forested GSB patch. Using the crown coverage thresholds adopted by the NFI (Gasparini and Tabacchi, 2011), the forested GSB were classified into: high (mean crown coverage higher than 80%), medium (mean crown coverage between 50 and 80%) and low (mean crown coverage lower than 50%) coverage, forested GSB.

(II.4) 2.3 The GEOSTAT population grid

Data on population density at national scale were derived from the GEOSTAT population grid at 2011, with a spatial resolution of 1 km. An overlap analysis was carried out to assign a mean population density value to each GSB patch. These data were aggregated, according to the DEGURBA classification system (Dijkstra and Poelman, 2014), into three population density classes as follows: *(i)* densely populated areas, with an average population density higher than 1,500 inhabitants/km^2; *(ii)* intermediate density areas, with an average population density between 300 and 1,500 inhabitants/km^2; *(iii)* thinly populated areas, with an average population density lower than 300 inhabitants/km^2.

(II.3) 2.4 GSB estimation

We adopted the methodology based on the design-based estimation approach developed by Baffetta *et al.* (2011) and already implemented by Corona *et al.* (2012a) and Sallustio *et al.* (2015) to assess coverage, abundance and average size of urban forests and sealed areas, respectively.

In this case, A, N and $\bar{a}$ being, respectively, the coverage, abundance and average size of the GSB patches or any sub-class according to the considered classification system, and Q the extent of the area formed by the n square grid cells completely covering the study area under the tessellation stratified sampling scheme adopted by IUTI. The estimate of A is given by

$$\hat{A} = \hat{p}Q \qquad [1]$$

where:

$$\hat{p} = \frac{n_u}{n} \qquad [2]$$

n_u = number of sample points classified as GSB (or their sub-classes).

The variance of $\hat{A}$ can be estimated as

$$v\hat{a}r(\hat{A}) = Q^2 \frac{n_u(n-n_u)}{n^2(n-1)} \qquad [3]$$

S and a_j are, respectively, the set of GSB patches selected at least once by the n sampling points and the size of the *j-th* GSB patch. If the values of a_j are negligible with respect to Q, the estimate of N is given by

$$\hat{N} = \frac{Q}{n}\sum_{j\in S}\frac{1}{a_j} \qquad [4]$$

with estimated variance equal to

$$\mathrm{v\hat{a}r}(\hat{N}) = \frac{1}{n(n-1)}\left(Q^2\sum_{j\in S}\frac{1}{a_j^2} - n\hat{N}^2\right) \qquad [5]$$

Accordingly, the estimate of $\overline{a}$ is given by $\hat{A}/\hat{N}$, i.e.,

$$\hat{\overline{a}} = \frac{n_u}{\sum_{j\in S}\frac{1}{a_j}} \qquad [6]$$

with estimated variance equal to

$$\mathrm{v\hat{a}r}(\hat{\overline{a}}) = \frac{Q^2}{\hat{N}^2 n(n-1)}\sum_{j\in S}\left(1-\frac{\hat{\overline{a}}}{a_j}\right)^2 \qquad [7]$$

(II.4) 3. Results

Sealed (consumed) surfaces occupy 54.8% (1,174,050 ha) of the built-up areas in Italy at 2008, while 43.5% (931,100 ha) is covered by unsealed (non-consumed) classes and 1.7% is unclassified (34,475 ha) (Figure 2). GSB cover approximately 170,215 ha and wetlands and permanent water bodies 11,825 ha (Figure 2). GSB are largely dominated by forested GSB (92%), while non-forested GSB cover about 8% of their total surface.

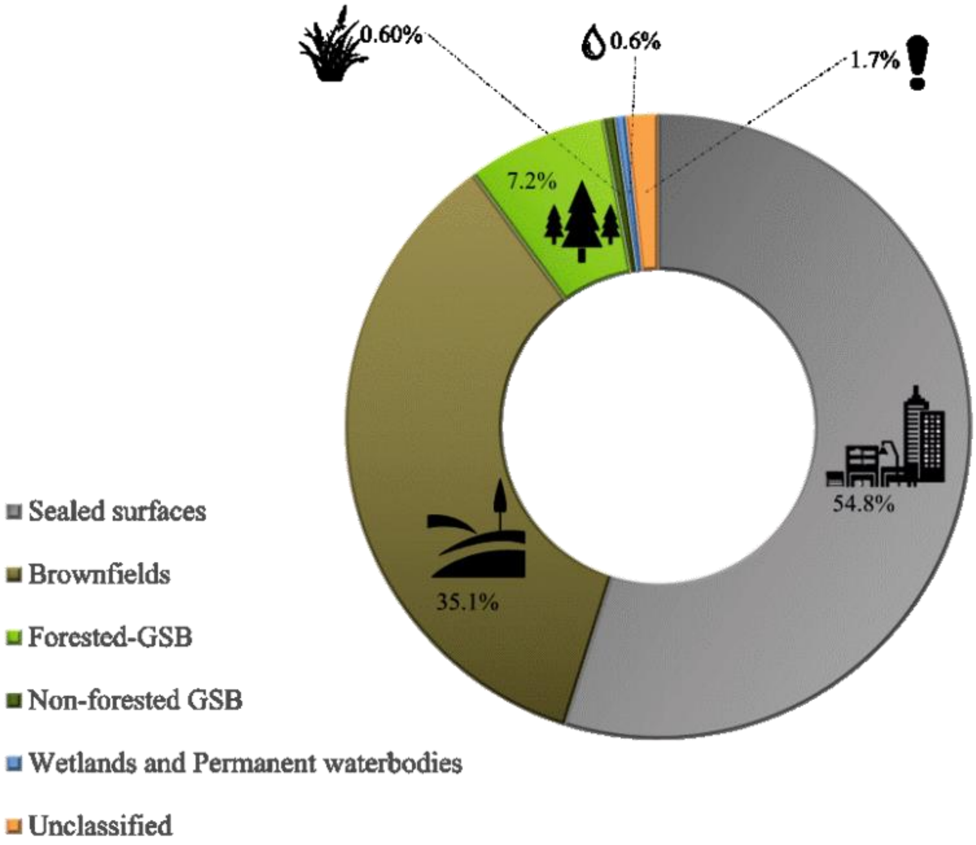


Figure 10: Built-up areas classified according to the High-Resolution Layers land cover classification.

F-GSB cover about 139,971 ha (±1.34%) representing 89% of the forested GSB, while the remaining is represented by SW (16,623 ha ±3.89%) (Table 2). Conversely, if considering their abundance in terms of number of patches, we exactly found the opposite situation with SW representing almost 89% of the total number of forested GSB. This is due to the difference in terms of average patch size, passing from 0.07 ha (±3.56%) in SW to 4.63 ha (±2.50) in F-GSB.

Table 6: Estimates of abundance $\hat{N}$, coverage $\hat{A}$ and average size $\hat{\bar{a}}$ of GSB, and their estimated relative standard errors (expressed in percent), distinguished by their type (forested GSB and non-forested GSB) and patch size (F-GSB and SW).

		$\hat{N}$	s.e. (%)	$\hat{A}$ (ha)	s.e. (%)	$\hat{\bar{a}}$ (ha)	s.e. (%)
	Total	**272,214**	**0.96**	**156,594**	**1.27**	**0.58**	**4.46**
Forested GSB	**SW**	241,996	1.02	16,623	3.89	0.07	3.56
	F-GSB	30,218	2.89	139,971	1.34	4.63	2.50

Non-forested GSB	**22,330**	**3.36**	**13,621**	**4.30**	**0.61**	**16.20**

The number of forested GSB decreases with the increase of the population density as well as their coverage (Table 3). The mean patch size of forested GSB is higher in thinly populated areas (0.85 ha), than in intermediate density (0.39 ha) and densely populated areas (0.30 ha). The highest average patch size was found in F-GSB sited in thinly populated areas (7.04 ha ±3.98%), while the lowest in densely populated ones (2.12 ±3.18%). Otherwise, the average patch size is almost stable for SW. The estimates of relative standard errors are satisfactory, unless those referred to the estimation of mean patch size for SW in intermediate density and densely populated areas, due to the relatively few SW within such areas (Table 3).

Table 7 Estimates of abundance $\hat{N}$, coverage $\hat{A}$ and average size $\hat{\bar{a}}$ of forested GSB, and their estimated relative standard errors (expressed in percent), distinguished by population density and patch size (F-GSB and SW).

		$\hat{N}$	s.e. (%)	$\hat{A}$ (ha)	s.e. (%)	$\hat{\bar{a}}$ (ha)	s.e. (%)
Thinly populated areas	**Total**	**120,806**	**1.44**	**102,513**	**1.57**	**0.85**	**6.94**
	SW	107,230	1.53	6,987	6.01	0.07	5.34
	F-GSB	13,576	4.31	95,525	1.62	7.04	3.98
Intermediate density areas	**Total**	**93,854**	**1.64**	**36,828**	**2.62**	**0.39**	**7.42**
	SW	83,476	1.74	5,549	6.74	0.07	47.65
	F-GSB	10,378	4.93	31,278	2.84	3.01	3.92
Densely populated areas	**Total**	**57,555**	**2.09**	**17,254**	**3.82**	**0.30**	**17.95**
	SW	51,290	2.22	3,985	7.96	0.08	77.00
	F-GSB	6,265	6.34	13,268	4.36	2.12	3.18

Forested GSB are generally composed by medium to high coverage forest stands (50.4 and 34.5%, respectively) (Figure 3). This trend is particularly enphasized in thinly populated areas, where high coverage forest stands represent 44.8% of the forested GSB surface. Conversely, while medium and low coverage stands tend to increase their relative coverage passing from thinly to intermadiate and densely populated areas, high coverage ones dicrease. The forested GSB with low crown coverage reach their highest relative surface in intermediate density areas (25.3% of the total forested GSB surface in this population class).

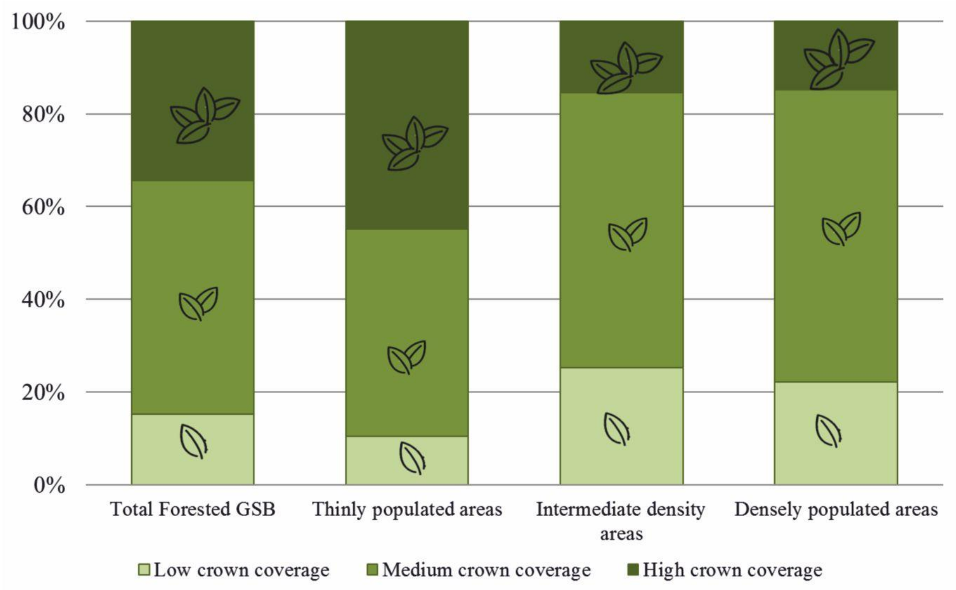


Figure 11: Distribution of the crown coverages' classes of forested GSB according to the population density classes.

(II.4) 4. Discussion and conclusions

(II.4) <u>4.1 Consistency and characteristics of greenspaces in built-up areas in Italy</u>

Results mainly indicated that, in Italy, more than half of the whole built-up area is sealed (54.8%). This value is similar to that observed in other EU countries (i.e., Germany = 52%; EEA, 2006), and rather consistent with available information on soil sealing within urban morphological zones in Europe, whose range is between 23% and 78% (EEA and JRC, 2010). A considerable portion of built-up areas in Italy is, however, unsealed (44.5%), of which more than 18% represented by GSB. On the other hand, more than 80% of the total unsealed area is covered by brownfields, which, in spite of their current low ecological value, are *de facto* available to "re-create", enhance and implement greenspaces (Haase *et al.* 2014).

Considering the current lack of information on GSB and the difficulty to refer to a unique classification system, comparing our estimates with other studies on the same topic is challenging (De La Barrera *et al.* 2016). The use of indicators can be helpful to overcome this issue. The square meters of greenspaces per capita is often used as a suitable indicator to describe the relevance of GSB for public health and wellbeing (World Health

Organization, 2012). We found that 27.2 m^2 of GSB is averagely available per inhabitant. Even considering the different classification systems, this value is proximal to that provided by national statistics in 2014 for UGS (approximately 31 m^2 of greenspaces per-capita; ISTAT, 2016), and it is similar to those in other European countries, such as Austria and Finland (Hansen *et al.* 2015). This average value is influenced by the high value of GSB per capita in thinly populated areas (72 m^2), while it considerably decreases in intermediate and densely populated ones (15.2 and 9 m^2, respectively). Moreover, we found that the average national value of GSB per capita is higher than the threshold fixed by the italian law to secure the quality of life in urban areas of 9-11 m^2/inhabitant (DM 14/01/2013 n°10), while the one referred to densely populated areas is exactly in line with it. The value of GSB per capita highlighted the necessity of being careful in evaluating urban densification hypothesis in Italy, which may lead to a worrisome decrease of this indicator especially in city centres, reducing their attractiveness for inhabitants (Nilsson *et al.* 2014).

Results also unravelled that GSB are widely dominated by forests and, secondarily, grasses. The combination of natural elements other than trees (i.e., bushes and grasses) strengthens the perceived restoration from greenspaces in urban contexts (Van den Berg *et al.* 2014). Moreover, although gardens and lawns are poorly considered while assessing greenspaces (Mathieu *et al.* 2007), they offer several benefits, such as urban water cycle improvement, increase of carbon sequestration (Davies *et al.* 2011) and reduction of surface runoff (Cameron *et al.* 2012).

F-GSB constitute a considerable part of the total GSB area, in comparison with that covered by SW. Larger patches of forested GSB have a key role in preserving biodiversity (Gonzalez *et al.* 2010), increasing cooling effect (Kong *et al.* 2014), and reducing nutrient leaching (Nidzgorski and Hobbie, 2016). Large crowns and foliar biomass help sequestrating gaseous pollutants from the urban atmosphere. Concentration of particulate matter (PM1) was found to be lower in urban parks, and urban and peri-urban forests were found to abate up to 20% of PM_1 emitted from anthropogenic sources (Fares *et al.* 2016). Among the others, the fragmentation and size of greenspaces were demonstrated to be discriminatory variables affecting their potentiality to provide services, especially in highly populated areas (Chang and Lee, 2016). It is worth noting that the ratio between SW and F-GSB tends to increase from thinly to intermediate and densely populated areas (7.3, 17.7 and 30%, respectively), thus highlighting the different contributions in socio-ecological terms attributable to small forested lots. This trend is also confirmed by the decrease of F-

GSB patch size when moving from thinly to densely populated areas. In spite of their little significance to cities' ecological conditions, small GSB patches in densely populated areas play a very important role in enhancing their liveability and quality of life, thus even increasing their attractiveness in the future (Nilsson *et al.* 2014). Other spatial processes of forest dynamics, such as the distance from non-forested and forested areas to nearest forest patches in a certain landscape, or the extension and characteristics of the so called trees outside forests (Fattorini *et al.* 2016), will be needed to quantify differences over space and time.

The proportion of forested GSB with high crown coverage decreases when passing from thinly to densely populated areas, while stands with low crown coverage (less than 50%) are more present in intermediate density areas, followed by densely and thinly populated ones. This behaviour may derive from the characteristics of forested GSB, when changing the type of urban pattern in relation to the population density. Accordingly, as a general trend, in densely populated built-up areas, GSB usually refer to small lots spreading over a high number of patches with intermediate crown coverages (i.e., private gardens, squares, vegetated roundabouts, tree rows; Del Tredici, 2010). Those in thinly populated areas are widely represented by more compact and larger patches with high crown coverage (i.e., parks, forests). The negative relationship between population density and crown coverage was demonstrated by Nowak *et al.* (1996). However, our results indicate that the relative contribution of forested GSB with low crown coverage with respect to the total forested GSB area spans from 10.4 to 25.3% in thinly and intermediate density populated areas (average of 15.1%), respectively. Considering that Heynen and Lindsey (2003) suggested 40% as a threshold value for crown coverage in forested GSB to provide all the possible ecological benefits, we conclude that the Italian forested GSB provide a relevant contribution in this sense.

(II.4) 4.2 Towards implementing Nature-Based solutions in built-up areas in Italy

The estimates of abundance, coverage and average size of urban forests provided by Corona *et al.* (2012a) are quite similar to F-GSB located in intermediate and densely populated areas (thus intuitively referable to urban and peri-urban contexts). Hence, for this aggregated class, we obtained estimates 16% lower in terms of number of patches (16,642 vs. 19,806), while 3.6 and 23.3% higher in terms of coverage (44,546 vs. 43,000 ha) and average size (2.68 vs. 2.17 ha) than estimates achieved by Corona *et al.* (2012a). It is worth noting that the coverage of F-GSB, located within environmental settings other than densely populated areas (i.e., continuous urban fabric), constitute approximately 90% of the whole F-GSB coverage, similar to the value (93%) provided by Corona *et al.* (2012a). Again, F-GSB in intermediate and densely populated areas represent 26.2 and 5.7% of the total coverage and number of GSB. These results demonstrate that the concept of built-up areas, instead of the urban ones, relies to different estimates. In light of this, we can conclude that enlarging information to GSB rather than only urban forests may have a crucial role to better and fully incorporate ecological principles within land use and urban planning processes. This is particularly relevant in Europe, where cities have progressively become much less compact during last decades (Nilsson *et al.* 2014), in favor of highly fragmented, scattered and dispersed patterns characterized by a relatively low population density (Romano *et al.* 2017). Therefore, our methodology may avoid potential underestimation, since it is not only limited to observation of urban areas, big cities and high density populated contexts.

Assessing and estimating abundance, coverage and average size of GSB, and referring such parameters to population density and crown coverage support a deep understanding on *(i)* how natural elements interact with people, and *(ii)* in which way built-up areas expand over peripheral areas following a human-nature gradient (i.e., urbanization pattern). At national scale, we were able to provide information on GSB, which represent a suitable basis for fostering the resilience concept in complex urbanized landscapes (Nesshöver *et al.* 2017), even through *i)* further large-scale research on their role to ameliorate the quality of life of inhabitants (i.e., ecosystem services provisioning), and *ii)* supporting the implementation of NBS in built-up areas, addressing the need of additional research to support implementation, functioning and cost-effectiveness aspects of NBS in cities. With particular regard to urban planning, the availability of adequate information on GSB is crucial in order to orient future decisions. In fact, on one hand, "built-up densification" can be a good solution for avoiding further urban sprawl in peripheries, but, on the other hand, it may shrink the availability of greenspaces (Nilsson *et al.* 2014).

Generally, NBS in urban environments should include climate change adaptation, community engagement, and social cohesion (Nilsson *et al.* 2014). In this context, Beninde *et al.* (2015) suggested that cities may become more sustainable if large forested areas are left, and corridors between them are created. Among other benefits, planting trees in urban environments may also foster the conservation of species that are considered rare and threatened (Willis and Petrokofsky, 2017). In a Mediterranean setting, Capotorti *et al.*

(2015) described an ecological-based approach to improve green infrastructures' network in Rome, and Tomao *et al.* (2017) proposed forest area per capita as a useful indicator to implement NBS in Athens and its periphery. With the primary purpose to maximize the provision of ecosystem services in urban areas, Pesola *et al.* (2017) highlighted the need to predict (in a standardized way at national scale in Italy) above-ground biomass-biodiversity relationships in urban green spaces. A further understanding of species composition and other stand characteristics (e.g., tree height, stand development) in urban green spaces may better orient management options towards ecologically-sound solutions, thus also improving public consciousness about environmental benefits by GSB (cf., the case of., in Milan, Italy; Marziliano *et al.*. 2013). Furthermore, Ugolini *et al.* (2015) highlighted that the multi-functionality of urban forestry and green infrastructure network should be fostered by bridging knowledge and communication gaps between individuals and institutional actors. Accordingly, we conclude that a coherent NBS framework for built-up areas in Italy should be based on: *(i)* balancing the spatial allocation of spaces for human settlements with green ones especially in densely populated areas through promoting re-use and requalification of existing settlements rather than urban densification options; *(ii)* guaranteeing the spatial integrity of greenspaces against urban sprawl in intermediate density and thinly populated areas through limiting uncontrolled urban sprawl as well as control their crown coverage in order to maximize the related environmental benefits; and (*iii*) improving trade-offs between services provided by greenspaces in densely populated areas (e.g., physical activity) and those available in thinly populated ones (e.g., biodiversity conservation). For example, enhancing the visits in a large green area in an urban environment may improve human well-being and resilience (cf., the case of Milan; Panno *et al.* 2017). Such strategies are also consistent with policy trajectories towards implementing NBS at EU scale (European Commission, 2015) and moving from grey to green infrastructures (EU Green Infrastructure (GI) – Enhancing Europe's Natural Capital; COM(2013) 249 final).

III Chapter- CONCLUSION & FUTURE PROSPECTIVE

For understanding the interaction of pollutants with woody plants, it is critical to thoroughly characterize their distribution and fate within plant tissues. The first research highlights a synergistic combination of ecological and physiological aspects for improving the knowledge of the mechanisms involved in metal uptake by woody plants. These results can be applied to biomonitor atmospheric pollution and to assess plant bioaccumulation potentials, especially when trees are near industrial plants or in urban areas. The research has importance for designing pollution monitoring plans with mitigation purposes, to ensure health and welfare of citizens and to promote a better coexistence of human activities with nature, especially in the urban environment.

Trees store pollutants into their tissues, from leaf and/or root adsorption, which may induce datable signals in tree rings. The second study reveals how dendrochemical analysis of oak trees grown in the city of Terni are useful bioindicators of environmental changes due to various pollution sources. The long-term tree-rings series represent useful archives to biomonitor past environmental contamination. The LA-ICP MS is a suitable technique that requires a quick sample preparation for high resolution analysis to detect trace elements in tree rings with annual resolution. The reconstruction of the environmental pollution through dendrochemistry can be a support for the policies needed to control airborne pollution, as well as input information for future air quality modelling.

Trees give quantitative or qualitative indications of pollution and are providers of ecosystem services. The third study provide informations on spatial distribution of green areas in urban environments. This information are required to formulate effective air quality policy, especially in the urban context, and support a deep understanding on how and to what extend natural elements interact with people. Moreover, the methodology applied for this last research also provides a reliable large-scale estimation of greenspaces, according to different population densities, contributing to further understand the role of greenspaces towards improving the quality of life, as well as to support the implementation of Nature-Based Solutions within urbanized landscapes.

New tools to address future environmental challenges in urban areas

Recently the development of technologies in environmental monitoring has exponentially increased the capacity to determine human-induced changes in ecological processes. A wide array of previously undetected contaminants can be now associated with pollution sources resulting from urbanization or industrial activities. The detection of these new pollutants require changes in the conventional approach to pollution detection, prevention and control. The implementation of monitoring tools and the intensification of environmental changes will help defining new environmental policy and regulations. In this framework, future challenges in urban environments are focused on the question: "*how do trees and urban infrastructure transformations in the 21st century advance the environmental sustainability and human well-being of our cities by taking advantage of the enormous potential offered by data science and technology*?" (Hartig and Kahn, 2016).

New technologies are already available for environmental researches and their application are being tested i.e. the computer-tomography imaging at the synchrotron radiation developed to evaluate relevant biological and chemical signals to detect environmental change in plant; the potential of chemical analysis on different biological matrixs (tree-rings and/or lichens) to provide indication and explanation of altered environmental conditions in temporal and spatial combination; the developed of a powerful analytical tools to estimate spatial indicators of green infrastructure in urban ecosystem.

In this regard, the H2020 – th EU R&I Framework Programme – and more particularly its Societal Challenges 5 on "Climate Action, Environment, Resources Efficiency and Raw Materials" recently suggested that the properties of natural ecosystems, and the ecosystem services provided by green infrastructures, should become the focus of specific research and innovation policies in order to find new viable solution to challenges faced by society. In this sense, the Nature-Based solutions may exert a pivotal role, since they promote important ecosystem services, including air quality amelioration through pollutant removal. This thesis explored the possibility to integrate ecological knowledge with spatial planning and territorial management to maximize ecosystem services provision making future cities more green and sustainable. This information can be useful to stakeholders for a better development and administration of the urban and peri-urban green infrastructure initiatives.

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I would like to finish this thesis in Italian, to express my feelings and emotions at best.

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1- Cocozza C, Perone A, Giordano C, Salvatici C, Pignattelli S, Raio A, Lasserre B, Tognetti R, Marchetti M, Shaub M, Sever K, Cherubini P (2017) **Following silver nanoparticles in woody species through foliar and root uptake**. In prep.
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Poster and oral communication

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