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MEDICINAL AND AROMATIC PLANTS AS INNOVATIVE AND SUSTAINABLE APPROACH FOR PHYTOREMEDIATION OF POLLUTED SOILS

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SUMMARY

ABSTRACT

Soil contamination by heavy metals and nitrates from industrial and agricultural sources poses critical environmental and health risks globally. This study examines the potential of medicinal and aromatic plants (MAPs)—specifically *Urtica dioica L.*, *Melissa officinalis L.*, and *Hypericum perforatum L.*—for phytoremediation of nitrate and nickel-contaminated soils. Phytoremediation is a sustainable and cost-effective method, based on the uptake abilities of non-edible plants to reduce contaminants in soil without introducing these pollutants into the food chain. *Urtica dioica L.*, a nitrophilous species, was evaluated for nitrate uptake in Nitrate Vulnerable Zones of Emilia Romagna, Italy, showing high biomass yields under nitrate-rich conditions and promising bioactive compounds content. Additionally, *Melissa officinalis L.* and *Hypericum perforatum L.*, tested for nickel uptake in controlled environments, exhibited tolerance and accumulation capacities, with nickel retained in roots. Both species showed high essential oil and biomass production as well as bioactive compounds production in Nickel-contaminate conditions.

Results indicate that MAPs are effective in absorbing and stabilizing soil contaminants while producing valuable secondary metabolites. This integrated approach highlights MAPs as promising agents for mitigating soil pollution, producing commercially valuable compounds, and supporting sustainable environmental management practices.

1 INTRODUCTION

The contamination of soils and groundwater has attracted worldwide attention, since many contaminants are poorly biodegradable and can accumulate in living organisms, causing implications for plants, animals, and human health. Healthy soils are crucial for food, biomass, fiber, and medical production, retaining and filtering water and they play a key role in carbon and nutrient cycles as well (EEA, 2022).

Population, technological development, and urbanization increase the demand for natural resources. These aspects are responsible for the high generation of waste, which is directly related to population growth and has a propensity to affect the economic, environmental, and safety aspects (Abdolali et al. 2017). From an environmental perspective, the contamination of soils and water is one of the main problems caused by the incorrect disposal of solid and liquid residues from agriculture, industry, and domestic activities (Marques et al. 2011). Soil pollution affects soil fertility and food security, both essential for human survival.

The term "contaminated site" refers to any area where human activities have led to a degradation of the quality of soil, surface water, or groundwater, with contaminants concentration exceeding regulatory limits. Contaminated sites are recognized as significant environmental hazards, with the potential to damage ecosystems by altering biogeochemical cycles or reducing biodiversity. These sites are major spots of pollution, and they might manifest through acute or chronic ecotoxicological effects. Soil contaminants, which may include organic and/or inorganic compounds such as heavy metals, metalloids, hydrocarbons, pesticides, and halogenated compounds, often exhibit high toxicity, environmental persistence, and mobility, potentially leading to the contamination of groundwater and the food chain. In particular, Organic contaminants are frequently characterized by high lipophilicity, facilitating bioaccumulation in food chains (EEA, 2022).

Numerous studies and epidemiological investigations have shown that the human exposure to soil pollution is estimated to contribute to more than 500,000 premature deaths globally each year (Landrigan et al. 2018).

Most of health problems occur in vulnerable groups, such as children and the elderly, affected by long-term exposure. Moreover, the health risks are related to the exposure to only a limited range of pollutants (EEA, 2022). Depending on the chemicals involved, soil pollutants can affect various organs, such as lungs, skin, gut, liver and kidneys. These pollutants may also affect the immune, reproductive, nervous and cardiovascular systems (Morrens et al. 2013; Levasseur et al., 2021).

Currently, it is estimated that 2.8 million potentially contaminated sites exist in the EU. A large proportion of these are legacy sites (so-called brownfield sites), often with unknown ownership (Payá and Rodríguez, 2018). This estimate is considered conservative, and the number of potentially contaminated sites across the EU is likely to be underestimated — depending on which polluting activities are considered (EEA, 2022). The proportion of unregistered sites and sites that have not been risk assessed is large (more than 50%). Figure 1 shows that recent trends in the management of contaminated sites are positive; however, the levels of national action strongly differ across the EU. The high number of undetected/suspected contaminated sites poses a serious risk to citizens and the environment.

The public health, environmental, moral and socio-economic case for the EU to lead the global fight against pollution is today stronger than ever. On 12 May 2021, the European Commission adopted the EU Action Plan: "Towards a Zero Pollution for Air, Water and Soil" (and annexes) - a key deliverable of the European Green Deal.

The European Green Deal announced the headline actions on zero pollution.

- Chemical strategy for sustainability to better protect citizens and the environment against hazardous chemicals.
- Zero Pollution Action Plan for water, air and soil - to better prevent, remedy, monitor and report on pollution.
- Revising measures to address pollution from large industrial installations, to ensure their consistency with climate, energy and circular economy policies.

The EU soil strategy for 2030 reiterates the zero-pollution target that, by 2050, soil pollution should be so low that it no longer harms human health (EC, 2021). The strategy priorities preventing pollution at its source, which aligns with the zero-pollution hierarchy. Furthermore air, water and soil pollution may be reduced to levels no longer considered harmful for health and natural ecosystems, thereby creating a toxic-free environment significantly reducing risks as a result. This is expected to include provisions on identifying, keeping an inventory of and remediating contaminated sites.

The key 2030 targets to reduce source of pollution include:

- improving air quality to reduce the number of premature deaths caused by air pollution by 55%;
- improving water quality by reducing waste, plastic litter at sea (by 50%) and microplastics released into the environment (by 30%);

- improving soil quality by reducing nutrient losses and chemical pesticides' use by 50%;
- reducing by 25% the EU ecosystems where air pollution threatens biodiversity;
- reducing the share of people chronically disturbed by transport noise by 30%, and significantly reducing waste generation and by 50% residual municipal waste.

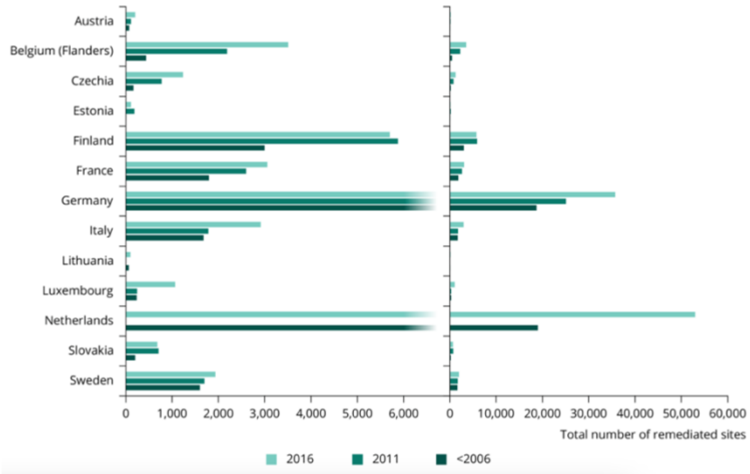

Figure 1. Progress in the management of contaminated sites in the EU, total number of recorded remediated sites (source EEA, 2022).

1.1 Definition of environmental pollution and contaminated sites.

The terms 'environmental pollution' is used when toxic level of chemicals is found in land, water and air. Soil contamination creates a significant risk to human health. For instance, heavy metals from industrial waste contaminate drinking water, soil, fodder, and food (EEA, 2022).

Soil pollution refers to the introduction of substances or energy into the environment that may pose a risk to human health, harm living organisms and ecosystems, damage structures and landscapes, and diminish the usability of natural resources (e.g., restrictions on fishing or swimming). A pollutant is defined as any substance that, even when it occurs at low concentrations, leads to toxic effects on living organisms and ecosystems. (EEA, 2022)

In order to minimize the differences in interpretation by individual countries of terms used EEA (2022) provided following definition:

- "Contaminated site" (CS) refers to a well-defined area where the presence of soil contamination has been confirmed and this presents a potential risk to humans, water, ecosystems, or other receptors. Risk management measures (e.g., remediation) may be needed depending on the severity of the risk of adverse impacts to receptors under the current or planned use of the site.
- "Potentially contaminated site" (PCS) refers to sites where unacceptable soil contamination is suspected but not verified, and detailed investigations need to be carried out to verify whether there is unacceptable risk of adverse impacts on receptors.
- "Management of contaminated sites" aims to access and, where possible, reduce to an acceptable level the risk of adverse impacts on receptors (remediate). The progress in management of CS is traced in 4 management steps starting with preliminary study, continuing with preliminary investigation, followed by site investigation, and concluding with implementation of site remediation (reduction of risk).

The large volume of waste and the intense use of chemicals during past decades have resulted in numerous contaminated sites across Europe. Contaminated sites could pose significant environmental hazards for terrestrial and aquatic ecosystems as they are important sources of pollution which may result in ecotoxicological effects (Paganos et al. 2013). Earlier industrialization and poor environmental management practices in the European Region have left a legacy of millions of contaminated sites. The hazards associated with contaminated sites are heterogeneous, and often diffuse and difficult to recognize.

Under the European Union (EU) Thematic Strategy for Soil Protection, the European Commission has identified soil contamination as a priority for the collection of policy-relevant soil data at European scale. According to data, the number of estimated potential contaminated sites is more than 2.8 million and the identified contaminated sites around 342 thousand (EEA, 2022). Municipal and industrial wastes contribute most to soil contamination (38%), followed by the industrial/commercial sector (34%). Mineral oil and heavy metals are the main contaminants contributing around 60% to soil contamination. In terms of budget, the management of contaminated sites is estimated to cost around 6 billion Euros (€) annually. According to the Environmental European Agency, contamination by metals in the European Union (EU) accounts for more than 37% of cases, followed by mineral oil (33.7%), polycyclic aromatic hydrocarbons (PAH, 13.3%) and others (Vamerali et al., 2009).

Soil contamination can be considered one of the most significant environmental problems in many areas of the world. In developed countries and particularly in Europe (EEA, 2022), heavy metals and Nitrogen inorganic compounds are counted among major soil contaminants and major food contaminants (Akinyele and Shokunbi, 2015), representing a significant risk for human health.

The environment is often contaminated with a mixture of compounds, such has heavy metals and polycyclic aromatic hydrocarbons, causing soil pollution (Zulfiqar et al. 2019).

Management of contaminated sites aims at assessing the adverse effects caused and taking measures to satisfy environmental standards according to current legal requirements. In particular, according to WHO, priority should be given to the pollutants based on toxicity, environmental persistence, mobility, and bioaccumulation. Due to this global advance in environmental protection and preservation, numerous concerns have been underlined recently. (Rostami and Azhdarpoor, 2019).

1.2 Nitrate pollution in soil and water

Nitrogen (N) is an essential element for plant growth and productivity. An increased crop yield is achieved by intensive use of synthetic N fertilizers. Nitrate (NO_3^-) is the oxidized form of dissolved N and the most usable form of N fertilizers. Moreover, other inorganic N fertilizers are rapidly converted to nitrate through microbial nitrification and together with non-agricultural sources of nitrate, such as septic tanks and dairy lagoons, contaminate groundwater (Norton and Ouyang 2019), and shallow aquifers (Burkart and Stoner 2002).

Nitrate belongs to the most frequently occurring contaminants; according to the European Community the permitted maximum concentration of nitrate is 50 mg in 1 L of water. The high intake of nitrate in an organism can be a source of different carcinogenic and mutagenic dysfunctions (Swann, 1975). Therefore, trends of nitrate pollution of freshwaters reflect legacies of current and past applications of fertilizers and manures, since nitrate pollution has been a direct consequence of applying large quantities of N fertilizer used in agriculture. (Norton and Ouyang 2019)

The nitrate leaching potential from soils is conditioned by several agricultural and environmental factors such as management practices (nitrogen fertilizer application doses and irrigation types), soil properties as texture and drainage, climatic conditions (Li et al. 2020).

In fertilized soils, ammonium is oxidized into nitrites and nitrates, which are easily dispersed from soils into groundwater and surface water bodies. Consequently, the excessive use of fertilizers determines an increase in these nitrogenous compounds, causing environmental and health problems. Moreover, studies have shown that N leaching and nitrous oxide emissions increase when N inputs largely exceed N crop removal (Zhang et al. 2015).

Denitrification is an important process in that it deletes fixed nitrogen—such as nitrate—from the ecosystem and returns it to the atmosphere in its biologically inert form (N2). In fact, nitrogen losses for denitrification in agriculture can represent up to 30% of the fertilizer applied and the nitrogen balance (N) provides an indication of the risk of N losses in the environment. (Cabello et al. 2009).

However, excess nitrates in groundwater might be related to other inputs such as waste industrial, untreated waste discharge, and sewage spilling. Substantial amounts of nitrates are also produced from the organic waste generated by farm animals and the sewage produced by cities, and these can also reach groundwater bodies. In areas such as intensive feedlots, livestock waste constitutes a potent source of excess nutrients flowing into the environment.

Trends of nitrate pollution in freshwaters, therefore, reflect legacies of current and past applications of fertilizers and manures. It was a direct consequence of applying large quantities of fertilizer N. Globally, 60% of areas with elevated nitrate-N in ground water occur in croplands (Bijay-Singh and Craswell, 2021).

As only 20% of the total cultivated land is under irrigated agriculture and accounts for about 40% of the global food production, fertilizer N use and loss of nitrate-N to natural water bodies from the irrigated cropland is much higher than from rain-fed agriculture (FAO, 2022).

Substantial amounts of nitrates are also produced from the organic waste generated by farm animals and the sewage produced by cities, and these can also reach groundwater bodies. In areas such as intensive feedlots, livestock waste constitutes a potent source of excess nutrients flowing into the environment. The uptake of fertilizer N in crop production, however, is only about 50% and the surplus may accumulate in soils or be lost to groundwater and surface water through various pathways (Mohammadzadeh and Hajiboland 2022).

In addition to all this, it was observed that elevated nitrate concentrations in drinking water are linked to health problems, such as methemoglobinemia in infants and stomach cancer in adults (Shuval and Gruener 2013). Together with phosphorus, nitrate causes eutrophication of surface waters, while their denitrification in the soil and emission in a form of N_2O or other nitrous oxides also cause harmful effects (Wang et al. 2009).

Nitrate removal from water or wastewater may be achieved through physicochemical or biological treatment methods. The most common physicochemical processes are reverse osmosis, ion exchange, electro-dialysis, activated carbon adsorption, and metallic iron-aided nitrate reduction. Green remediation approaches, such as bioremediation and phytoremediation are generally seen as being more sustainable in terms of conserving natural resources (Paz-Alberto and Sigua 2013).

During phytoremediation, plant-based mechanisms are exploited to destroy, eliminate, transfer, or stabilize the environmental pollutants for cleaning up organic and inorganic pollutants. Numerous studies have been undertaken on the phytoremediation of heavy metal-contaminated soils (Shah and Daverey, 2021) and organic contaminations (Newman and Reynolds 2004).

However, till now, only a few works studied the potential of plants for phytoremediation of nitrate-contaminated soils. As the success of phytoremediation is strictly related to the ability of the plant to take up contaminants, cultivation of Nitrophilous plants may be an efficient solution for nitrate phytoremediation. In addition, the application of high biomass production plants is crucial for the achievement of phytoremediation process.

Urtica dioca L. is a is a neglected species, with potential as a future multi-purpose crop. It may grow in a wide range of habitats and it predilates nitrogen-rich soils. In particular, the positive effect of Nitrate rich cultivation medium on the increase in biomass production have been widely demonstrated (Radman et al. 2015).

1.2.1 Legislation on Nitrate Vulnerable Zones

According to the Food and Agriculture Organization of the United Nations (FAO) report (2017) nitrate of agricultural origin is the most common chemical pollutant in the world's groundwater aquifers; its contamination influences the quality of drinking water resources and crops, with adverse effects on ecological and human health (Carrey et al. 2021).

As mentioned before, the nitrate leaching potential from soils is conditioned by several agricultural and environmental factor.

Excessive use of fertilizers leads to the oxidation of ammonium into nitrites and nitrates, which can contaminate groundwater and surface water, posing environmental and health risks. Nitrogen leaching and nitrous oxide emissions increase when nitrogen inputs exceed crop uptake. Denitrification plays a vital role in removing nitrates, returning nitrogen to the atmosphere, but up to 30% of applied nitrogen can be lost this way. Additionally, nitrate contamination in groundwater may also result from industrial waste, untreated sewage, and organic waste from farm animals and cities. In areas such as intensive feedlots, livestock waste constitutes a potent source of excess nutrients flowing into the environment.

The EU Water Framework Directive (WFD 2006/118/EC) has set thresholds for achieving good chemical status in groundwater and good environmental status in surface water (European Parliament, 2006). In particular, the Council Directive had referred to as "Nitrates Directive" (ND), the European Union intervened to ensure that the Member States protect the quality of the water, since 1991 (European Parliament, 1991). As defined by the Directives, it is necessary to control the concentration of nitrates in freshwater (surface and groundwater) and the eutrophic state caused by nitrogen in surface freshwater. The criticality limit identified by World Health Organization (WHO, 2003) is 50 mg/l of nitrate, considering human health and environmental risks.

In addition, water bodies monitoring regarding nitrate concentrations, designation of Nitrate Vulnerable Zones (NVZs), and establishing codes of good agricultural practices and measures to prevent and reduce water pollution from nitrates were fixed by the directives (Massarelli et al. 2021).

In more details:

I. the identification, every four years of the territorial areas particularly susceptible to being polluted, so-called "Nitrates Vulnerable Zones" (NVZs);
II. the preparation, within one year from the designation of the NVZs, of mandatory measures (Action Programs) that must be adopted by farmers; especially by those

carrying out activities concerning livestock production and practices related to nitrogen fertilization.

III. the application, by farmers, of a set of critical interventions for the correct management of agricultural activities to protect the soil and water resource, which must be referred to the Code of Good Agricultural Practice defined by the Ministry of Agriculture and Forestry Policies.

IV. training and information initiatives aimed at agricultural operators concerning agronomic practices to protect the environment (Massarelli et al. 2021).

1.3 PTE and Nickel Contamination

Potentially Toxic Elements, or PTEs, are a group of inorganic chemical contaminants, from both natural and anthropogenic sources can end up in the soil. Improper disposal of industrial and domestic waste, spills during industrial operation, overuse of agrochemicals, and mining and smelting operations are the most common activities leading to accelerated soil pollution (Palansooriya et al. 2020).

Soil contamination with potentially toxic elements (PTEs) (i.e., antimony (Sb), arsenic (As), barium (Ba), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), silver (Ag), tin (Sn), titanium (Ti), vanadium (V), and zinc (Zn)) has been widely reported and has led to growing concerns regarding severe negative effects on living organisms, including humans (Wang et al. 2018). As a result, these elements may create acute and chronic toxic effects throughout the trophic chain, depending on their concentration in the environment. Any metal (or metalloid) species may be considered a “contaminant” if it occurs where it is unwanted, or in a form or concentration that causes a detrimental human or environmental effect. Globally 20 million ha of land is contaminated by heavy metal or metal(loid)s As, Cd, Cr, Hg, Pb, Co, Cu, Ni, Zn, and Se, with present soil concentrations higher than the geo-baseline or regulatory levels (Liu et al. 2018). For instance, the most frequent elements causing environmental pollution are considered Cd, Cu, Cr, Ni, Zn, Pb and As. These elements are often introduced into the environment as mixtures, for this reason it is possible to find areas with higher concentrations of more than one metal or contaminant (US. EPA, 2009).

Numerous industrial, home, agricultural, medical, and technological applications of these metals have led to their widespread distribution in the environment and have raised concerns about their potential effects on human health and the environment. Heavy metals are accumulated in the environment from the processes of pedogenic weathering as well as through human activities. The most important natural resources include mineral erosion, weathering, and volcanic activity (Kordrostami et al. 2019). Soil may be contaminated by heavy metals and metalloids from industrial areas, mines, waste disposal, diesel and lead paint, agricultural fertilization applications, animal manure, sewage sludge, pesticides, sewage irrigation, coal combustion residues, petrochemicals, and atmospheric sediments (Yadav et al. 2017).

For plants or living organisms' growth and development, metals play fundamental roles thanks to their chemical and biochemical properties. However, when present in excess in the environment, they induce adverse effects on biological systems, producing oxidative and genotoxic stresses or causing secondary deficiency of essential elements because of competition (Waldron et al., 2009; Andresen et al., 2018; Corso et al., 2021)

In particular, Nickel is the 22nd most abundant element on earth crust (twice as Cu) and an important trace metal Chemical and physical processes and biological transport mechanisms found in living species introduce Ni into the environment and distribute it across the ecosystem (WHO, 1991).

As Nickel is abundant in Earth crust, humans are constantly exposed to it, and depending on the dose and length of exposure, as an immunotoxic and carcinogenic agent, it may be responsible of a huge number of health effects, such as contact dermatitis, cardiovascular disease, asthma, lung fibrosis, and respiratory tract cancer. However, the exposure of human beings mainly concerns oral ingestion through water and food as nickel may be a contaminant in drinking water and/or food (Sinicropi et al. 2012) (Chen et al., 2017).

Although the molecular mechanisms of nickel-induced neurotoxicity are not yet clear, an important role is due to oxidative stress and mitochondrial dysfunctions. Nickel-induced mitochondrial damage can occur, due to impairment of mitochondrial membrane potential, reduction of mitochondrial ATP concentration and destruction of mitochondrial DNA. The use of antioxidant molecules, such as L-carnitine, taurine and melatonin, molecules that stimulate and amplify antioxidant enzyme activity, can prevent nickel-induced neurotoxicity and carcinogenicity (Carocci et al. 2014).

Accumulation of nickel and nickel compounds in the body through chronic exposure may be responsible for a variety of adverse effects on the health of human beings, such as lung fibrosis, kidney and cardiovascular diseases and cancer of the respiratory tract (Seilkop et al. 2003).

A small fraction of nickel is dermally absorbed, and Ni2+ ions and nickel particles penetrate the skin at sweat ducts and hair follicles. Moreover, dermal absorption of this metal is affected by solubilizing agents, such as detergents, and clothes and gloves that behave as a barrier to the skin. Nickel nanoparticles are associated with reproductive toxicity (Zambelli et al., 2016).

As mentioned before, because of its low environmental mobility, Nickel tends to accumulate in ecosystems and living organisms and, unlike organic contaminants, cannot be degraded by microorganisms in soils, it can be only mobilized or stabilized. The harmfulness of this metal, as well as ease of assimilation by living organisms, depends on its concentration, oxidation state, electrical charge, and more generally on their chemical and physical characteristics.

Concerning plants growth and development Nickel is an essential element and plays important roles in a wide range of morphological and physiological functions, such as germination seeds and productivity. However, at high levels nickel alters the metabolic activities of the plants by the inhibition of enzymatic activity, photosynthetic electron transport and chlorophyll biosynthesis (Merkens et al., 2008).

Nickel adverse effects on organic molecules, such as proteins, enzymes, and lipids, as the inactivation of proteins and enzymes, oxidation of lipids, and disruption of sugar metabolism was demonstrated by Kordrostami et al. (2019).

High concentrations of heavy metals cause stress and damage plant cells by producing oxygen-free radicals, leading to lipid peroxidation (Corso et al., 2021). To reduce and scavenge the formation of reactive oxygen species (ROS) and avoid oxidative damage in plants, the activity of antioxidant enzymes such as catalase, deoxothase superoxide, and peroxidase is increased (Mafakheri and Kordrostami, 2021).

The stress of heavy metals by induction of defense responses also stimulates the biosynthesis pathway of the elicitors and accumulation of secondary metabolites (Lajayer et al., 2017).

However, metalliferous soils of geogenic or anthropogenic origin are colonized by specific floras adapted to high metal concentrations, so called metallophyte species.

While the majority of metallophyte species exclude metals from their tissues, more than 700 species have acquired the capacity to accumulate and tolerate huge amount of metals in shoots or roots (Van der Ent et al., 2013; Reeves et al., 2018).

Plants hyperaccumulating nickel (> 1000 mg kg-1 shoot DW) represent, counting 520 species, most of the metal hyperaccumulators (Reeves et al., 2018), probably because of the frequent occurrence in the Mediterranean basin of outcrops originating from ultramafic rocks, including serpentine soils (Garcia de la Torre et al., 2021). The large phylogenetic distribution of nickel hyperaccumulators in various plant families further suggests that nickel hyperaccumulation appeared independently several times during plant evolution (Kramer, 2010)

For instance, several organic molecules, including histidine, nicotianamine and organic acids, have been shown to form complexes with nickel and have been proposed to play a role in hyperaccumulation (Garcia de la Torre et al., 2021).

Thus, physiological mechanisms that protect plants from unfavorable environmental conditions make medicinal plants suitable for phytoremediation processes, and a viable option for removing pollutants from contaminated soil and water.

Physiological mechanisms may be useful for extracting, immobilizing, or removing salts, metals, organic compounds and radionuclides from soil and water. Certain properties may allow the safe use of medicinal plants for human consumption while removing contaminants from soil or water (Shmaefsky and Husen 2023).

However, the application in phytoremediation has not been widely investigated. In contrast to biomass crops, effect of contaminants on yield and biomass production is important, but quality is also assessed concerning the content of Specialized Metabolites in medicinal species (Murch et al. 2003).

Therefore, the environmental effects of nickel contamination on specialized metabolites biosynthesis and physiological mechanisms involved in phytoremediation processes should be investigated. The two medicinal plants *Hypericum perforatum* L. and *Melissa officinalis* L. are considered Nickel tolerant and hyperaccumulating plants, valuable for soil restoration processes. *Hypericum perforatum* L. (St. John's wort) is a medicinal species used in the treatment of neurological disorders including mild to moderate depression and recently found to be useful for the inhibition of cancer tumor cell growth (Schempp et al., 2002).

Hypericin, pseudohypericin and hyperforin are the three principal specialized metabolites mainly involved in pharmaceutical application (Murch 2003). *Melissa officinalis* L. (lemon balm) is an important medicinal plant in the family of Lamiaceae, containing three major groups of phytochemical compounds, namely protocatechuic acid, caffeic acid and rosmarinic acid (Maivan et al. 2017). Lemon balm exhibited a wide scope of pharmaceutical properties such as sedative, carminative, antispasmodic, antibacterial, antiviral, antifungal, anti-inflammatory and antioxidative. Furthermore, *M. officinalis* is used to treat Graves', Alzheimer's and thyroid diseases (Kim et al. 2010).

The content of these metabolites has been shown to be mainly influenced by environmental conditions (Saeidnejad et al. 2012; Poutaraud et al., 2001; Briskin and Gawienowski, 2001), biotic and abiotic stress, but also from genotype and storage conditions (Buter et al. 1997).

Therefore, little information about the physiological and biochemical behavior of *M. officinalis* L. and *H. perforatum* L. under Nickel abiotic stress conditions are present (Maivan et al. 2017).

Moreover, both plants were notices as Ni tolerant species. For these reasons they have been selected as important aromatic and medicinal plants for this study.

1.3.1 Legislation on Remediation of Contaminated sites

Actually, the issue of contaminated sites is highly relevant at the present time and is extremely important in the context of environmental risk and public health. Soil contaminants, in fact, can be characterized by high level of toxicity, environmental persistence, and mobility that may led to contamination of groundwaters and trophic chain, becoming a danger for human health.

Contaminated sites, therefore, require remediation efforts according to site-specific characteristics, economic factors, and remediation objectives.

Currently, remediation interventions are only marginally focused on soil quality. The first objective of the remediation interventions is to reduce the residual pollutant content below the levels required by regulations, with no focus on the possible consequences that the technologies employed may have on soil quality.

Concerning Italian National legislation on the remediation of contaminated sites, it was introduced by Ministerial Decree 471/99, and then deeply modified by Legislative Decree 152/06 and ss.mm.ii.

"Regulations on environmental issues," dealing more specifically with "Remediation of contaminated sites" in Part Four Title V. In details, it regulates the remediation and environmental restoration of contaminated sites and defines the procedures, criteria, and methods to remove the sources of pollution and lower pollutants concentrations.

For the legislation, “contaminated site” refers to areas where past or present human activities led to an alteration in the qualitative characteristics of the environmental matrices soil, subsoil and groundwater, posing a risk to human health.

Specifically, the legislation indicates as "**site**" the area or portion of land geographically defined including the different environmental matrices (soil, landfill materials, subsoil, and groundwater) and any building or structures eventually present.

Moreover, national legislation indicates and sets contamination threshold according to two indexes: **CSR** and **CSC**. Briefly, the CSC indicates a threshold value above which a site characterization should be carried out, while CSR identifies an acceptable level of residual contamination needing remediation and/or safety interventions.

In Legislative Decree No. 152 of 2006, remediation (art 240, paragraph 1, letter p) refers to "the series of interventions designed to eliminate the sources of pollution and pollutants or to reduce the concentrations of the same in the soil, subsoil and groundwater to a level equal to or lower than the values of risk threshold concentrations (CSR).

Actions on contaminated soil can be carried out in relation to different objectives, and in this sense a distinction can be made in remediation interventions and permanent safety interventions.

In particular, while the remediation of a polluted site is aimed at eliminating pollution or bringing the concentrations of pollutants back within the contamination threshold values (CSCs), permanent safety interventions are aimed at removing and isolating pollutant sources and containing the spread of pollutants to prevent their contact with humans and surrounding environmental receptors.

1.4 Phytoremediation Technologies

In 2002, the European Union predicted that partially cleaning up its hazardous sites would cost more than 100 billion dollars (Mishra and Chandra, 2022). Soil decontamination is time-consuming and expensive, and the procedures now available are insufficient for the task.

Phytoremediation is gaining traction as a strong, alternative, environmentally friendly, and practical approach for remediation, decontamination, and industrial waste/environmental waste stabilization. It is a cost-effective treatment approach in which pollutant/heavy metal toxicant is removed from polluted soil by various plants. Phytoremediation is a large category of plant-based technology that purifies the environment by using natural or genetically altered plants (Mishra and Chandra, 2022).

The high cost of conventional remediation processes stimulates research for the development of innovative and more sustainable techniques. Likewise, phytoremediation is a cheap technology that uses plants to absorb, transform, and detoxify contaminants through *in situ* (phytoextraction, phytotransformation, and phytovolatilization) and *ex situ* mechanisms (phytostabilization and phytostimulation). Recently, phytoremediation has been adopted as a more profitable technique than physicochemical processes. Otherwise, the existence of variables, such as interactions between climate, soil, and plants, requires analysis methods for its implementation, which ensure the reduction of time and cost and improve its efficiency. Research on the application of different phytoremediation techniques is still in progress, and therefore, this study evaluated the main advantages of phytoremediation through a literature overview, comparing the most adequate remediation models in terms of economic, social, and environmental aspects.

Between conventional methods for removing contaminants from soil are involved incineration, volatilization, adsorption, electrocoagulation, and selective leaching processes (Boparai et al., 2011).

However physical and chemical techniques may be costly and led to soil degradation, in particular chemical remediation may produce secondary pollution (Lan et al. 2021).

Conventional technologies are more based on transferring pollutants, creating new wastes that may develop unknown toxic intermediates (Kuppusamy et al. 2016). In addition, these techniques are not suitable for phytoremediation processes where large areas and relatively low concentration of pollutants are involved (He et al. 2015). In recent years, is gaining more relevance and it has been considered as a cost-effective green technology that utilizes the capacity of hyperaccumulator plants to extract heavy metals from the soil (Chaney et al. 2020).

Phytoremediation is a plant-based technology for the remediation of contaminated sites affected by different contaminants, working in different environmental compartments (soil, water, sediment, deep and surface water, atmosphere).

Phytotechnology can be alternative or complementary remediation techniques to conventional techniques for removing contamination from environmental matrices.

These techniques can be applied both *in* situ and *ex situ*, comprising mainly *in situ* applications, by taking advantage of the intrinsic physiological abilities of plant organisms.

Phytoremediation as a technology based on plants for the removal of pollutants from the environment, might be an effective, low-cost tool for the degradation of organic compounds or accumulation of heavy metals (Macek et al., 2008). Phytoremediation is a plant-based application that uses the controlled interactions of plants with groundwater, inorganic and organic molecules to achieve site-specific remedial goals in contaminated locations.

Hydrocarbons, gas condensates, crude oil, chlorinated compounds, and pesticides. Inorganic contaminants include salts (salinity), heavy metals, metalloids, and radioactive materials are included in the target organic contaminants for plants application. Parameters of fundamental importance for the application of this technology are the effective mobility and bioavailability of contaminants in the soil.

In the case of metals in the soil, they can be only slightly released into the water and thus mobile and potentially "bioavailable" to living organisms, or largely strongly bound to the soil, making them immovable and not accessible to organisms. The bioavailability of elements is correlated with the constitutive characteristics of the soil being caused by the affinity of certain elements for soil components. Phytotechnologies for remediation can be based on different physiological active mechanisms related to species-specific physiological aspects.

Furthermore, it improves soil quality over time. Organic and inorganic pollutants can accumulate in the root zone, and plants can promote microbial breakdown of organic contaminants (Arthur et al., 2005).

As previously stated, plants mitigate pollution by absorbing certain toxicants, chemicals, or pollutants, such as heavy metals or nitrate through their roots and collecting or translocating them in less toxic forms in various sections and cell organelles. Thus, various plant species have biochemical and biophysical processes for the detoxification of xenobiotic compounds, as such as translocation, absorption, transportation, acclimatization, hyperaccumulation, and mineralization are reported in plants involved in the remediation process of the toxicants from the contaminated soil/water (Mishra and Chandra, 2022).

Despite its advantages, phytoremediation has numerous drawbacks, including low biomass production, short plant roots and difficulties in controlling the growth of hyperaccumulators. However, organic phytoremediation appears to be a promising approach for removing toxins from polluted soil (Maestri et al., 2010). As phytoremediation mechanisms, plants may utilize phytoextraction, phytodegradation, phytostabilization, phytovolatilization, and rhizo-degradation (**Table 1, Figure 2**).

-Phytoextraction or Phytoaccumulation: Based on the ability of specific plant species to survive in contaminated soils and accumulate high concentrations of metals or salts in tissues (stem, leaves, root system), decreasing their total concentration in the polluted soil.

Phytoextraction involves the uptake of pollutants in soil or water by roots, subsequent translocation and accumulation in aerial biomass (Ehsan et al. 2016).

The translocation process simplifies the process by making the collection of contaminated tissue very easy, likened to a normal agronomic practice (Ali et al. 2013).

This approach is mainly exploited in case of heavy metal contamination, due to their high bioavailability or saline soils. Plant species with phytoextractive capabilities can be further distinguished into accumulator, tolerant and hyperaccumulator. Plants passively accumulating the contaminants present, species tolerating high concentrations of pollutants, and plants absorbing or hyperaccumulating elements in tissues, due to physiologic mechanisms, as accumulator, tolerant and hyperaccumulator species respectively (Bonomo, 2011).

Desirable characteristics for a plant species in phytoextraction are considered fast growth and high biomass; extended root system for exploring large soil volumes; good tolerance to high concentrations of metals in plant tissues; high translocation factor; adaptability to specific environments/sites; and low-input agricultural management (Vamerali et al. 2010).

-Rhizodegradation: The Rhizodegradation refers to the breakdown of organic pollutants by microorganisms in the soil and rhizosphere, extended about 1 mm around the root and penetrates within the plant's influence (Maiti and Subodh, 2010). Root exudates are sources of C and N for the microbiota and create a nutrient-rich environment that stimulates microbial enzymatic degradative activity (Ali et al. 2013). This mechanism is based on the biological activity of the Rhizosphere related to proteins and enzymes produced and exuded by the plants root system or/and soil microorganisms (bacteria, yeasts, and fungi). Organic contaminants as hydrocarbons may be metabolized, degraded, and mineralized by proteins and enzymes in the rhizosphere or in plant roots.

-Phytotransformation: Plants capacity to uptake and store contaminants in their tissues thanks to the chemical modification of molecules. The phytotransformation may be dividend in phytodegradation and phytostabilization.

-Phytodegradation concerns the uptake and transformation of soil contaminants by plant physiological processes. Phytodegradation is the result of the action of enzymes and enzymatic pathways on pollutants in plants tissue after their uptake. Organic pollutants by plants produced enzymes are depredated in plant tissues and the presence of rhizosphere microorganisms may support contaminants uptake even if their presence is not essential (Ali et al. 2013). The application of Phytodegradation mechanisms on synthetic herbicides and insecticides have been reported (Doty et al. 2007).

-Phytostabilization approach aims at contaminants stabilization in soils through reduction of mobility and environmental bioavailability of the former, preventing their percolation and entry into the trophic chain (Erakhrumen et al. 2007).

Pollutants are immobilized by various processes: radical uptake, precipitation, complexation, or metal valence reduction in the rhizosphere. In more details, plant species immobilize contaminants in soil, sediment, and groundwater through the uptake and accumulation within the roots, or outside the roots, or through immobilization and precipitation within the rhizosphere. It does not aim at removing contaminants from the medium, but it may reduce risks for human health and environment (Ali et al. 2013).

In this approach the application of mineral soil conditioners may yield positive results in PTE stabilization

For example, polyannual species manage to store at root level a fraction of contaminants, contributing to long-term stabilization of pollutants. (Vamerali et al. 2009)

Phytostabilisation improves the chemical and biological characteristics of contaminated sites by increasing the amount of organic matter, nutrient levels, cation exchange capacity and biological activity (Arienzo et al. 2004).

Table 1. Differing Areas of phytoremediation (Vamerali et al. 2010).

Technology	Description
Phytoextraction	Uptake of pollutants from environment and their concentration in harvestable plant biomass
Phytostabilisation	Reduction of mobility and bioavailability of pollutants in environment
Phytovolatilisation	Removal of pollutants from soil or water and their release into air, sometimes as a result of phytotransformation to more volatile and/or less polluting substances
Phytotransformation	Chemical modification of pollutants as a result of plant metabolism, both in planta and ex planta, often resulting in their inactivation, degradation or immobilization (phytostabilisation)
Rhizodegradation	Use of plant roots to absorb and adsorb pollutants or nutrients from water and wastewater (e.g. buffer strips)

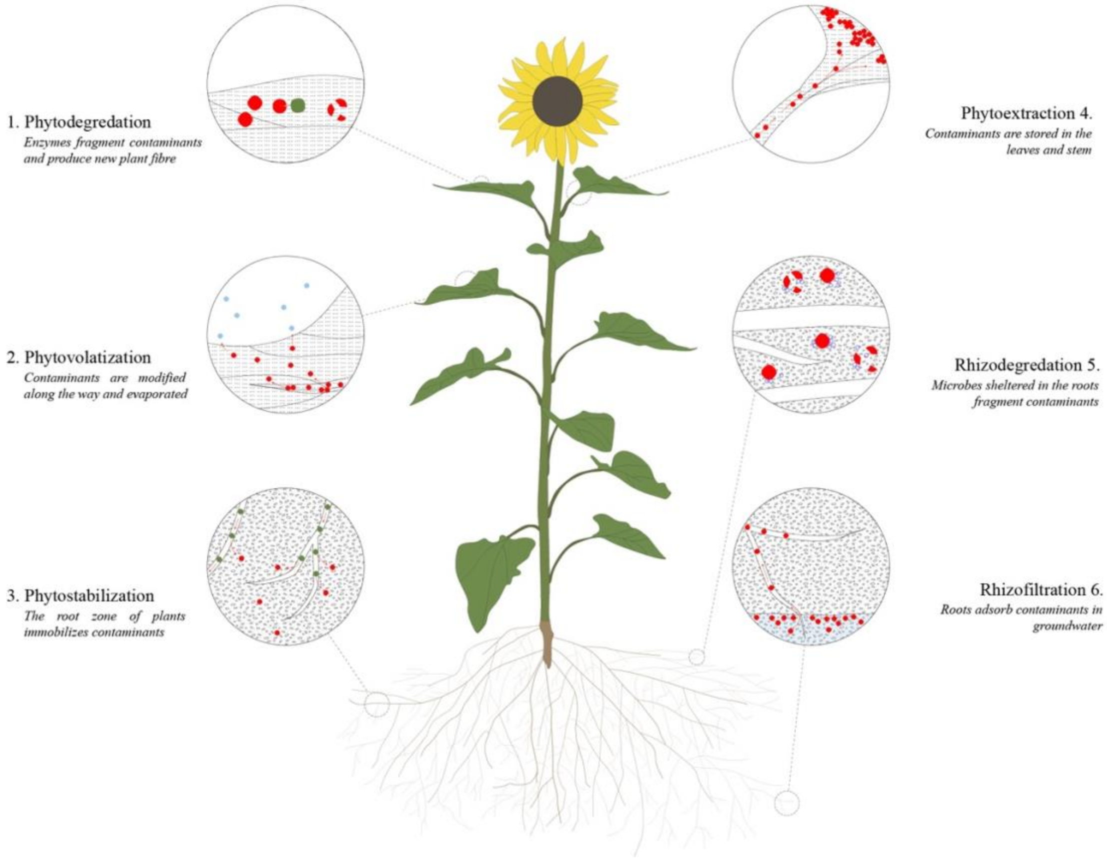

Figure 2. Mechanisms used by plants to clean up, or remediate, contaminated sites. To remove pollutants from soil, sediment and/or water, plants may break down, or degrade, organic pollutants or contain and stabilize metal contaminants by acting as filters or traps. (UTS, 2018) (https://powerplantsphytoremediation.com/bio-1).

1.5 Medicinal and aromatic plants (MAPs) in phytoremediation

As already mentioned, soil decontamination with plants is time-consuming, however the current existing physical and chemical procedures are still inadequate and expensive. Phytoremediation is gaining interest as an alternative, environmentally friendly, and practical approach for remediation, decontamination, and industrial stabilization. It is a cost-effective treatment approach in which pollutant/heavy metal toxicant is removed from polluted soil by various plants. Phytoremediation is a large category of plant-based technology that purifies the environment by using natural or genetically altered plants. The finding of heavy metal accumulation in plants is a critical milestone in the development of heavy metal soil phytoremediation (Angelova, 2017). The use of edible plants as phytoremediation crops (such as pulses, grains, and vegetables) has certain downsides, as heavy metals may enter the food chain and cause serious consequences. As a result, non-edible medicinal and aromatic plants (MAPs) are suggested to be potential sources of long-term phytoremediation. MAPs are essential oils and bioactive compounds producing plants, with possible applications in agro-food, pharmaceutical and cosmetic industries.

Furthermore, no risk of contaminant accumulation and subsequent entry into the food chain during the synthesis and extraction of essential oils and bioactive compounds from these plants (Angelova, 2017; Pandey et al., 2019). Medicinal plants used in phytoremediation offer a dual advantage: they effectively decontaminate polluted soils while producing valuable secondary metabolites. These metabolites, such as alkaloids, flavonoids, and terpenoids, can be harnessed for medicinal and industrial applications, creating an economic incentive for their cultivation in contaminated areas. Additionally, the cultivation of non-edible MAPs on polluted lands can help restore degraded ecosystems without compromising food security. By integrating phytoremediation with the production of bioactive compounds, MAPs provide a sustainable and economically viable solution to soil contamination, addressing both environmental and socio-economic challenges. Hence, the cultivation of MAPs in contaminated soils proposes a novel approach for phytoremediation. *Poaceae, Geraniaceae, Asteraceae,* and *Lamiaceae* are some of promising MAPs families identified for phytoremediation.

The danger in the final product consumption of the plant components is crucial when using MAPs for phytoremediation, and aromatic crops give a safer, cheaper, and far more eco-friendly solution. In addition, MAPs products are primarily used for non-edible purposes. The last century has witnessed an increase in the demand for essential oils procured from aromatic plants. Accordingly, growing aromatic plants on polluted soils for phytoremediation may facilitate restoring the soil and provide economic benefits (Mishra and Chandra, 2022) (**Figure 3**). MAPs are high- value economic crops that provide financial benefits by being grown in polluted regions rather than food crops. Furter studies supported that after contaminants exposure, the percentage of essential oil content and bioactive compounds in certain MAPs, not severely compromised by pollutants, progressively increased (Angelova, 2017; Pandey et al., 2019).

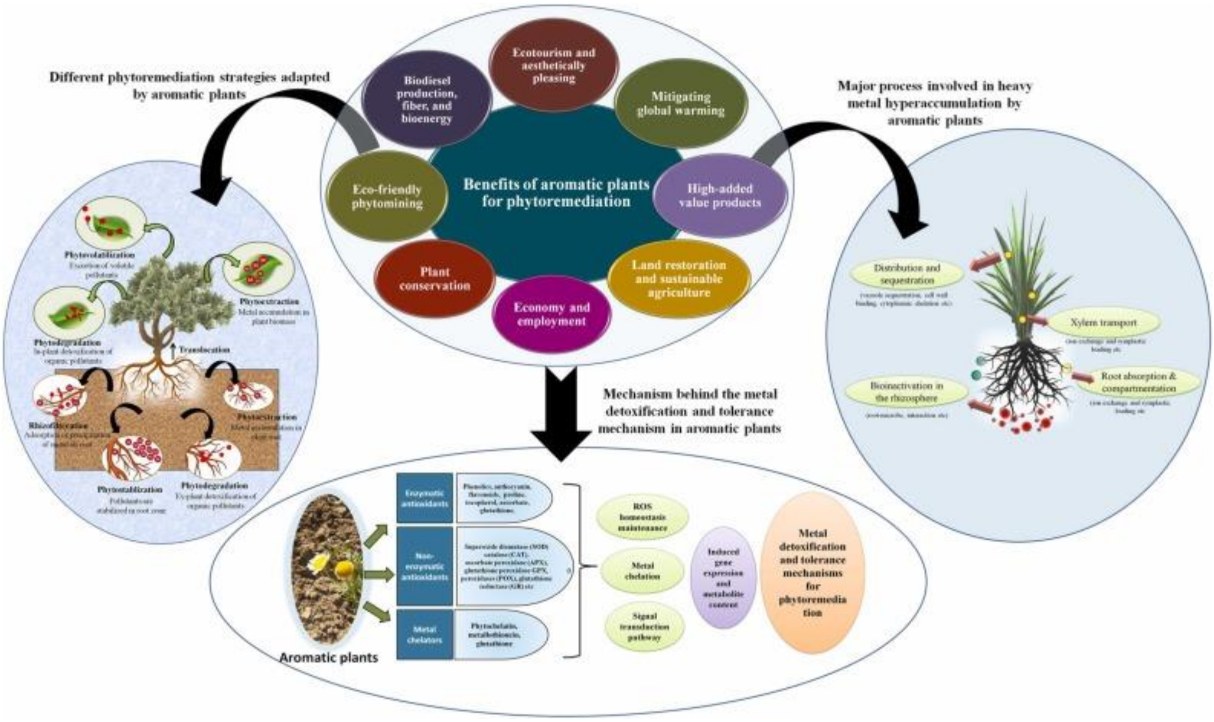

Figure 3. Benefits of aromatic plants for sustainable phytoremediation: Their different strategies for phytoremediation and metal hyperaccumulation and their adaptive mechanism behind the metal hyperaccumulation (Mishra and Chandra, 2022).

1.6 Medicinal and aromatic plants (MAPs) case study: Urtica dioica L., Melissa officinalis L. and Hypericum perforatum L.

1.6.1 *Urtica dioica* L. and Nitrate pollution

Urtica dioica L., known as stinging nettle, is an herbaceous perennial plant and belonging to *Urticaceae* family. Stinging nettle grows in a wide range of habitats, even if it favors nitrogen-rich soil (‘‘nitrophile’’ species). The plant belongs to phytoalimurgic vegetables, and its application is growing in recent years because of its unique chemical composition and positive correlation with human health (Grauso et al. 2019 a, b).

Stinging nettle (*Urtica dioica* L.) stands out as a valuable species that is neglected as a food source, as it has a significant content of specialized metabolites, and thus has an extremely high potential for use both nutritionally and pharmacologically. All parts of the plant (flowers, stems, leaves, and roots) can be used in food, cosmetic and pharmaceutical industries for their health-promoting properties, as nettle is rich in various biologically active compounds and specialized metabolites (SM). The phytochemical profile of nettle leaves can be divided into several categories: terpenoids, chlorophylls and carotenoids, fatty acids, phenolic acids and polyphenolic compounds, essential amino acids, vitamins, tannins, carbohydrates, sterols, polysaccharides, isolectins (Bombardelli and Morazzoni, 1997) and minerals like Potassium, iron, zinc and selenium (Özcan et al 2008).

Main phenolic components had been identified in neochlorogenic acid, chlorogenic acid and caffeoylmalic acid as well as quercetin, kaempferol and isorhamnetin glycosides (as rutinosides and glucosides), with trace kryptochlorogenic acid, 2-caffeoyltartaric acid and p-coumaroylquinic acid (Moreira et al. 2020).

Clinical studies have confirmed the effectiveness of Stinging nettle and report positive effects on prostatic hyperplasia (Lopatkin et al. 2005; Koch, 2001), alleviation symptoms associated with allergic rhinitis (Mittman, 1990) and arthritic rheumatisms (Randall et al., 2000).

Additional published research initiatives on medicinal uses, attributable to distinctive constituents (phenolic compounds, antioxidant activity, ascorbate, carotenoids, and fatty acids), have evidenced numerous benefits, including anti-inflammatory, antimicrobial, antioxidant, cardiovascular, chemo-preventative, diuretic, hepatoprotective (Chrusbasik et al. 2007; Grauso et al., 2020; Kregiel et al., 2018; Di Virgilio et al., 2015).

The latter studies provide the alternative use for bioactive content of stinging nettle. Recent research has also investigated the potential as food additives (Chakravartula et al. 2021; Shonte et al., 2020), including the biofortification of wheat- and maize-based breads (Diddana et al., 2021; Maietti et al., 2021; Man et al., 2019) which are comparatively poorer in bioactive contents (Adhikari et al., 2016).

The important bioactive component concentrations are affected by different agronomical factors, including location (climate, soil, and agricultural practices), the phenological stage of the plant and time of harvest. In the case of stinging nettle, the choice of the main product, whether stem fibers or bioactive leaf components, constitutes an additional determinant on when and how nettles are harvested (Di Virgilio et al., 2015).

Due to its documented medicinal and functional properties, *U. dioica* holds significant economic importance. However, nettle cultivation is insufficient to meet the demand, resulting in most of the material being sourced from natural stands. This reliance on wild harvesting is concerning because *U. dioica* is known to bioaccumulate nitrates and heavy metals when grown in contaminated soils (Coodling and Rutto, 2014).

Elevated level of Chromium (Cr), copper (Cu), Nickel (Ni) and Zinc (Zn) in tissues of nettles growing on polluted sites had been reported (Güleryüz et al. 2008 and Spongberg et al. 2008).

Moreover, stinging nettle is a nitrophilous plant species that prefers soils rich in nitrogen and organic matter, lead to the accumulation of potentially harmful nitrates in plant material

(Radman et al. 2015; Opacic et al. 2022). Nettle is able to accumulate and store nitrate ions from the environment in vacuoles, mostly from the soil (Horikawa et al. 2005) (Szabo et al. 2006). Stinging nettle is recommended as a potential species for ecotoxicological tests to measure the transfer of heavy metals to primary consumers (Sinnett et al., 2009) and for phytoremediation of contaminated soils (Shams et al., 2010).

The ability to accumulate heavy metals and nutrients, such as nitrogen and phosphorus, the high biomass and multiple harvests within crop cycle make this species suitable for phytoremediation purposes (Burges et al. 2018) (Petrovic et al. 2019) (Viktorova et al. 2016).

It is recognized that more research is necessary at a field scale for contaminants uptake and plants response. In particular, there are not reports on the performance of the stinging nettle grown on soils with high nitrate content and contamination effects has still not been widely investigated compared to heavy metals one. Hence more studies are needed to elucidate the uptake pathway of nitrate from soil to plants and the transport mechanisms.

1.6.2 *Hypericum perforatum* L. and PTE

Hypericum perforatum L. (Hypericaceae) is a perennial herb that is commonly known as St. John's Wort. It is a glabrous perennial, erect and usually woody at the base plant.

Extracts from Hypericum aerial part contain six major active compound groups: naphthodianthrones, phloroglucinols, flavonoids, biflavones, phenylpropanes, and proanthocyanidins. Identified flavonoids aglycone in *H. perforatum* L. include Kaempferol, luteonin, myricetin and quercetin (Naeem et al. 2010)

Additionally, lesser amounts of tannins, xanthones, essential oils, and amino acids are present. Naphthodianthrones compounds are typical of the genus *Hypericum,* where hypericin and its derivatives represent the most abundant components (Marrelli et al. 2016).

Hypericin and pseudohypericin occur in the flowers and leaves of the crude plant material in concentrations of 0.03% to 0.3% of dry weight, with significant variation depending on the developmental stage of the plant (Cellarova et al., 1994). Hypericin is reported to be the most abundant lipophilic compound, and it shows different accumulation trend depending on the species, chemotype and phenological phase/stage (Filippini et al. 2010).

Hypericum perforatum L. has important biological and chemical perspectives and its use in the treatment of infectious diseases has been documented in ethnobotanical reports. Most recent interest in *H. perforatum* has focused on its antidepressant effects, and only recently has its antimicrobial activity been evaluated against several bacterial and fungal strains (Saddige et al. 2010).

The chemical composition and pharmacological activities of *H. perforatum* have been well studied, no lot of data are available concerning toxic element absorption and effect on secondary metabolites biosynthesis.

In particular, the presence of trace elements, especially heavy metals, in *H. perforatum* for pharmaceutical application was evaluated. The *H. perforatum* accumulation capacity has been tested for Cr, Co, and Ni, focusing on soil geochemistry and soil-plant relationships have been carried out (Pavlova and Karadjova, 2013). Plant uptake of trace elements was found positively correlated with the soil extractable fraction and their accumulation distinctly different and species-specific. Moreover, recent studies on *H. perforatum* L. absorption of Cu, Ni, Zn elements, to evaluate potential toxic effect on medicinal products, have been carried out (Bonari et al. 2019). In addition, the total heavy metal content (Mn, Zn, Cu, Pb, Ni and Cd) in randomly collected herb material (25 cm from the top of plant and for yarrow – 30 cm) was determined. A relationship between heavy metals content and soil composition, in particular pH, was found. Thus Mn, Zn, Cd and Ni decreased linearly with the increase of soil pH (Radanovic et al. 2001). Effects of Ni contamination on growth and secondary metabolite composition of St. John's wort seedlings have been evaluated by Murch et al. (2003). In this study the accumulation of secondary metabolites in seedlings was significantly decreased by the presence of nickel in the culture environment.

Hypericin and pseudohypericin concentrations were significantly decreased by 21- and 15-fold, respectively, in nickel-exposed seedlings. Hyperforin concentrations decreased to below the detection limits in the Ni-exposed tissues (Murch et al. 2003). Heavy metals absorption and presence in aerial parts was widely demonstrated for medicinal use, but data concerning toxic element absorption and effect on secondary metabolites biosynthesis in adult plants is not deeply studied.

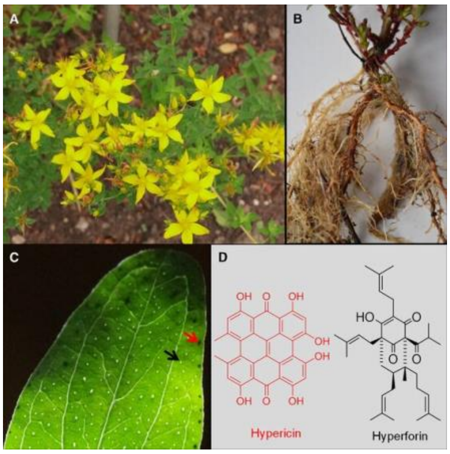

Figure 4 A) Hypericum perforatum entire plant. (B) Root system of field-grown plant. (C) Leaf with translucent glands spread over the lamina (hyperforin-rich; black arrow) and marginal dark nodules (hypericin-rich; red arrow). (D) Formulae of hypericin which accumulates in the dark nodules and hyperforin which is housed in the translucent glands (Gaid et al., 2019).

1.6.3 *Melissa officinalis* L. and PTE

Genus *Melissa* (*Melissa* L.) belongs to Family Lamiaceae and Subfamily: Nepetoideae, with numerous species. *Melissa axillaris* (Benth.) Bakh. f., *Melissa flava* Benth., *Melissa officinalis* L. - lemon balm; *Melissa yunnanensis* are recognized as the most frequent in the world (Taiwo et al. 2012), furthermore M. Officinalis L. is known as the only species growing in Europe (Sawicka et al. 2020).

Melissa officinalis L. is a perennial, herbaceous plant (Turhan, 2006), widely cultivated for the amounts of phenolic metabolites contained in plant tissue and leaves essential oils (**Figure 5**). Like other species from the Lamiaceae lemon balm is rich in polyphenols, in particular leaves are supposed to be richer in total phenolics than the stems (Chizzola et al. 2018). Actually, phenolic acids are the main components of this medicinal plants, and they could be divided in two classes: cinnamic acid and benzoic acid derivates.

The phenolic compounds most present in the plant are hydroxycinnamic acid derivatives such as Rosmarinic acid, Caffeic acid esters and flavonoids (e.g., luteolin and apigenin for the flavones group, and hesperidin, hesperetin, and naringenin for the flavanones one) (Scimone et al. 2024). Triterpenoids are also reported (Hansel and Sticher, 2010).

Rosmarinic acid is confirmed the major phenolic compound in *M. officinalis* L., reaching 85% and 69% of total phenolics in leaves and stems respectively (Fecka et al. 2007). These compounds are supposed to play an active key role within lemon balm antioxidant mechanisms (Shakeri et al. 2016) (Hawrylak-Nowak et al. 2021).

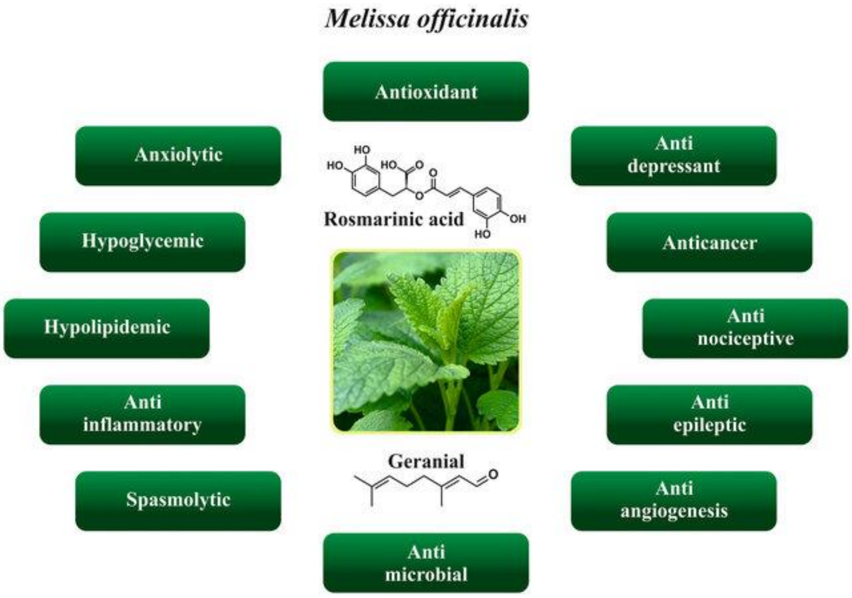

Figure 5. Health properties of Rosmarinic acid and Geranial in *Melissa officinalis* L. (Zam et al. 2022)

Plant tolerance to oxidative stress relies on biochemical components, including the ability of leaves to activate detoxification mechanisms. Specialized metabolites play a crucial role as a "secondary" antioxidant system, protecting plants against both biotic and abiotic stress factors (Pellegrini et al. 2019). Alterations in phenylpropanoid compounds contribute to detoxification by facilitating repair processes, scavenging reactive oxygen species (ROS), and preventing water loss. Additionally, carotenoids enhance the antioxidant activity of phenylpropanoids by dissipating excess energy and suppressing lipid peroxidation (Brunetti et al. 2015).

In literature various phenolic compounds besides rosmarinic acid are reported. Lyophilized ethanolic extracts were rich in flavonoids such as rutin, quercetin, quercitrin and isoquercitrin. Among phenolic acids caffeic acid is the most abundant, followed by ellagic and gallic acid respectively. The antioxidant activity of *M. officinalis* L. extracts is attributed to the presence of phenolic acids, mainly hydroxycinnamic acid derivatives such as rosmarinic acid (RA) (Scimone et al., 2024).

Modern pharmacological studies demonstrate that *M. officinalis* has several biological activities including antioxidant, hypoglycemic, hypolipidemic, antimicrobial, anticancer, antidepressant, anxiolytic, antinociceptive, anti-inflammatory and spasmolytic properties (Moradkhani et al. 2010; Miraj et al. 2016; Shakeri et al. 2016). Special attention should be given to antiviral effects (Moradi et al. 2016).

Due to *M. Officinalis* L. antimicrobial and antioxidant properties the application of extracts in the food industry had been widely investigated (Moradkhani et al. 2010).

In particular, the main active constituents of *M. officinalis* L. are volatile compounds (e.g. geranial, neral, citronellal and geraniol), triterpenes (e.g. ursolic acid and oleanolic acid), and phenolics (e.g. cis-and trans-RA isomers, caffeic acid derivatives, luteolin, naringin and hesperidin), as reported (Shakeri et al. 2016). Even if the plant contains low amounts of essential oils (EO), they are rich in geraniol, citronellal, geranial and neral as citrus aroma compounds and sesquiterpenes, mainly β-caryophyllenene and caryophyllene oxide in varying proportions (Chiozzola et al. 2018).

EO from *M. Officinalis* L. is obtained from fresh or dried flowers, leaves, and branches. It has commercial importance owing to its applications in the pharmaceutical and food industry. *M. officinalis* EO is used as an additive in foods, herbal teas, cosmetics, and in ornaments. The production cost and price of the oil are very high, because of the low yield of EO extraction (Sari and Ceylan, 2002; De Sousa et al., 2004). The EO is considered the mainly responsible for the antibacterial and antifungal activities of the plant (Mimica-Dukic et al., 2004).

Melissa officinalis L. strategies for the uptake of heavy metals from soil, how metals enter and migrate in the plant and their effects on plant physiology have been investigated (Bernal et al. 2006; Dubey et al. 2018). Of special interest are the effects of heavy metals stress conditions on phenolic compounds and essential oil yield. (Adamczyk-Szabela et al. 2019; Adamczyk-Szabela et al. 2022; Zheljazkov et al. 2008; Adamczyk-Szabela et al. 2023).

Accumulation of Cd, Mn, Cu and Zn, their effect on element absorption and translocation factor had been deeply investigated (Adamczyk-Szabela et al. 2019) (Adamczyk-Szabela et al. 2022). Concerning the effects of Cd and Zn on antioxidant activity and photosynthesis efficiency had been evaluated by Adamczyk-Szabela et al. (2023). Cd and Zn mostly accumulated in roots, and they led to a significant reduction in photosynthetic yield. Although a positive effect of contaminants on phenolic compounds biosynthesis was demonstrated. Herbal extracts shown high antioxidant capacities and scavenger ability for free radicals and ROS (Adamczyk-Szabela et al. 2023).

Further studies on lemon balm's application for heavy metal polluted soil restoration, without contamination of the final product had been demonstrated in Cd, Pb, Cu, Mn and Zn enriched soils (Zheljazkov et al. 2008). In this case yield in biomass and essential oil content and yield remained unchanged by contaminants. Even if Cd, Pb, Cu and Zn concentrations in plants tissues were relatively high, no toxicity symptoms were observed. (Zheljazkov et al. 2008).

Studies investigating on Nickel toxicity have been carried out (Rahimi et al. 2019; Maivan et al. 2017; Turon, 2019). In details, it was investigated the mediatory effects of Salicylic Acid (SA) in alleviating Nickel toxicity. The high potential for Ni accumulation in roots and high translocation factor may be introduced this plant as an excluder medicinal plant for Ni. An excess of this metal in *Melissa officinalis* L. inhibited growth indices and increased Ni content in leaves and roots dramatically. Although, the mitigation of Ni effects and the decrease in metal transport to the shoots thanks to SA foliar application was demonstrated (Maivan et al. 2017).

The effect of salinity, Nickel and their combination on Melissa officinalis L. antioxidant response was evaluated (Rahimi et al. 2019).

2 Aim of the thesis

As part of the biosphere, soil plays an important role in food production and environmental sustainability (Visser et al. 2019). Worldwide, a higher number of degraded soils as a result of increasing industrial, agricultural and civil activities is occurring. Soil contamination, both diffuse and localized, may lead to damage to several soil functions and contamination of surface- and groundwater (Vamerali et al. 2010). Thus, environmental and soil contamination is one of the major problems facing developing and industrialized countries (Mafakheri and Kordostami, 2021). Environmental pollution is defined as the entry of pollutants into the natural environment that causes adverse changes (Mahar et al. 2016). Soil contamination is often caused by human activities such as industrial development, urbanization, sewage sludge consumption, compost and chemical fertilizers in agriculture. Several factors may affect the physiological pathways and agronomic performance of plants, including the presence of heavy metals or high nitrate levels in farmland (Mafakheri M. and Kordostami M. 2021).

The contamination of agricultural lands and irrigation water with toxic pollutants such as heavy metals and nitrate poses an environmental risk to humans and animals.

Heavy metals including nickel, zinc, cadmium, chromium, copper and cobalt, may accumulate naturally in soil or may originate from industrial and mining processes (Sandeep et al. 2019).

Soil contamination by PTEs is a growing global crisis affecting the environment and human health; there are approximately 3,000,000 contaminated sites in the European Economic Area (Paya-Perez and Rodriguez, 2018).

Nickel is one of the more mobile and bioavailable heavy metal ions that may be present in both industrially contaminated and pristine soils. The natural presence of Ni in soils derived from sandstones, limestone or acid igneous rocks is generally less than 50 mg of Ni kg^{-1}. From the LUCAS (European Largest Soil Dataset) the mean Ni concentration in European topsoil in 2016 was determined to be about 18 mg kg^{-1}, with high variability. Concerning agricultural land, a considerable percentage of soils sampled from the Mediterranean region, Ni exceeded 50 mg kg^{-1} (Fernandez-Ugalde et al. 2022). Therefore, the risk for crop production and ecological and human health could be expected to be due to Ni in agricultural soil. The increase in this element in the environment at low or moderate levels may occur in agricultural lands due to the presence of this element in chemical fertilisers, pesticides and sewage and their excessive application in crop fields (Environment Agency, 2009). Furthermore, the ecotoxicological risk associated with nickel contamination on soil biota is addressed (Vischetti et al. 2022). Nitrate pollution of soil, ground and surface water bodies all over the world is generally linked with continually increasing global fertilizer nitrogen (N) use. After 1990, with more fertilizer N consumption in developing and industrialized countries, nitrate pollution is increasingly becoming a pervasive global problem (Bijay-Singh and Craswell 2021).

Faced with the increasing nitrate pollution, in-situ remediation has been widely studied and applied on field-scale as an efficient, economical and less disturbing remediation technology.

Among green technologies addressed to metal and nitrate pollution, phytoremediation has received increasing attention from the discovery of hyperaccumulator plants, which are able to concentrate high levels of specific metals in the above-ground harvestable biomass (Vamerali et al. 2010).

In order to address the challenge of remediation and management of the increasing number of areas considered as contaminated sites, the use of Medicinal and aromatic plants (MAPs) for phytoremediation purposes may be a considered as a commercial opportunity for phytoremediation (Pandey and Souza-Alonso 2019). The remediation of contaminated sites with appropriate and economically valuable crops is a dynamic strategy to provide additional benefits in the form of phytoproducts, such as aromatic essential oils, pulp–paper biomass, biochar, energy and biodiesel, ameliorated crops through biofortification, ornamental plants, or differently derived wood products (Pandey and Souza-Alonso 2019).

MAPs are rich sources of chemically active constituents which are used as raw materials in nutraceutical, fragrance, dyes, cosmetic and pharmaceutical. The constituents commonly known as SMs are used for adaptation by the plant during stress condition such as temperature, carbon dioxide, ozone, light and soil (Pant et al. 2021). These abiotic stresses not only modify plant structurally and anatomically but also lead to fluctuation in their chemical constituents' quantities. Thus, knowledge of abiotic stress and SMs help to protect the plant sources which are under pressure due to excessive exploitation. This clearly showed the synthesis of natural products can be altered by different abiotic stresses (Pant et al. 2021).

The critical part of phytoremediation is the selection of phytoremediation species allowing the removal of contaminants from soils. MAPs with phytoremediation capabilities owing to the biosynthesis of secondary metabolites in addition to distinctive morphological characteristics are eventually becoming the preferred choice for effective phytoremediation, in particular for heavy metals (Mafakheri and Kordrostami, 2021). In this case, Essential oil extraction process is stem distillation, which does not allow the contaminants moving to the oil. After harvesting the oil, residual biomass may be utilized for energy production. This energy may be produced by direct burning of biomass or production of biogas through the gasification of biomass. This integrated approach will not only reduce the cost of oil but also will help to develop a sustainable model which may mitigate many environmental issues (Jisha et al. 2017).

In recent studies, Ni was shown to accumulate in a variety of medicinal plants including *Hypericum perforatum* L. and *Melissa officinalis* L. (Sussa et al. 2022; Rahimi et al. 2019). Both plants may be suitable for phytoremediation purpose, thanks to their high biomass production and their tolerance to heavy metal, in particular Nickel. *Hypericum* spp. tend to accumulate Nickel in shoots and aerial part, instead Melissa in roots as previously demonstrated (Kováčik et al. 2022). St. John's wort (*Hypericum perforatum* L.) is a MAP used in the treatment of neurological disorders and recently identified as a possible treatment for cancer tumors, and its principal medicinally important secondary metabolites are hypericin, hyperforin and pseudohypericin. Lemon balm (*Melissa officinalis* L.) is a medicinal plant used for its antioxidant, antiviral and calming activities treat, and its principal medicinally relevant metabolites are Rosmarinic acid, Caffeic acid and Luteolin.

Concerning nitrate contamination, the application of plants well adapted to high nitrate content in soil and water is necessary for the phytoextraction process.

The medicinal plants *Urtica dioica* L. is a nitrophilous perennial species, that prefers soils rich in nitrogen and organic matter, which can lead to the accumulation of potentially harmful nitrates in plant material (Radman et al. 2016; Opacic et al. 2022). It may be a potential candidate for phytoremediation, since high biomass and multiple harvests within crop cycle (Burges et al. 2018). In particular, the positive effect of Nitrate rich cultivation medium on the increase in biomass production have been widely demonstrated (Radman et al. 2021).

This PhD thesis has been focused on two mainly soil contaminants Nitrate and Nickel. For the purpose different medicinal and Aromatic Plants species (MAPs) had been tested, *Urtica dioica* L., and *Melissa officinalis* L. and *Hypericum perforatum* L. for nitrate and nickel respectively. MAPs had been evaluated for their agronomic adaptability to contaminants, growth, yield, uptake capacity and physiological response in term of secondary metabolites biosynthesis.

Nitrate uptake by *Urtica dioica* L. in two different field located in Nitrate Vulnerable Zones of Emilia Romagna Region had been evaluated (Chapter 1). On the other hand, Nickel uptake capacity and physiological response for *M. officinalis* and *H. perforatum* under greenhouse conditions/in controlled environment had been studied (Chapter 2).

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Chapter I - Evaluation of Agronomic performance and health-beneficial properties of *Urtica dioica L.* cultivated in Nitrate Vulnerable Zones

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ABSTRACT

Nitrogen, essential for crop growth, contributes to water pollution when present in excess from agricultural sources. Current EU measures to reduce nitrate pollution emphasize Nitrate Vulnerable Zones (NVZs) monitoring and good agricultural practices, underscoring the need for targeted cultivation methods to ensure high-quality biomass with safe nitrate levels. Stinging nettle (*Urtica dioica* L.), a nitrophilous plant with significant bioactive and phytoremediation properties, can accumulate nitrates, heavy metals, and other nutrients, making it suitable for soil improvement as well as a valuable resource for nutraceutical applications. This study addresses the potential of cultivating stinging nettle in NVZs to improve soil and water quality and obtain a valuable final product from these marginal areas. Stinging nettle was cultivated in hilly and plan areas located in Emilia Romagna Region (Italy), Tresigallo and Ozzano respectively, categorized as locations belonging to NVZs. Samples were analyzed over three harvests for two cultivation years. Furthermore, bioactive compound levels, nitrate uptake, and elements content in stinging nettle were evaluated.

Stinging nettle was successfully cultivated with satisfactory yields and high levels of bioactive compounds, including polyphenols, flavonoids, DPPH, FRAP, and folic acid, particularly in July cut and Nitrate levels remained below EFSA (European Food and Safety Authority) thresholds for leafy vegetables for all cuts, locations and years. Notably, the Ozzano site produced optimal yields for polyphenols and antioxidants in May and July, demonstrating the importance of location-specific agronomic practices such as consistent irrigation and weed control. Over two years, nettle cultivation positively impacted total nitrogen levels in the first 50 cm soil and groundwater without compromising leaf nitrate thresholds. With multiple

harvests, NVZ-grown nettle yields a high-quality product rich in elements (iron, selenium, potassium), vitamin C, folate, phenols, and antioxidants. This shows promise for stinging nettle cultivation in NVZs with a high value final product suitable for medicinal/herbalist sector.

1 INTRODUCTION

Nitrogen is a vital nutrient helpful to plants and crop growth. However, among the leading causes of water resources pollution is the excess nitrogen from agricultural sources (Massarelli et al. 2021).

According to the Food and Agriculture Organization of the United Nations report (FAO, 2017), nitrate of agricultural origin is the most common chemical pollutant affecting the quality of water resources and crop production. Directives on nitrates regulation has been approved form the European Union countries to reduce Nitrate content in land and water bodies. The adopted measures concern monitoring of water bodies nitrate concentrations, designation of Nitrate Vulnerable Zones (NVZs), and establishing codes of good agricultural practices and measures to prevent and reduce water pollution from nitrates (Massarelli et al. 2021).

Nitrate Vulnerable Zones are a huge environmental problem for agricultural land and for human health. Increasing use in Nitrogen based fertilizers may led to an increasing Nitrate level in soil and underground water. In that way it could enter the human chain and if excessively consumed it might lead to health diseased.

Urtica dioica L. (stinging nettle) is a perennial herbaceous plant, belonging to *Urticaceae* family and to the group of phytoalimurgic vegetables.

It may grow in a wide range of habitats preferring nitrogen-rich soil as it is a “nitrophile’’ plant. Due to the high nutritional value and functional properties, as well as the wide use of stinging nettle, the demand for its fresh biomass is actually increasing. Stinging nettle is a plant with great nutritional value, containing biologically significant classes of chemical compounds.

The lack of information concerning good agricultural practices led to questionable quality and inconsistent chemical composition and may not adapt for human consumption. Nettle is mentioned as hyperaccumulating plant with a strong tendence to collect heavy metals and nutrients as nitrogen and phosphorous from soil. This ability to accumulate heavy metals and nutrients is used to purify the soil, which is why nettle is considered a phytoremediation plant (Petrovic et al. 2019) (Viktorova et al. 2016). Nettle is a nitrophilous plant species that prefers soils rich in nitrogen and organic matter, which may lead to accumulate potentially harmful nitrates in plant material. These characteristics make *U. dioica* L. suitable for cultivation in NVZs and for its application for soil amelioration. Since research studies demonstrate nitrate negative effects for human health, it may be necessary to set up nettle good agricultural practices if cultivated in NVZs. Such evidence indicates the need to introduce nettle into agricultural production, but the technology of nettle cultivation is still insufficiently researched (Maggini et al. 2014). Since most research studies analyze the chemical composition of wild-collected nettles, it is important to focus on cultivation techniques in NVZs located fields and the nutrient quality of plant material. The Crop cultivation in nitrate-rich soils may led to contamination and excess in nitrate in the food chain, as widely demonstrated (Romano et al. 2013, EFSA 2020, Yousefi et al 2023).

The cultivation of stinging nettle for medicinal purposes (with a high yield of bioactive compounds) is cited as a compromise between yield and the content of polyphenol and flavonoid content in the harvested product (Grevsen et al., 2008). So far numerous studies were conducted on wild nettle (Özkan et al., 2011; Kukrić et al., 2012; Otles and Yalcin, 2012; Andualem et al. 2015); however, in recent years interest about cultivated nettle is also increasing (Biesiada and Wołoszczak 2007; Grevsen et al., 2008; Biesiada et al., 2009). The reason for such trend is the raising concern about health, since the wild plant material collected from natural habitats may be of questionable nutritional and medicinal value. Nettle preference for loose soil with organic matter rich in Nitrate and Phosphate. Previous studies on cultivated nettle have shown that nutritional quality and chemical composition of the plants might be affected by the age, harvest time (Biesiada et al., 2010) and nitrogen (Biesiada et al., 2009; Rutto et al., 2012).

Therefore, this study aimed to assess the potential of stinging nettle (Urtica dioica L.) for phytoremediation in nitrate-rich soils by evaluating its growth and nitrate uptake capacity. The present study focused on quantifying the plant's ability to absorb excess nitrates while analyzing its bioactive and mineral compound content over multiple harvest periods in two Nitrate Vulnerable Zones (NVZs) in Emilia Romagna, Italy (plains and hilly regions). Furthermore, the nitrate uptake capacity of Nettle and its possible application for soil restoration as an alternative for remediation. To achieve these objectives, crop growth parameters, nitrate accumulation, and soil characteristics (nitrate and element content) before and after cultivation were examined.

2 Materials and Methods

2.1 Experimental locations

The experimental trials were conducted on two farms located in Nitrate Vulnerable Zones (NVZ) at different altitudes (plan and hilly) within the region of Emilia-Romagna, Italy. The locations of the two farms were as follows: Tresigallo (latitude 44◦49’26"N longitude 11◦54’42"E, − 1 m above sea level [a.s.l.]) and Ozzano dell ’Emilia (latitude 44◦23′ 16′ N, longitude 11◦25′ 54′ E, 110 m a.s.l.).

The experimental farm located in Ozzano was organic, otherwise the farm located in Tresigallo was conventional. The soil texture for Tresigallo and Ozzano was silty clay loam and sandy loam respectively (**Table 1**). Crops that were grown prior to *Urtica dioica* L. were soyabean and horticultural onion in Tresigallo and Ozzano respectively. Soil parameters at 0-30 depth for each location were analyzed prior and posterior to the stinging nettle experimental trial by Agriparadigma (https://www.agriparadigma.it/) laboratories and Chemistry laboratories of University of Bologna, respectively (**Table 1** and **Table 2**).

Prior to *Urtica dioica* L cultivation, the soil of Ozzano was characterized by a slightly higher C and organic matter content and a lower N and exchangeable K content compared with the values of Tresigallo (**Table 1**). The soil of Tresigallo was characterized by higher cation exchange capacity compared with the Ozzano location (**Table 1**).

Concerning total elements Tresigallo location shown higher content of Ba, Be, Ni and V compared to Ozzano location. Otherwise, higher Sr content was observed in Ozzano compared to Tresigallo. For the remaining analyzed elements comparable values were observed in the two locations. In addition, any significative effects of nettle on elements were not observed in 2022 (**Table 2**).

The meteorological data (temperature and precipitation) for the entire duration of the experimental trial, comprising the multiple cycles in each location, was obtained from the Arpae weather station, located in Emilia Romagna (https://simc.arpae.it/dext3r/) (**Figure 2**). Using the temperature data, the mean growing degree days (GDD) was calculated (Hykkerud et al., 2018).

Table 1. Main physico-chemical soil properties (0-30 cm depth) of Tresigallo and Ozzano fields before and after *Urtica dioica* L. cultivation.

	Tresigallo		**Ozzano**	
Soil characteristics	2020	2022	2020	2022
Clay (%)	37	37	12	12
Loam (%)	55	55	18	18
Sand (%)	8	8	70	70
Cation exchange capacity (meq 100 g-1)	31.9 ± 1.56	28.5 ± 1.0	27.8 ± 0.73	13.0 ± 1.2
Total N (g kg-1)	1.8 ± 0.28	1.2 ± 0.14	1.6 ± 0.28	1.1 ± 0.14
Assimilable P (mg kg-1)	21.0 ± 4.24	20 ± 4.00	20.0 ± 9.90	11.0 ± 4.40
Exchangeable K (mg kg-1)	270 ± 5.7	123 ± 4.6	342 ± 8.7	195 ± 0.6
Total Organic C (g kg-1)	12.5 ± 0.71	16.0 ± 1.0	16.5 ± 1.21	13.0 ± 0.2
C/N	6.9	13.3	10.3	11.8
Mg/K	2.3	2.7	1.6	1.7
Organic matter (%)	2.2 ± 0.11	2.7 ± 0.11	2.8 ± 0.35	2.2 ± 0.35
Ammoniacal N (g/kg)	3 ± 0.21	< 2	< 2	< 2
Nitrate (g/kg)	83 ± 3.7	64 ± 2.2	41± 1.6	10 ± 0.6

Table 2. Soil element composition (0-30 cm depth) of Tresigallo and Ozzano in Emilia Romagna, prior to and after the cultivation of *Urtica dioica* L

	Ozzano		Tresigallo	
Elements	2020	2022	2020	2022
Ag µg kg-1	428,9	419,3	476,2	468,1
Al g kg-1	27,7	27,3	39,2	33,8
As mg kg-1	6,3	5,2	7,9	7,1
B mg kg-1	31,6	28,9	39,1	31,3
Ba mg kg-1	96,7	90,9	163,1	142,7
Be µg kg-1	188,2	244,8	514,1	408,6
Ca g kg-1	44,4	39,4	41,0	41,4
Cd µg kg-1	363,8	380,6	501,2	484,7
Co mg kg-1	11,1	11,8	16,2	15,8
Cr mg kg-1	86,3	92,8	129,1	120,6
Cu mg kg-1	34,9	34,2	46,0	46,1
Fe g kg-1	25,3	26,2	31,3	30,1
Hg µg kg-1	≤LOD	≤LOD	≤LOD	≤LOD
K g kg-1	5,0	4,8	6,0	4,9
Li mg kg-1	39,8	40,3	48,0	43,8
Mg g kg-1	7,8	8,0	13,8	13,2
Mn g kg-1	695,2	708,5	747,5	721,8
Mo µg kg-1	≤LOD	≤LOD	≤LOD	≤LOD
Na mg kg-1	433,7	388,5	545,7	440,3
Ni mg kg-1	46,7	50,5	101,6	97,6
P mg kg-1	718,0	702,7	731,6	687,8
Pb mg kg-1	17,0	18,0	20,8	18,7
S mg kg-1	208,9	216,3	200,5	203,1
Sb mg kg-1	1,4	1,9	2,4	2,7
Se mg kg-1	2,5	2,5	4,0	3,6
Sn mg kg-1	101,4	122,9	198,3	155,5
Sr µg kg-1	776,8	1144,9	824,6	451,4
Ti mg kg-1	182,7	158,9	178,2	176,0
Tl mg kg-1	207,8	165,9	388,8	312,3
V µg kg-1	771,2	779,3	1657,2	1316,9
Zn mg kg-1	52,5	51,0	64,3	56,0

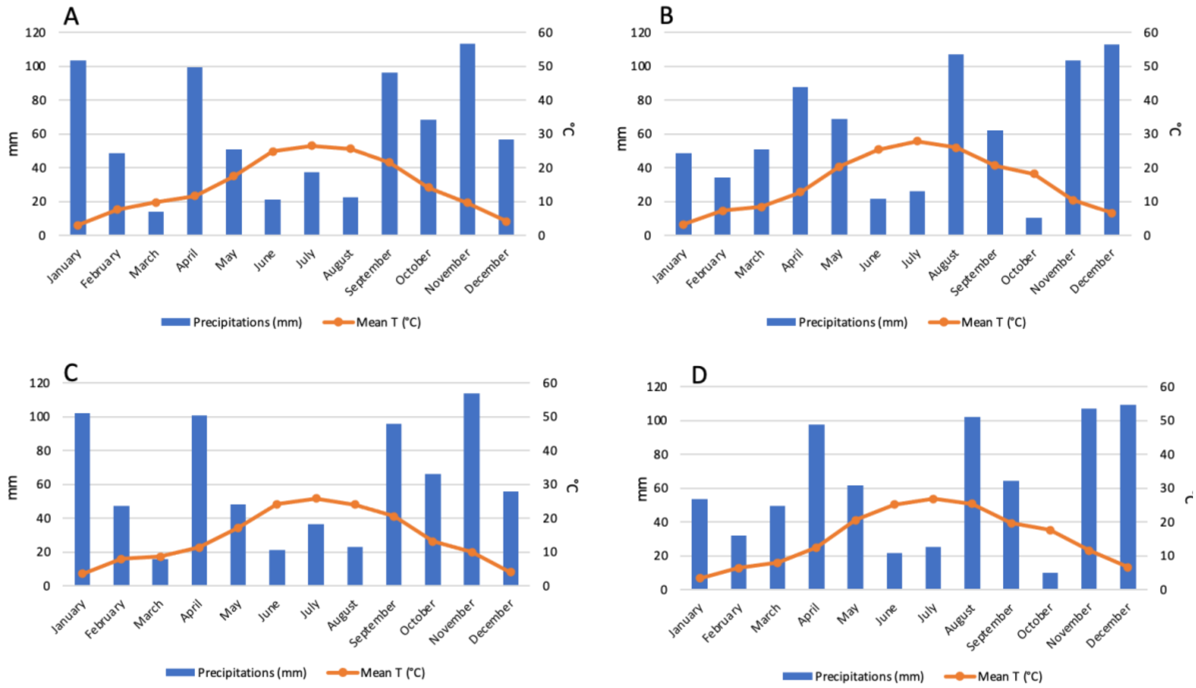


Figure 1. Monthly precipitation (mm) and average temperature (°C) at each location for all cultivation cycles between 2020 and 2022.

A= Ozzano 2021, B= Ozzano 2022, C= Tresigallo 2021 and D= Tresigallo 2022. Meteorological data supplied by the Arpae weather station, located in Emilia Romagna (https://simc.arpae.it/dext3r/).

2.2 Plant material, field trial conditions and harvesting

Seed material of a commercial variety of *Urtica dioica* L., specifically selected for the harvest of bioactive compounds, was purchased from Fluxias GmbH (Baden-Württemberg, Germany). The seeds were germinated in multicell trays at the end of April 2020 and seedling establishment performed under greenhouse conditions at 25 ◦C. This procedure was previously shown to improve final leaf yield at harvest within the first year of cultivation (Biesiada et al., 2009). After 40 days, the seedlings were transplanted in the field at a recommended plant density of 6.66 plants/m2 (Jankauskiene and Gruzdeviene, 2015). Within each location, the total cultivation area was 5000 m^2 for Tresigallo and 3500 m^2 for Ozzano (**Figure 2**). Each area was, respectively, composed of three replicate blocks. Planting distances within the assigned areas were 75 cm inter-row at Tresigallo and twin-row with 100:50 cm alternate rows at Ozzano. At transplantation in both location, seedlings were irrigated with a sprinkler system with 200 L ha-1. Tresigallo was rainfed during the cultivation period. Instead at Ozzano,

irrigation (200 L ha-1) was only implemented under emergency conditions twice a week. At all locations, no pesticide or herbicide treatments were performed on the crop. Weeding was performed twice and five within each growing cycle for Tresigallo and Ozzano respectively, and it was realized with milling cutter and manually to remove certain species. For each location, three leaf harvests were made for each year of cultivation (2021-2022) respectively, in May, July and September, in an approach adopted previously by Biesiada et al. (2010).
The leaves were harvested from a 3 m^2 area within in each replicate block at each location. Leaf material was removed manually from the top 10 – 15 cm of the plant. Leaf biomass was determined by weighing the fresh leaves. Dry mass was calculated after drying the fresh leaves to a constant weight in an oven at 40 °C. After each leaf harvest, the plants were allowed to resume growth over next growth cycle.

Figure 2. Field experimental locations of *Urtica dioica* L. On the left Ozzano location and on the right Tresigallo location are represented.

2.3 Soil preparation and analysis

Soils of both locations were collected randomly in the field, before and at the end of nettle crop cycle. The first 20 cm (discarding first 5 cm) of soil were sampled, dried, sieved (<2 mm) and then homogenized in laboratory. An aliquot of <2 mm was finely ground for elemental analyses. The element content (Ag, Al, As, B, Ba, Be, Ca, Cd, Cl, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, Sn, Sr, Ti, V, Zn) was determined according to Vittori Antisari et al. (2013). Briefly, the finely ground soil (0.25 g) was treated with *aqua regia* (2 mL HNO3 65% plus 6 mL HCl 37%, suprapur grade Carlo Erba) in a microwave oven (Start D 1200, Milestone, USA) and the element concentrations were determined by ICP-MS. The analysis of each sample was replicated three times and compared with analyses of the International Reference

Materials (BCR 141) and laboratory internal standards (MO and ML), which was run after every 10 samples to check changes in sensitivity. Controls with only reagents were also determined (**Table 2**).

2.4 Leaf quality analyses

For the analysis of functional quality parameters, the fresh leaf material was ground to produce a fine powder and three replicates performed for leach location and cut, respectively. The functional quality analyses included polyphenol and flavonoid content, ferric reducing antioxidant potential (FRAP) and anti-radical activity using the 1,1- diphenyl-2-picrylhydrazyl (DPPH) radical, Ascorbate, Folic, Malic, Citric and Oxalic Acids.

Just for the analysis of functional quality parameters such as insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) the dry leaf material was ground to produce a fine powder and three replicates performed for leach location and cut, respectively.

2.4.1 Polyphenol and flavonoid content and antioxidant activity analyses

Polyphenols, comprising both free and bound constituents, were extracted as described previously (Dinelli et al., 2011). Free (FP) and bound polyphenols (BP) were then measured according to the Folin-Ciocalteau spectrophotometric (765 nm) method using gallic acid (GA) as a reference standard (Singleton et al., 1999). Likewise, the free (FF) and bound flavonoids (BF) were individually measured using a spectrophotometric (510 nm) colorimetric assay with catechin (CA) as a reference standard (Adom et al., 2003).

The total amount of polyphenols and flavonoids had been calculated from the free and bound content, respectively. The DPPH assay was performed by measuring the reduction (515 nm) of DPPH• to 1,1-diphenyl-2-picryl hydrazine (Floegel et al., 2011) and FRAP (reduction of Fe2+) was determined using a spectrophotometric (593 nm) method reported previously (Benzie and Strain, 1996). The antioxidant activity in the free and bound fraction were summed and expressed as total DPPH and FRAP, respectively.

2.4.2 Ascorbic acid content

Ascorbic acid was extracted from the leaf material as previously reported (Shivembe et al., 2017). Separation was achieved with a Waters e2695 Alliance HPLC System combined with a Waters ACQUITY QDa Mass Detector. A Luna Omega C18 (250 × 4.6 mm, 5 µm)

(Phenomenex, CA, USA) column was used at a temperature of 40 ◦C. The isocratic mobile phase was water containing 0.05% CH3COOH/CH3CN (90:10, v/v). The flow rate and the injection volume were 0.85 mL/min and 10 µL, respectively. Single-ion recording (SIR) was used in the Electrospray Ionization ESI negative mode, with the following parameters: cone voltage (8 V), probe (600 ◦C), sampling rate (10 points/sec), capillary (Pos: 0.8, Neg: 0.8). The monitored negative ions were m/z 175.2, 173.2 for ascorbic acid and dehydroascorbic acid, respectively. Data acquisition and instrument control were performed using the Empower 3 software.

2.4.3 Folic acid content

Folic acid was extracted from the leaf material with an Ascorbic Acid aqueous solution (pH 9) as previously reported (Meng et al., 2021). Separation was achieved with a Waters e2695 Alliance HPLC System combined with a Waters ACQUITY QDa 2489 UV/Vis Mass Detector. A Luna Omega C18 polar (250 × 4.6 mm, 5 µm) (Phenomenex, CA, USA) column was used at a temperature of 40 ± 2 ◦C. The isocratic mobile phase was composed of water containing 0.05% CH_3COOH (solution A) and CH_3OH (solution B), in 75:25 v/v respectively. The flow rate and the injection volume started with 0.4 mL/min until 0.5 ml/min and 10 µL, respectively. Single-ion recording (SIR) was used in the Electrospray Ionization ESI negative mode, with the following parameters: cone voltage (8 V), probe (600 ◦C), sampling rate (10 points/sec), capillary (Pos: 0.8, Neg: 0.8). The monitored negative ions were m/z 365 and 295. Data acquisition and instrument control were performed using the Empower 3 software.

2.5 Dry Leaves and roots analysis

For the analysis of element content and antinutritional compound as Nitrate, the dry leaf and root material was ground to produce a fine powder and then homogenized in laboratory. Three replicates for each location, cut and year were performed for nettle aerial parts. Root samples in three replicates for each location were harvested and analyzed at the end of the crop cycle (October 2022). Samples from each location, harvest cut, and year were evaluated. Moreover, roots samples were harvested and analyzed at the end of the cultivation cycle and compared with the results concerning the cut of September 2022 of the aerial part. Concerning element content in aerial parts, for each cut (May, July and September) the impact of location and year, and the interaction of these factors was evaluated.

Therefore, a focus on elements with health beneficial properties as Fe, Mg, Se, K, Ca and P and with toxic effects on human health as Ni, Cd, Cr, Pb was done for PCA analysis.

2.5.1 Nitrate content

Given that nitrate content in the leaves is considered an antinutritional component and in order to evaluate uptake capacity, nitrate was extracted from the leaf material according to the method of Raimondi et al. (2006). As reported, grinded leaves material was extracted in ultra-pure water and incubated for 1 hour in boiling water-bath. Afterwards, activated charcoal (WVR) was added and filtered. Filtered nitrates extracts were added to Nitraver®5 Nitrate Reagent Powder Pillow, 10-mL (Hach) brought to volume and measured using a spectrophotometric (500 nm) colorimetric assay (Raimondi et al., 2006). A Nitrate Nitrogen Standard (NO_3N) was used as the reference standard. Nitrate content in roots and aerial parts, for each location, cut and year was evaluated.

2.5.2 Dietary fiber Content

Insoluble dietary fiber (IDF), soluble dietary fiber (SDF), were extracted and measured according to the instruction protocol provided with the Megazyme Total Dietary Fibre Assay Procedure kit (Megazyme International, Ireland), that was based on previously reported methods (Lee et al., 1992; Prosky et al., 1988).

2.5.3 Mineralization

Dry tissues of different organs (roots and leaves) of nettle plants for each location, cut and year, were finely grounded and digested using a nitric acid and oxygen peroxide solution in the microwave oven according to the United States Environmental Protection Agency – USEPA (2009) method, modified by Vittori Antisari et al. (2014). Approximately 0.25 g sub-sample of plant tissue was treated with 6 mL of concentrated ultrapure nitric acid (Merck) plus 1.5 mL of hydrogen peroxide (Carlo Erba for electronic use). The mineralization was carried out in PTFE vessels in the microwave oven, and both the content of nutrients (Ca, Mg, K, Na, P, S) and metals (Ag, Co, Ce, Fe, Ni, Sn, Ti) in leaves, stems and roots, were quantified by ICP-OES. Blank and International Reference Materials were analyzed to validate the method. In addition, standard solutions were analyzed every 10 samples for quality control/quality assurance purposes (Vittori Antisari et al. 2018).

2.6 Statistical analysis

Statistical analyses were conducted using the Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA). A one-way analysis of variance (ANOVA) in conjunction with Tukey's honest significant difference was performed to compare the three growing locations. Significant differences between means were determined by least significant difference values for $p < 0.05$. Pearson's correlation coefficient (r) was calculated at significance level of $p < 0.01$.

The principal coordinate analysis (PCA) was used in the present study. PCA is an unsupervised clustering method and is a powerful tool for analysis of multivariate data, without requiring any knowledge of the dataset (Jambu, 1991). PCA was used to transform numerous correlated variables into a smaller number of uncorrelated variables called principal components (Tabachnick and Fidell, 2001). In the present study, the correlation method was preferred over covariance since PCA on the covariance matrix is not invariant to a component-wise change of scale (Bilodeau and Duchesne, 2002). With this method the original space for variable measurements was projected down onto two low-dimensional subspaces. One of these was case-related stinging (nettle samples collected in the two growing locations), the other was variable-related. The fifteen variables were as follows: total polyphenols (TP), total flavonoids (TF), DPPH and FRAP antioxidant activities, total dietary fibers (TDF), ascorbate (ASC), Folic Acid (FA), nitrate (NITR) and elements as Fe, Se, Zn, Mg, K, P and Cu. The variable-related subspace was analyzed (factor loading) to understand the correlation between the variables and factors (principal component).

3 Results and Discussion

Nettle is perhaps one of the most widely distributed wild plants, found in all regions of the temperate zones and growing in all seasons (Grauso et al. 2020).

All parts of the plant (flowers, stems, leaves and roots) might be used in food, cosmetic and pharmaceutical industries for their health-promoting properties (Kregiel et al. 2018; Garcia et al. 2021), as nettle is rich in various biologically active compounds and specialized metabolites (SM). The phytochemical profile of nettle leaves may be divided into several categories: terpenoids, chlorophylls and carotenoids, fatty acids, polyphenolic acids and compounds, essential amino acids, vitamins, tannins, carbohydrates, sterols, polysaccharides, isolectins and minerals (Di Virgilio et al. 2014).

According to various studies, the presence of chemical components in plants is influenced by many abiotic, biotic and anthropological factors as well as on the conditions of raw material storage (Biesiada et al., 2010; Grevsen et al., 2008; Nencu et al., 2013; Radman et al., 2014).

Provide Nettle with the necessary nutrients, through an appropriate and, above all, balanced fertilization is important for its cultivation and development, since it requires various nutrients for its growth and development, especially nitrogen, as it is considered a nitrophilous plant species (Radman et al. 2015; Stepanović et al. 2009). However, there is comparatively less information on the bioactive contents of commercial stinging nettle cultivated in Nitrate Vulnerable Zones or contaminated sites compared with wild nettle.

To expand on the earlier studies by including Nettle cultivation with multiple locations and/or phenological stages, more recent studies on wild *Urtica dioica* L. also showed variation in health promoting compounds based on location, phenological stage and harvest time. The parameters measured were yield, and of specific interest, the contents of polyphenols (flavonoids), antioxidant activities (DPPH and FRAP), ascorbate, folic acid and Nitrate content. Given that stinging nettle is a NUS with potential for main-stream agriculture (Ulian et al., 2020), the present study was aimed addressing agronomical aspects influencing bioactive production in NVZs, considering the effects of Nitrate on plant development. In addition, the effect of the cultivation of nitrophilous plant on the soil in terms of Nitrate and mineral content. This was considered important towards prioritizing this crop for future research, development, and innovation for medicinal/herbalist products.

3.1 Yield and growth parameters

Yield (biomass) is one important determinant influencing the potential success of a MAP and phytoremediation operation. In the present study, the total biomass was shown to increase in relation to crop age for both May and July cuts as previously reported (Marotti el al. 2022). However, the highest biomass level was evident in first year of cultivation coinciding with September 2021 cut (**Table 3**).

The applied plants density (Jankauskienė and Gruzdeviene, 2015) and the leaf biomass levels recorded at the end of the first year of crop age (September 2021) were higher to the overall above-ground green biomass (stems, leaves, inflorescences) for commercial stinging nettle reported by Jankauskienė and Gruzdevienė (2015). The lower biomass production might be mainly due to the different pedoclimatic conditions and cultivation techniques adopted. Leaf biomass of stinging nettle seeded in multiwell plates (prior to transplantation to the field) was shown to reach 3000 kg/ha within the first year (Biesiada and Wołoszczak, 2007) in Poland areas. In the present study, for the cuts of May and July similar values were obtained only in the second year (**Table 3**) for July and May cuts, otherwise in September cut double yield production compared to Biesiada and Wołoszczak (2007) was obtained in both cultivation year, confirming September as the best harvest season for nettle cultivated in Emilia Romagna Region (Italy). It should be underlined that even if the planting condition were the same, pedo-climatic conditions, due to the cultivation areas and agronomic management (water supply and weeds), strongly influenced the total yield. In addition, exogenous application of N has also shown to be an important determinant of stinging nettle yield (Grevsen et al., 2008; Radman et al., 2015). Thus, the natural high N level in soil and ground water characterizing NVZs in Italy allow to obtain higher biomass level compared to the one obtained in the previously reported literature.

The impact of different location was evident in this study as well, since the yields were significantly lower for Tresigallo location compared to Ozzano, for all cuts and total biomass respectively. This trend was associated with a significantly increased leaf DM percentage in Ozzano rather than in Tresigallo (**Table 4**). This evidence was related to Tresigallo field that was severely compromised by the presence of weed and ununiform supply of water.

Indeed, emergency irrigation supplied on requirement at Ozzano shown to be effective in ensuring competitive biomass yields for all cuts in 2021 and 2022 (**Table 3**) compared to reported data (Jankauskienė and Gruzdeviene, 2015; Biesiada and Wołoszczak, 2007; Marotti et al., 2022).

This result corroborated previous research highlighting the need for intensive weed management and uniform supply of water over the growing period (Di Virgilio et al., 2015; Bacci et al., 2009). Precipitations were extremely low in both location and cultivation years (2021 and 2022) (**Table 5)**. Thus, increasing drought spring season in Italy lead to an higher request of emergency irrigation supply to maximize yield.

Despite better results in terms of GI (Growth Index) were obtained for the May and July cut in 2021 and 2022, respectively (**Table 4**). This indicates that the growth index value observed in different cuts is not representative of the final yield value of the crop, since seed production phase may affect green biomass production leading to higher GI and DM but lower biomass production. DM values were comparable to previously reported data (Radman et al., 2015), for Ozzano location, for all cuts and years, but Tresigallo location showed higher DM results compared to cited ones.

Hence, from the present study, yield under organic farming conditions in NVZs thanks to natural supply of Nitrogen is favored with agronomic practices that collectively include effective weed removal, a more uniform supply of water (whether consistent or emergency irrigation). The obtained high biomass may also enable greater uptake of nitrates from the soil.

Table 3. Leaf biomass (on a fresh weight basis) and Dry Matter percentage (DM) of *Urtica dioica* L. in Tresigallo and Ozzano in relation over two years of cultivation (2021 and 2022) for three harvesting periods (cuts) from May, July and September. Data were obtained from cuts by the sum and mean, respectively. Value p ≤ 0.05, Tukey's least significant difference test. The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, DM % = Dry Matter. L = Location, Y = year of cultivation, LxY = interaction between the two variables Location and Year

	May		July		Sept.		Total	
	Yield Kg ha^{-1}FW	DM %	Yield Kg ha^{-1} FW	DM %	Yield Kg ha^{-1} FW	DM %	Yield Kg ha^{-1} FW	DM %
Tresigallo	1533.18	34.00	1610.97	37.5	1409.47	36.5	4563.62	36.00
Ozzano	2464.48	29.00	3510.03	33.0	10158.3	32.6	16132.81	31.53
2021	980.17	28.5	1360.63	35.5	6886.83	34.1	9227.63	32.70
2022	3017.49	34.5	3760.37	35.0	4680.97	35.0	11458.83	34.83
Mean	1998,83	31,5	2560,5	35,25	5783,9	34,55	10345.72	33.8
LSD 0.05	709.3	0.4	465.0	0.4	2458.2	0.2	2138.2	1.6
L	*	***	***	***	***	***	***	***
Y	***	***	***	ns	ns	***	ns	*
L x Y	ns	***	***	***	*	ns	ns	ns

Table 4. Growth Index, Surface and high (h) of *Urtica dioica* L. in Tresigallo and Ozzano in relation over two cultivation years 2021 and 2022 for the three harvesting periods (cuts) May, July and September and Mean value. $p \leq 0.05$, Tukey's least significant difference test. The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. ns = not significant.

L = Location, Y = year of cultivation, LxY = interaction between the two variables Location and Year

	May			July			Sept.			Mean		
	h (cm)	Surface (cm^2)	GI	h (cm)	Surface (cm^2)	GI	h (cm)	Surface (cm^2)	GI	h (cm)	Surface (cm^2)	GI
Tresigallo	36.95	1592.15	32.28	25.18	1121.39	29.86	27.72	1071.03	31.10	30.20	1277.93	31.10
Ozzano	37.12	1796.55	39.16	37.40	1727.12	38.69	27.94	2223.43	40.68	34.33	1904.25	39.46
2021	44.63	1787.32	41.48	24.94	1099.19	30.31	27.36	1678.40	36.19	31.24	1483.11	35.38
2022	28.77	1572.97	28.77	44.39	2093.64	42.57	28.82	1662.05	35.92	33.93	1767.54	35.17
Mean	37.0	1557.5	34.9	31.7	1442.95	34.5	27.8	1673.0	36.1	32.3	1592.5	35.3
LSD 0.05	4.8	404.5	4.6	4.7	300.8	3.0	4.7	444.1	4.4	4.2	235.3	3.0
L	ns	ns	**	***	***	***	ns	***	***	*	***	***
Y	***	ns	***	***	***	***	ns	ns	ns	ns	**	ns
L x Y	***	**	***	***	***	***	*	*	ns	***	***	***

Table 5. Total rainfall, mean temperature and growing degree days, comprising the growing seasons of Urtica dioica L. for 2021 (January-December period) and 2021 (January-December period) within the two locations (Tresigallo and Ozzano).

	Rainfall		Mean T		GDD	
Month (2021)	Tresigallo	Ozzano	Tresigallo	Ozzano	Tresigallo	Ozzano
January	102.2	103.6	3.6	3.7	-44.1	-39.7
February	47.5	48.7	8.0	8.1	84.3	85.8
March	15.8	14.1	8.6	9.7	112.5	144.4
April	100.7	99.5	11.4	11.9	192.7	206.7
May	48.4	50.8	17.2	17.8	379.3	395.5
June	21.4	21.4 + 60	24.2	24.9	576.2	595.5
July	36.4	37.4 + 60	25.8	26.7	645.3	671.7
August	23.2	22.6 + 60	24.0	25.6	589.0	639.3
September	95.9	96.2	20.5	21.5	464.1	495.4
October	66.0	68.4	13.2	14.1	228.3	243.9
November	113.8	113.2	10.0	9.7	125.2	121.4
December	55.8	56.8	4.1	4.1	-28.0	-28.0
Month (2022)						
January	53.8	48.4	3.4	3.3	-50.9	-48.5
February	31.9	34.4	6.5	7.3	41.1	46.2
March	49.5	50.8	7.9	8.4	92.3	98.1
April	97.5	87.8	12.5	12.8	225.8	231.2
May	61.9	68.7	20.6	20.3	466.6	459.8
June	21.9	21.5 + 60	25.2	25.4	604.5	609.3
July	25.5	26.3 + 60	26.9	27.8	679.2	701.9
August	102.3	106.8	25.4	25.9	509.0	519.0
September	64.3	62.2	19.7	20.7	412.7	433.6
October	10.3	10.2 + 60	17.6	18.2	391.0	404.3
November	107.3	103.6	11.6	10.4	145.9	130.8
December	109.5	112.8	6.7	6.6	46.2	45.5

3.2 Content of Bioactive compounds for Location and Harvest Period

Phenolic compounds and total flavonoids in the extracts of stinging nettle leaves were higher in July and May cut, respectively (**Table 6**). The present results corroborated previous findings showing higher levels of polyphenols (Biesiada et al., 2010, Grevsen et al., 2008; Koszegi et al., 2020; Paulauskiene˙ et al., 2021; Repajic et al., 2021). DPPH be higher between April – July harvest periods in *Urtica dioica* L. and are in accordance with previously reported data about Nettle cultivation in high Nitrogen condition as well (Radman et al. 2015).

Antioxidant activity of extracts and phenolics (polyphenols and flavonoids) were impacted form the location more than cultivation years, with better results obtained for Tresigallo location, probably related to the water stress induced in this location due to the absence of emergency irrigation.

The highest level of additional health promoting product (Ascorbate and Folate), were recorded in 2021 for September cut and 2022 for July cut, respectively. Moreover, Ascorbate was impacted by the location, and it was higher for Ozzano location, on the other hand no impact of location was observed for Folate. Variable ascorbate levels through different stinging nettle cuts have been reported (Nencu et al., 2013, Paulauskiene˙ et al., 2021, Radman et al., 2015), and additional water supply may be a contributory factor in determining increased ascorbate content, corroborating present results (Paulauskiene˙ et al. 2021) (**Table 7**). Although ascorbate levels were previously shown to be inversely related to N supplementation, it is evident that the Ascorbic lever is lower than the data reported (Radman et al., 2015).

As regards, insoluble and soluble dietary fibers, there were found to be higher in the second year of cultivation, with not statistical difference for location. Interestingly, harvesting in May and July, coinciding with the increased polyphenol content and antioxidant activity, also coincided with increased fiber content (**Table 7**). Increased number of harvests resulted in an increase of ascorbic acid content and the lowest average value of this vitamin was recorded in May harvest with 32.9 and 36.9 mg/100g fw, for Tresigallo and Ozzano respectively, while the highest in September (49 mg/100g fw). According to the conducted research cultivated nettle, besides minerals and phenols, is a valuable source of vitamin C. Comparatively, wild nettle contains smaller amounts of ascorbic acid, such as 13.0 mg/100g (Otles and Yalcin, 2013), 13.7 mg/100g fw (Stepanović et al., 2009; Radman et al., 2015) and 36.40 mg 100 g-1 fw (Skalozubova and Reshetova, 2013).

For each cut, the interaction between year and Location had been analyzed.

In May cut, TP, TF, Ascorbate and Folate (**Table 6**) in stinging nettle leaves were not significantly different from the lower yielding location of Tresigallo and the higher yielding location of Ozzano, respectively. Bound components of Flavonoids and Polyphenols (FB and PB) and DPPH were significantly lower in Ozzano. The year impacted Polyphenols (PF and PT), Flavonoids components, Ascorbate and TDF, which were significantly higher in 2021.

In contrast with these results, in the cut of July, Phenolic compounds, FRAP, DPPH and Folate were significantly higher in Tresigallo than in Ozzano. Instead, Ascorbate content was significantly higher in Ozzano compared with Tresigallo (**Table 6**). In July cut the second year of cultivation (2022) impacted positively on Phenolics (PF and PT), Flavonoids (FF and FT), DPPH, Ascorbate, Folate and TDF.

In September cut no notable significant differences were found, except for Ascorbate and Folate higher level in Ozzano than Tresigallo. In the first cultivation year Ascorbate and Folate were positively impacted, instead in 2022 PF, PT, FF, FT, FRAP and DPPH were significantly higher than in the first cultivation year (**Table 6**).

The highest TP and TF obtained in July cut was shown to be analogous to the levels extracted previously from both commercial (Biesiada et al., 2010; Radman et al., 2015; Shonte et al., 2020) and wild nettle (Ko˝szegi et al., 2020; Paulauskiene˙ et al., 2021 Repaji´c et al., 2021), respectively. For the extraction of high levels Ascorbate and Folate, is recommended in September and July, respectively (**Table 7**). Similarly, the highest levels of Ascorbate obtained in September were analogous to that obtained in both commercial (Radman et al., 2015; Shonte et al., 2020) and wild stinging nettle (Nencu et al., 2013; Paulauskiene˙ et al., 2021) respectively.

Table 6. Polyphenol and flavonoid content as well as antioxidant activity in the leaf material of *Urtica dioica* L. within each location (Tresigallo, Ozzano) for three consecutive harvest cuts and two years of cultivation (2021 and 2022).

Different letters within each column for each location represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, FP = free polyphenols, FB = bound polyphenols, TP = total polyphenols, FF = free flavonoids, BF = bound flavonoids, TF = total flavonoids, FRAP = ferric reducing antioxidant potential, DPPH = 1,1- diphenyl-2-picrylhydrazyl anti-radical activity, FW = fresh weight, DW = dry weight, GAE = gallic acid equivalent, CE = catechin equivalent and TE = Trolox equivalent, IDF = insoluble dietary fiber, SDF = soluble dietary fiber, TDF = total dietary fiber.

MAY		PF	PB	PT	FF	FB	FT	FRAP	DPPH	Ascorbate	Folate	IDF	SDF	TDF	Nitrate
		(mg GAE 100 g^{-1} FW)			(mg CE 100 g^{-1} FW)			(mmol Fe^{2+} 100 g^{-1} FW)	(mmol TE g^{-1} FW)	(mg 100 g^{-1} FW)	(mg 100 g^{-1} DW)	%	%	%	(mg kg^{-1} DW)
Location	Tresigallo	395.75	95.48	491.23	268.48	22.10	290.57	17.30	82.11	32.95	24.06	55.53	17.5	73.03	1020.25
	Ozzano	554.16	36.80	470.57	348.70	3.22	292.33	26.6	26.85	36.92	26.54	52.28	14.4	66.68	748.70
Year	2021	710.93	50.11	640.64	430.88	7.01	378.28	10.86	50.49	41.08	24.09	59.26	16.2	75.46	943.40
	2022	212.86	79.96	292.82	172.60	17.70	190.30	35.70	54.53	27.42	26.93	48.55	15.8	64.35	825.55
	Mean	481.04	63.9	480.1	311.7	11.9	291.5	291.5	52.3	34.8	25.4	53.9	15.9	69.9	884.5
	Field	***	***	ns	**	***	ns	***	***	ns	ns	ns	*	*	***
p	Year	***	***	***	***	***	***	***	ns	***	ns	**	*	**	***
	C x Y	***	*	***	***	***	***	***	***	***	***	*	ns	*	***
JULY		**PF**	**PB**	**PT**	**FF**	**FB**	**FT**	**FRAP**	**DPPH**	**Ascorbate**	**Folate**	**IDF**	**SDF**	**TDF**	**Nitrate**
		(mg GAE 100 g^{-1} FW)			(mg CE 100 g^{-1} FW)			(mmol Fe^{2+} 100 g^{-1} FW)	(mmol TE g^{-1} FW)	(mg 100 g^{-1} FW)	(mg 100 g^{-1} DW)	%	%	%	(mg kg^{-1} DW)
Location	Tresigallo	660.42	81.77	742.19	701.39	49.99	751.38	10.88	67.63	25.15	49.56	55.88	18.10	73.9	547.95
	Ozzano	510.11	65.32	530.80	436.05	23.19	418.43	9.80	59.98	39.08	34.07	54.25	16.70	70.9	468.5
Year	2021	496.60	100.50	488.39	541.50	35.27	529.17	9.77	55.76	32.82	23.21	52.16	15.55	67.7	366.40
	2022	648.60	43.86	749.37	551.71	33.44	585.15	10.73	70.58	57.84	57.84	57.97	19.26	77.23	650.00
	Mean	572.7	72.2	618.9	546.6	34.4	557.2	10.3	6.3	34.8	25.4	55.07	17.4	72.4	508.2
	Field	**	*	**	***	***	***	ns	ns	***	***	ns	ns	ns	ns
p	Year	*	***	***	ns	*	ns	ns	*	***	***	ns	*	*	**
	C x Y	ns	***	ns	***	***	***	ns	ns	*	**	ns	ns	ns	ns
SEPT.		**PF**	**PB**	**PT**	**FF**	**FB**	**FT**	**FRAP**	**DPPH**	**Ascorbate**	**Folate**	**IDF**	**SDF**	**TDF**	**Nitrate**
		(mg GAE 100 g^{-1} FW)			(mg CE 100 g^{-1} FW)			(mmol Fe^{2+} 100 g^{-1} FW)	(mmol TE g^{-1} FW)	(mg 100 g^{-1} FW)	(mg 100 g^{-1} DW)	%	%	%	(mg kg^{-1} DW)
Location	Tresigallo	485.87	30.61	516.47	426.07	11.67	437.74	8.58	41.43	27.50	20.84	52.66	15.59	68.25	1865.2
	Ozzano	435.66	24.51	462.17	450.21	10.83	461.04	8.42	40.19	37.29	37.93	57.65	15.98	73.63	965.4
Year	2021	354.25	17.63	371.88	356.26	13.03	369.29	7.49	31.14	44.10	36.80	52.64	12.74	65.38	1507.3
	2022	531.78	24.18	555.95	492.73	10.07	502.79	9.17	47.26	24.54	24.44	57.67	18.83	76.50	1323.3
	Mean	460.8	21.6	482.3	438.1	11.3	449.4	8.5	40.8	32.4	40.8	55.1	15.7	70.8	1415.2
	Field	ns	ns	ns	ns	ns	ns	ns	ns	***	***	*	ns	*	***
p	Year	**	ns	***	***	ns	***	***	***	***	***	*	*	*	ns
	C x Y	ns	ns	ns	**	ns	**	***	ns	**	***	*	ns	ns	**
MEAN		**PF**	**PB**	**PT**	**FF**	**FB**	**FT**	**FRAP**	**DPPH**	**Ascorbate**	**Folate**	**IDF**	**SDF**	**TDF**	**Nitrate**
		(mg GAE 100 g^{-1} FW)			(mg CE 100 g^{-1} FW)			(mmol Fe^{2+} 100 g^{-1} FW)	(mmol TE g^{-1} FW)	(mg 100 g^{-1} FW)	(mg 100 g^{-1} DW)	%	%	%	(mg kg^{-1} DW)
Location	Tresigallo	506.62	70.92	577.54	453.01	27.56	480.57	156.75	64.87	28.20	31.02	54.68	17.06	71.74	1144.47
	Ozzano	506.75	40.91	486.87	407.59	12.58	383.18	126.38	42.57	37.55	32.31	54.72	15.71	70.43	727.53
Year	2021	551.36	40.26	523.67	452.37	18.39	429.42	96.83	47.80	38.88	26.77	54.68	14.80	69.48	938.98
	2022	464.51	68.26	532.71	405.68	20.41	426.08	186.35	57.45	22.93	36.40	54.71	17.96	72.67	932.95
	Mean	506.7	54.6	528.3	428.4	19.4	427.7	14.3	52.8	30.7	31.7	54.7	16.4	71.1	935.98
	Field	ns	***	*	ns	***	*	ns	***	**	ns	ns	ns	ns	***
p	Year	*	***	ns	ns	ns	ns	***	ns	***	**	ns	*	*	ns
	C x Y	ns	**	**	***	ns	***	*	ns	**	**	ns	ns	ns	**

Table 7. Polyphenol and flavonoid content as well as antioxidant activity, ascorbic and folic acids, total dietary fiber and Nitrate in the leaf material of *Urtica dioica* L. within each location (Tresigallo, Ozzano) for three consecutive harvest cuts and two years of cultivation (2021 and 2022). Different letters within each column for each location represent significant different values (p ≤ 0.05, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, FW = fresh weight, DW = dry weight, GAE = gallic acid equivalent, CE = catechin equivalent and TE = Trolox equivalent, TDF = total dietary fiber.

		Polyphenols		Flavonoids		DPPH		FRAP		NITRATE		Ascorbic Acid		Folic Acid		TDF	
		mgGAE/100g FW		mgCE/100g FW		µmolTE/g FW		mmolFe^{2+} 100g^{-1} FW		mg/kg DW		mg/100g FW		mg/100g FW		%	
Trials	Tresigallo	577.5	a	480.6	a	27.4	a	48.46	a	1152.8	a	33.2	a	35.7	a	70.0	a
	Ozzano	486.9	b	383.2	b	22.9	b	41.98	b	772.5	b	10.4	b	32.3	a	71.2	a
	p	***		***		**		**		***		***		ns		ns	
Year	2021	523.7	a	429.4	a	4.8	b	9.68	b	942.4	a	18.6	b	26.8	b	66.5	b
	2022	532.7	a	426.1	a	44.1	a	78.24	a	982.9	a	22.9	a	40.5	a	71.6	a
	p	ns		ns		***		***		ns		*		***		**	
Cut	May	500.5	b	322,5	c	9.2	c	21.08	c	884.5	b	19.4	b	23.8	c	71.7	a
	July	615.9	a	563,8	a	44.1	a	67.63	a	575.7	c	18.2	b	46.0	a	72.7	a
	Sept.	482.3	b	449,4	b	29.6	b	58.04	b	1427.8	a	25.7	a	36.8	b	67.8	b
	p	***		***		***		***		***		***		***		**	

3.3 Nitrate content

Nitrogen strongly stimulates growth and it's the most important element in nutrition (Rutto et al., 2012), its excessive absorption may result in accumulation of potentially harmful nitrates in plants. Nitrates are part of the nitrogen cycle and have an important role in the nutrition and function of plants (Shimada et al., 2004; EFSA, 2008). However, very high concentrations can increase the occurrence of methaemoglobinemia and possibly gastric cancer (Cárdenas-Navarro et al. 1999). According to Santamaria (2006) nitrate accumulation in plants is affected by the following factors: genetic, environmental (atmospheric humidity, substrate water content, temperature, irradiance, photoperiod) and agricultural (nitrogen doses and chemical forms, availability of other nutrients, use of herbicides).

Nitrate content in the stinging nettle leaves varied from a maximum of 1865 mg/kg of dry weight (dw) to a minimum of 366.40 mg/kg. Namely, leafy vegetables have relatively high levels of nitrate, and Rocket is allowed to contain 4800 mg kg-1 fw, lettuce 2130 mg kg-1 fw and spinach 785 mg kg-1 fw (EFSA, 2008) (**Table 6**, **Table 7**).

Hence, nitrate content was not a deterring factor in evaluating the success of stinging nettle for the harvest of medicinal products. The low concentration of Nitrate may be due to the multiple harvest cuts made in the cultivation period. For each cut the Nitrate content was higher for Tresigallo location and second cultivation year (**Table 6**). The amount of Nitrate may be strictly related to soil characteristics and water supply, since in Tresigallo no irrigation had been supplied. July was shown to be the cut with lowest accumulation of N in aerial part of Nettle plants. This result combined with the high polyphenol content obtained in July further enhances the use of nettle for pharmaceutical purposes without health risks, while cultivating the species in NVZs.

3.4 Element content in aerial parts and roots

Element content in aerial parts (**Table 8**) and roots (**Table 9**) had been analyzed for each location, harvest cut and year.

In May, significant effects on element content for both examinate location and year except for Ag, As, B, Ca, Cu and Mo were observed. Focusing on health beneficial and toxic elements, Tresigallo location showed higher concentration of Fe, Pb, Cr compared to Ozzano. 2022 cut showed statistically higher concentration of some elements as Ba, Be, Cr, Cu, Fe, Li, Mg, Na, Ni, Pb, V and Zn . In July cut, significant effects on mineral content for both examinate location and year except for As, B, Be, Fe, Li and P, were observed. Focusing on health beneficial and toxic elements, Tresigallo location showed significantly higher concentration of P, Ni, Pb compared to Ozzano. Moreover, focusing on these elements, higher concentration of Ni, Pb and K minerals in second year July cut was observed. In September cut, significant effects on mineral content for both examinate location and year except for Ca, Na, Ni, S, and Sr were observed. Focusing on health beneficial and toxic elements, Tresigallo location showed higher concentration of Mg, P, Se, and Cu and Cr, respectively, compared to Ozzano.

Furthermore, concerning September cut higher concentration of Cd, Se and Fe in first year and Cr, Cu, K and Mg in second year were observed. Consequently, the effect of Location on total element content is not only connected to soil characteristics since the trend of elements concentration in aerial parts was changing within the cuts.

Further studies reported the influence of N fertilization (soil or with foliar application) on elements content in leaves. The different proportions of N influenced significantly the leaf contents of N, Ca, Mg, S, Mn, Zn, B, and Cu.

Higher availability of N-NO_3^- in the soil might result in increased absorption of K, Mg, and Ca by plants, supposedly by ionic competition (Ueda et al. 2017), and it may be related to species adaptation and preference (Ying et al. 2017, Barrow et al. 2023, Li et al. 2013).

The iron content in dry weight Nettle varied from 867 ppm to 3094 ppm in aerial parts, in the present study. As previously reported (Radman et al. 2015) high Nitrogen concentration in soil had an adverse effect on iron content. The average value of iron amounted 1892 ppm Fe dw, which was a much higher value than data reported (Başgel and Erdemoğlu 2006 and Rutto et al. 2013). In addition, according to Bergmann (2006), potassium uptake by plants depends largely on diffusion and mass flux, meaning that its availability is severely reduced in dry periods. Plants may consequently exhibit signs of potassium deficiency after long dry periods even on soils with a good potassium status. In Tresigallo only 78 mm (mean value) of precipitation occurred in summer period each year, meanwhile in Ozzano emergency irrigation was applied.

Roots and aerial parts samples had been examinate and the impact of location on the translocation (part of plant), and the interaction of these factors was evaluated (**Table 9**).

Some minerals as B, Ca, Cl, K, Mo, P, S, Sr (63,6 mg kg-1, 57,3 mg kg-1, 14,8 g kg-1, 26,9 g kg-1, 2,4 mg kg-1, 2,2 g kg-1 , 3,1 g kg-1 and 30 mg kg-1, respectively) were significantly higher in aerial parts of plants (in September 2022) than in roots. No significative difference in distribution (leaves or roots) was observed for Ag, Mg, Sb and Sn (not reported data).

Mn, Ni, Pb, Se, Cr, Fe (23.2 mg kg-1, 31,7 mg kg-1, 8,7 mg kg-1, 2,7 mg kg-1, 62,7 mg kg-1, and 7,8 g kg-1, respectively) values were more than twice higher in roots than in aerial parts.

Results showed a significative impact of the Location on translocation only concerning few elements as Mo, Na, P, Si, Sn, B, Ba and Cl; in details B, Cl, Si and Sn were significantly higher in Ozzano than Tresigallo location. The interaction between factors (Location x Portion in **Table 9)** was highly significative (***) only for Cu. As highlighted by the present results, for most of the elements investigated, there is no evidence of an effect of environment on element distribution between aerial part and roots (**Table 9**). This suggests that the element distribution within the different plant organs is influenced more by genotype than by pedo-climatic conditions investigated.

Table 8 Total mineral content in aerial parts, per harvest cut. May described in Table A, July in Table B and September in Table C, respectively. In each cut the impact of year and location was evaluated, with a 2Way anova. Significant different values ($p \leq 0.05$, Tukey's least significant difference test) indicated with the number of stars representing significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant.

A	Locations (L)			Years (Y)					
MAY	Tresigallo	Ozzano	*p* (L)	2021	2022	*p* (Y)	Mean	LSD 0.05	*p* (L x Y)
Ag µg kg-1	67.8	69.4	ns	66.4	70.8	ns	68.6	3.3	ns
Al g kg-1	2.6	2.3	***	2.0	2.9	***	2.4	0.3	ns
As mg kg-1	1.1	1.2	ns	1.0	1.3	ns	1.2	0.1	ns
B mg kg-1	61.4	50.4	ns	59.1	52.8	ns	55,9	4.6	ns
Ba mg kg-1	45.3	41.0	***	40.1	46.2	**	43.1	2.6	***
Be µg kg-1	112.7	103.4	***	91.3	124.8	***	108	12.3	***
Ca g kg-1	51.0	54,5	ns	56,6	48,9	ns	52,8	5.5	ns
Cd µg kg-1	316.2	279.1	**	308.8	286.5	*	297.6	15.3	*
Cl g kg-1	14.7	14.1	ns	14.0	14.8	ns	14.4	0.3	ns
Co mg kg-1	1.5	1.5	ns	1.2	1.8	ns	1.5	0.2	ns
Cr mg kg-1	29.3	14.7	***	17.2	26.8	***	22.0	6189.2	***
Cu mg kg-1	37.8	62.0	**	34.0	65.7	**	49.9	14.1	**
Fe g kg-1	3.1	2.4	***	2.4	3.1	***	2.7	0.3	***
K g kg-1	22.4	23.2	ns	23.5	22.1	ns	22.7	1.1	ns
Li mg kg-1	5.7	5.6	ns	4.7	6.7	***	5.7	2.2	ns
Mg g kg-1	3.1	4.1	***	3.1	4.1	***	3.6	0.05	***
Mn µg kg-1	222.1	148.0	**	206.7	164.0	*	185.4	7.3	*
Mo mg kg-1	849.3	1365.5	**	819.9	1394.9	**	1107.4	79.0	**
Na g kg-1	232.4	250.8	**	151.9	331.3	***	241.6	5.5	***
Ni mg kg-1	9.0	9.9	**	6.2	12.7	***	9.4	0.6	***
P g kg-1	1.2	2.1	**	1.5	1.9	*	1.7	0.3	*
Pb mg kg-1	3.5	3.0	ns	2.9	3.6	***	3.2	0.3	ns
S g kg-1	2.5	2.4	ns	2.3	2.6	ns	2.4	0.1	ns
Se mg kg-1	1.4	1.6	ns	1.5	1.5	ns	1.5	0.07	ns
Si mg kg-1	44.9	34.2	**	47.4	31.7	**	39.6	0.7	**
Sn µg kg-1	1118.0	847.9	**	976.9	989.0	ns	983.0	95.6	ns
Sr mg kg-1	158.5	223.7	***	182.2	199.9	*	191.1	23.9	ns
Ti mg kg-1	15.8	14.7	ns	11.9	18.6	*	15.2	2.4	ns
V mg kg-1	5.2	4.9	*	4.1	6.0	**	5.0	0.7	ns
Zn mg kg-1	43.7	44.4	ns	29.6	58.6	**	44.1	1.0	*

B JULY	Location (L)			Years (Y)					
	Tresigallo	Ozzano	*p* (L)	2021	2022	*p* (Y)	Mean	LSD 0.05	*p* (L x Y)
Ag µg kg-1	70.2	73.7	**	80.1	63.8	***	73.9	6	***
Al g kg-1	1.0	1.1	*	1.1	1.0	*	1.1	0.05	*
As mg kg-1	587.5	912.3	***	706.8	793.0	ns	753.1	118.8	ns
B mg kg-1	65.5	79.6	ns	83.1	62.0	**	74.6	9.0	ns
Ba mg kg-1	37.6	37.9	ns	37.9	37.5	ns	37.8	0.2	ns
Be µg kg-1	44.9	49.3	ns	47.1	47.2	ns	49.1	1.8	ns
Ca g kg-1	60.0	46.1	**	49.3	56.8	*	56.5	5.8	ns
Cd µg kg-1	141.7	243.7	***	174.1	211.3	**	212.7	39.4	*
Cl g kg-1	10.2	18.1	***	11.0	1.7	***	14.4	3.6	**
Co mg kg-1	461.8	684.7	***	562.7	583.8	ns	595.5	79.8	**
Cr mg kg-1	11.2	8.2	***	8.6	10.8	***	9.7	1.3	***
Cu mg kg-1	40.7	11.6	***	37.2	15.1	***	26.4	12.9	***
Fe g kg-1	0.9	1.2	***	1.0	1.0	ns	1.0	0.1	**
K g kg-1	23.3	25.1	*	22.3	26.1	*	24.3	1.5	ns
Li mg kg-1	2.0	2.8	***	2.4	2.4	ns	2.4	0.3	ns
Mg g kg-1	3.6	4.3	***	4.6	3.3	***	4.0	529	***
Mn µg kg-1	106.8	60.2	***	51.9	115.1	***	83.7	27.8	***
Mo mg kg-1	1.6	3.0	***	2.9	1.7	***	2.3	0.7	***
Na g kg-1	142.8	132.9	***	148.8	126.9	***	143.3	8.9	***
Ni mg kg-1	4.1	6.5	***	5.6	5.0	**	5.51	0.9	***
P g kg-1	1.9	1.87	ns	2.0	1.8	*	2.1	0.1	ns
Pb mg kg-1	1.4	1.7	***	1.5	1.6	**	1.6	0.1	*
S g kg-1	3.7	3.3	**	3.9	3.0	**	3.9	0.4	*
Se mg kg-1	1.3	1.5	ns	1.4	1.4	ns	1.4	0.08	ns
Si mg kg-1	45.1	33.7	***	42.1	36.7	**	39.7	4.5	***
Sn µg kg-1	1000.3	933.4	**	929.2	1004.5	**	1002.7	39.1	**
Sr mg kg-1	266.8	264.7	ns	275.1	256.4	ns	268.8	6.8	ns
Ti mg kg-1	8.2	10.3	***	10.9	7.6	***	9.2	1.4	***
V mg kg-1	1.7	2.2	***	2.1	1.8	**	2.4	0.3	*
Zn mg kg-1	24.2	21.0	***	22.3	22.9	ns	23.0	1.2	*

C SEPT	Location (L)			Years (Y)					
	Tresigallo	Ozzano	*p* (L)	2021	2022	*p* (Y)	Mean	LSD 0.05	*p* (L x Y)
Ag µg kg-1	77.9	53.3	***	61.3	86.5	*	68.4	13.1	**
Al g kg-1	1.7	2.2	***	2.2	1.7	***	1.9	0.2	***
As mg kg-1	785.9	1073.1	***	1019.2	839.7	***	932.7	119.8	***
B mg kg-1	64.2	78.2	***	78.8	63.6	***	73.2	7.4	***
Ba mg kg-1	45.1	42.4	*	41.5	46.0	*	43.8	1.8	*
Be µg kg-1	67.3	92.2	***	93.8	65.7	***	81.7	13.3	***
Ca g kg-1	56.7	54.3	ns	53.7	57.3	ns	59	2.2	ns
Cd µg kg-1	171.7	258.5	***	264.1	166.1	***	235.1	47.2	***
Cl g kg-1	16.2	17.3	*	18.7	14.8	***	17.0	1.5	*
Co mg kg-1	889.5	1252.6	***	1270.4	871.6	***	1093.3	190.9	***
Cr mg kg-1	15.3	13.4	ns	13.9	14.7	ns	14.4	0.7	ns
Cu mg kg-1	14.8	12.5	ns	12.1	15.2	ns	13.9	1.4	ns
Fe g kg-1	1.5	2.1	*	2.1	1.4	*	1.8	0.3	ns
K g kg-1	27.1	27.3	ns	27.4	26.9	*	27.3	0.2	ns
Li mg kg-1	4.1	5.9	***	6.0	4.1	***	5.2	0.9	***
Mg g kg-1	4.8	3.8	***	3.8	4.7	***	4.3	0.5	***
Mn µg kg-1	89.6	112.3	***	112.8	89.1	***	101.2	11.6	***
Mo mg kg-1	2.4	1.7	***	1.6	2.4	***	2.0	0.4	***
Na g kg-1	317.3	342.9	ns	342.3	317.9	ns	335.6	12.7	ns
Ni mg kg-1	6.2	6.2	ns	6.3	6.2	ns	6.2	0.1	ns
P g kg-1	2.1	1.6	**	1.6	2.2	**	2.1	0.3	ns
Pb mg kg-1	2.0	2.8	***	2.8	2.0	***	2.4	0.4	***
S g kg-1	3.1	3.2	ns	3.2	3.1	ns	3.6	0.2	ns
Se mg kg-1	1.4	1.38	ns	1.48	1.33	ns	1.46	0.06	ns
Si mg kg-1	28.0	30.6	***	31.8	26.8	***	29.6	2.0	***
Sn µg kg-1	992.6	1045.2	***	1052.2	985.7	***	1054.8	34	***
Sr mg kg-1	298.7	258.4	ns	254.9	302.2	*	281.6	22	ns
Ti mg kg-1	16.5	12.9	***	12.99	16.4	***	14.7	1.8	***
V mg kg-1	3.37	4.64	***	4.7	3.3	***	4.4	0.7	***
Zn mg kg-1	21.8	25.3	***	24.2	22.9	***	23.97	1.3	***

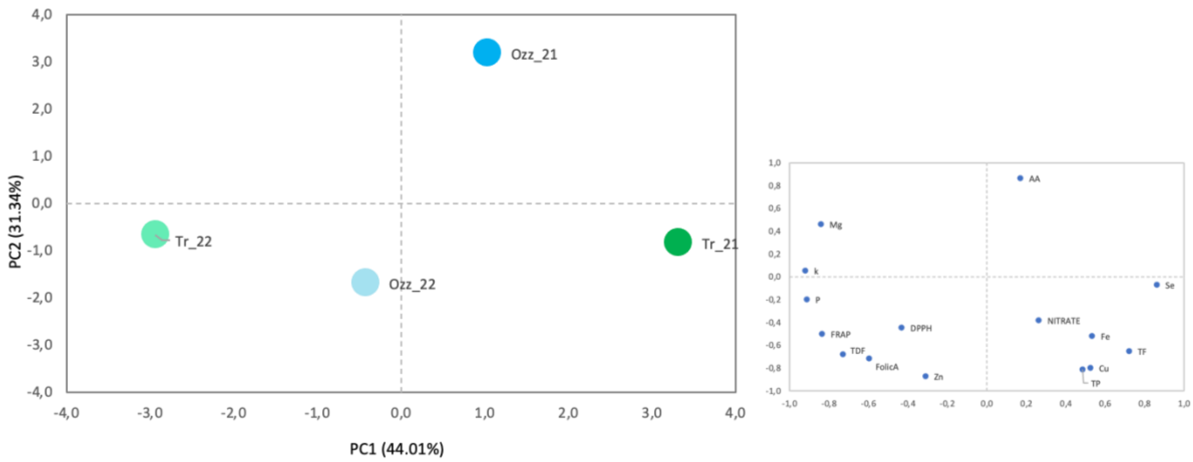
Table 9. Total mineral content in aerial parts at final cut (September 2022) and roots (collected at the end of crop cycle). Significant different values (p ≤ 0.05, Tukey's least significant difference test) indicated with the number of stars representing significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant.

	Location (L)			Portion (P)					
	Ozzano	Tresigallo	*p* (L)	Root	Aer. Pt	*p* (P)	Mean	LSD 0.05	L x P
Al g kg-1	5.5	4.8	ns	8.6	1.7	**	5.1	4.3	*
As µg kg-1	1807.1	1234.8	ns	2202.2	839.7	***	1520.9	889.5	ns
B mg kg-1	58.0	37.5	*	31.9	63.6	**	16.0	18.4	*
Ba mg kg-1	54.9	72.8	**	81.6	46.0	***	55.1	18.4	ns
Be µg kg-1	231.4	189.1	ns	354.8	65.7	***	210.2	182.2	*
Ca g kg-1	39.0	39.2	ns	20.9	57.3	***	39.1	12.1	ns
Cd µg kg-1	504.3	429.4	ns	767.6	166.1	**	466.8	366.6	ns
Cl g kg-1	14.3	8.3	*	7.8	14.8	**	11.3	6.8	**
Co µg kg-1	3182.9	2598.2	ns	4909.5	871.6	***	2890.6	2555.2	*
Cr mg kg-1	35.6	41.8	ns	62.7	14.7	***	38.7	29.2	ns
Cu mg kg-1	19.4	18.8	ns	23.0	15.2	*	19.1	8.1	***
Fe g kg-1	5.1	4.2	ns	7.8	1.4	***	4.6	2.6	*
K g kg-1	17.0	15.6	ns	5.7	26.9	***	16.3	12.3	ns
Li mg kg-1	11.7	9.0	ns	16.6	4.1	***	10.3	8.0	*
Mg g kg-1	4.8	5.4	ns	5.5	4.7	ns	5.1	1.4	ns
Mn mg kg-1	169.6	151.9	ns	232.5	89.1	**	160.8	98.6	*
Mo mg kg-1	1.3	1.9	*	0.8	2.4	***	1633.4	1020.4	ns
Na mg kg-1	333.8	220.5	**	236.4	317.9	*	277.1	95	*
Ni mg kg-1	20.7	17.2	ns	31.7	6.2	***	18.9	10.5	*
P g kg-1	1.6	2.0	**	1.5	2.2	***	1.8	0.5	ns
Pb mg kg-1	5.9	4.8	ns	8.7	2.0	***	5.4	2.8	ns
S g kg-1	2.3	1.8	ns	1.0	3.1	***	2.1	1.0	ns
Se mg kg-1	2.2	1.8	ns	2.7	1.3	***	1.9	0.8	ns
Sn µg kg-1	935	1108.5	***	1057.9	985.7	ns	1021.8	76.5	ns
Sr mg kg-1	200.3	229.2	ns	127.3	302.2	***	214.7	104.5	ns
Ti mg kg-1	22.0	35.4	*	40.9	16.4	**	28.7	11.4	*
V mg kg-1	10.3	9.5	ns	16.5	3.2	***	9.9	8.2	ns
Zn mg kg-1	27.4	24.2	ns	28.8	22.9	*	25.8	5.6	**

3.5 PCA

To examine a possible grouping of the nettle leaf samples analyzed components and location for each year, PCA was carried out (**Figure 3**). According to the preliminary PCA, a communality value of ≥ 0.5 described all variables, thus they were all included in the test. The first two components (PC1 and PC2) explained the 75.35% of the variance (**Figure 3**).

A distinctive separation for location and year was evident. The quadrant with positive branch of PC1 and negative branch of PC2 was associated with Nitrate; TP, TF, and minerals as Se, Fe and Cu, distinctive for Tresigallo in 2021 cut, inversely related to K and Mg content. 2022 cuts for both locations Tresigallo and Ozzano were placed on the negative branch PC1 and negative branch of PC2 and associated with P, Zn, antioxidant activity (FRAP and DPPH), TDF, Folic Acid and negatively associated with Ascorbic Acid. The quadrant with a positive PCA 1, and positive PCA 2 was distinctive for Ozzano 2021 cut and primarily associated with Ascorbic Acid content. Based on yield, health promoting components, and nitrate the second cultivation year may be a good alternative/choice for Nettle harvest to obtain high antioxidant activity, Folic Acid content, fiber, Zn and P with a lower Nitrate content. Tresigallo location shown higher phenolics may be due to water stress in 2021 and due to the irrigation deficiency lower Mg and K content. On the other hand, Ozzano location showed high yield and Ascorbic Acid content thanks to supply emergency irrigation in summer months. Evidently, meteorological factors and irrigation impacted on determining both the expression of bioactive components as well as the yield in each of the locations. To determine whether and to what extent temperature may have impacted on the expression of bioactive components, the mean temperature over each growing cycle was calculated from the data presented in **Table 3**. for each location. GDD, calculated from temperature, is a useful parameter to predict plant development rate (ultimately affecting yield). GGD was then correlated to the respective bioactive components. Inevitably, a significant inverse relationship between GDD and the expression of FP (hence TP), FRAP and IDF (hence TDF) was also evident (Results not showed).



A B

Figure 3. A - B. Principal component analysis based on *Urtica dioica* L. leaf bioactive contents. The scatter plots report the projection of cases (stinging nettle samples) and variables (bioactive compounds) on the first two principal components (PC1 and PC2). For location: (TR_21 = Tresigallo 2021, (TR_22) = Tresigallo 2022, (Ozz_21) = Ozzano 2021 and (Ozz_22) = Ozzano 2022 (**Figure 3A**) TP = total polyphenols, TF = total flavonoids, FRAP = ferric reducing antioxidant potential, DPPH = 1,1-diphenyl-2-picrylhydrazyl anti-radical activity, AA = ascorbate, FolicA = folic acid, TDF = total dietary fiber (**Figure 3B**).

4 CONCLUSIONS

The potential for the cultivation of stinging nettle under sustainable organic farming practices (low-input and herbicide/pesticide free) in Nitrate Vulnerable Zones in Emilia Romagna, Italy, for harvesting leaf bioactive components and decrease the Nitrate impact in soil and ground water was evaluated. The present study showed that stinging nettle cultivation was feasible. Satisfactory yields in combination with high levels of polyphenols and flavonoids, as well as DDPH and FRAP, and Folic Acid were obtainable in July. The highest Nitrate level was obtained in September, even if it was lower than the threshold fixed by EFSA for leafy vegetables. Moreover, high levels of ascorbate, in combination with high yields, were obtainable in September. Only Ozzano location met the yield criteria for the harvest of polyphenols (flavonoids), with associated antioxidant activities in May and July. In so doing, the requisite for agronomic practices that collectively include effective weed removal, a more uniform supply of water (consistent or emergency irrigation) and the necessity of high soil nitrate was clearly demonstrated.

The influence of the cut on elements content was not evident, otherwise it was more influenced by the location. As a depauperative crop total mineral content in soil was higher previous *U. dioica* L. cultivation for all elememts and for both the examined locations. As previously researches demonstrated, Stinging Nettle is very effective in accumulating nutrients and mineral from soil, leading to a reduction of these elements. Therefore, due to its nutrient storage capacity and polyannual habit, it may be identified as a species with soil-depleting characteristics. Assuming this, Nettle cultivation might be included in a rotation crop system with a renewal culture as improver as legumes or alfalfa to restore soil condition.

Thus, the cultivation in these marginal areas does not affect the potential application of the plant for nutraceutical and cosmetic purposes and no additional fertilization are needed or recommended to achieve a desirable quality and chemical composition.

Moreover, multiple harvest cuts through the growing season allowed to maintain Nitrate levels in above ground organs lower than legal fixed thresholds (EFSA, 2008). Through this management, peculiar to MAPs and polyannual species, it is possible to obtain a marketable product with high content in bioactive compounds in NVZs.

In conclusion, NVZs cultivated nettle is a valuable source of bioactive compounds for pharmaceutical, agro-food and cosmetic industries, thanks to it is richness in minerals (iron, selenium, potassium), Vitamin C, Folate, phenols and high antioxidant capacity.

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Chapter II - Phytoremediation potential of Medicinal and Aromatic Plants *Hypericum perforatum* L. and *Melissa officinalis* L. for nickel-contaminated soils

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ABSTRACT

Heavy metal contamination of soil and water due to industrial, agricultural, and urban activities is a growing global concern. Traditional chemical and physical methods of soil decontamination are time-consuming and expensive, and the available procedures and technologies are insufficient for the demand. This led to an increasing interest in phytoremediation as an efficient, eco-friendly solution. This study investigates the feasibility of cultivating two non-edible medicinal and aromatic plants (MAPs), *Hypericum perforatum* L. and *Melissa officinalis* L., in nickel-contaminated soils to evaluate their physiological and metabolic responses, together with their metal accumulation capacity. Plants were grown in controlled environment and subjected to two different nickel concentration (300 and 100 ppm) for two consecutive years. Both species showed high tolerance to elevated nickel concentrations (up to 300 mg/kg), maintaining stable biomass and essential oil yields and producing high concentration of bioactive compounds (e.g., rosmarinic acid and hypericin) even under moderate to high contamination levels. *Hypericum* showed an increase in essential oil production following nickel exposure, while *Melissa* remained stable. Morevoer, plants of the two species exhibited distinct nickel tolerance mechanisms, including increased proline content, selective chlorophyll content, and organic acid adjustments to mitigate stress. Notably, both MAPs showed good

BAF and limited nickel translocation from roots to aerial parts, resulting in low translocation factors (TF), which reduce the risk of nickel toxicity in the edible part of harvested plant, thus enabling safe plant use in phytoremediation. Thus, *H. perforatum* L. and *M. officinalis* L. offer promising potential for nickel-contaminated soil remediation, contributing to soil restoration, and providing economic benefits through biomass, essential oil and bioactive compounds production.

1 INTRODUCTION

The contamination of agricultural lands and irrigation water with toxic pollutants such as inorganic contaminants pose an environmental risk to humans and animals. Trace metal elements (TMEs) including nickel, zinc, cadmium, chromium, copper and cobalt, may accumulate naturally in soil or may originate from industrial, mining processes and bad agricultural practices. Nickel is one of the more mobile and bioavailable TME present in both industrially contaminated and pristine soils. The natural presence of Ni of lithogenic origin in soils had been demonstrated. As reported typically soils evolving on ophiolites and serpentinites are characterized by very high Nickel content. (D'Amico, 2009; Amorosi et al. 2022). Ni derived from sandstones, limestone or acid igneous rocks is generally less than 50 mg of Ni/kg of soil (Bonari et al. 2019).

Nickel is a transition element extensively distributed in the environment, air, water, and soil. It may derive from natural sources and anthropogenic activity. Although nickel is ubiquitous in the environment. Environmental pollution from nickel may be due to industry, the use of liquid and solid fuels, as well as municipal and industrial waste (Genchi et al. 2020)

Nickel is an essential element necessary for the growth and development of the plants and affect a wide range of morphological and physiological functions, such as germination seeds germination and plant yield. However, similarly to other essential TME as Zinc, excessive Nickel concentration alters the metabolic activities of the plants inhibiting enzymatic activity, photosynthetic electron transport, chlorophyll biosynthesis (Sreekanth et al. 2013) respiration, and increasing lipid peroxidation and proline content (Singh et al., 2012).

Concerning human health, as an immunotoxic and carcinogen agent, Ni may cause a variety of diseases, such as contact dermatitis, cardiovascular disease, asthma, lung fibrosis, and respiratory tract cancer, depending on the dose and length of exposure (Chen et al. 2017).

MAPs may be cultivated as alternative crops in TME polluted soils without contamination of the final marketable produce. Furthermore, medicinal crops may offer a phytoremediation option for mildly heavy metal polluted agricultural soils.

Lemon balm (*Melissa officinalis* L., Lamiaceae) and St. Johan worth (*Hypericum perforatum* L.) are important medicinal plants studied for their beneficial effects for human health (Brunakova et al. 2021; Boneza and Niemeyer, 2018) and essential oil production.

Lemon balm and Hypericum studies were mostly related to antioxidants activity and phenolic compounds evaluation under abiotic stresses (heavy metals, salinity, and drought) or elicitors application. Both crops demonstrated an efficient Nickel accumulation, and they may be use for phytoremediation purposes (Kováčik et al., 2022; Murch et al., 2003; Pavlova et al., 2015; Soltani Maivan et al., 2017; Rahimi et al. 2019; Torun 2019).

However, phytoremediation capacity and the impact of nickel stresses on physiological parameters as antioxidant defense system and Specialized Metabolites (SMs) accumulation in *M. officinalis* and *H. perforatum* was not still studied in detail.

The current study was aimed to evaluate the potential impact of Ni contaminated soil on the adaptability, growth, and specialized metabolites, phenylpropanoids in particular accumulation of two selected medicinal plants *Hypericum perforatum* L. and *Melissa officinalis* L.. Ni translocation, plants metal uptake capacity and effect on soil have been investigated. For this purpose, plants were grown in controlled environment since numerous environmental variables may be eliminated/avoided.

Plants aerial parts had been quantified for phenolic content, antioxidant activity and stress parameters (Proline content, Chlorophyll), and yield in terms of biomass and essential oil production were evaluated. Further, a deeper study on Nickel influence on SMs biosynthesis with untargeted metabolomic analysis focusing on Amino acids, Alkaloids, Cinnamic acids, Flavonoids, Lipids and fatty acids, Organic acids, Phenolic acids, Polyketides, Terpenoids and Carbohydrates, for both species was carried out.

2 Material and methods

2.1 Soil preparation and analysis

Agricultural soil was collected from 0 to 30 cm in an experimental farm located in Ozzano dell' Emilia (Bologna, Italy) and analyzed for physicochemical parameters (**Table 1**) and total elements content (**Table 2**), before the beginning of trials. A part of the total amount of soil, was contaminated with Ni $(NO_3)_2$ $6H_2O$ to reach a concentration above and one below the legislative threshold fixed in D.Lgs. 152/2006, 300 mg kg-1 and 100 mg kg-1 respectively. A control for each species was provided and a randomized plot scheme with 3 replicated for each condition (Control and Contaminated) was adopted. At the end of the trial total elements in soil were quantified for each treatment for monitoring their final content (**Table 3**).

Trials and measurements were repeated for two years of cultivation 2023 and 2024, respectively.

Table 1. Soil chemical and physical characteristics (0-30 cm) before nickel implementation and trials (± standard deviation).

Soil characteristics	
Clay (%)	12
Loam (%)	18
Sand (%)	70
Cation exchange capacity (meq 100 g-1)	13.0 ± 1.2
Total N (g kg-1)	1.1 ± 0.14
Assimilable P (mg kg-1)	11.0 ± 4.40
Exchangeable K (mg kg-1)	195 ± 0.6
Total Organic C (g kg-1)	13.0 ± 1.2
C/N	11.8
Mg/K	1.7
Organic matter (%)	2.2 ± 0.35
Ammoniacal N (g/kg)	< 2
Nitrate (g/kg)	10

Table 2. Soil total elements (0-30 cm) before Nickel implementation and trials (± standard deviation).

Soil mineral content Before-treatment	
Al g kg-1	27,3 ± 2,7
As mg kg-1	15,16 ± 2,6
B mg kg-1	28,9 ± 1,1
Ba mg kg-1	90,9 ± 6,3
Be mg kg-1	244,8 ± 8,7
Ca g kg-1	39,4 ± 0,3
Cd mg kg-1	5,81 ± 0,1
Co mg kg-1	11,8 ± 4,2
Cr mg kg-1	9,23 ± 0,8
Cu mg kg-1	54,2 ± 7,9
Fe g kg-1	26,2 ± 4,8
K g kg-1	4,78 ± 1,4
Mg g kg-1	7,97 ± 0,5
Mn mg kg-1	708,5 ± 14,8
Na mg kg-1	388,5 ± 9,4
Ni mg kg-1	50,5 ± 2,1
P mg kg-1	702,7 ± 32,1
Pb mg kg-1	18,0 ± 0,4
S mg kg-1	216,3 ± 49,3
Se mg kg-1	2,53 ± 0,3
Si mg kg-1	277,0 ± 32,8
Tl ppm	165,9 ± 4,5
V µg kg-1	350,1 ± 19,5
Zn mg kg-1	251,0 ± 10,3

Table 3. Medium final element content for all treatment and both analyzed species *Hypericum perforatum* L. and *Melissa officinalis* L..

	***Hypericum perforatum* L.**			***Melissa officinalis* L.**		
	Ctr	**N100**	**N300**	**Ctr**	**N100**	**N300**
Al g kg-1	17,3	15,4	16,9	13,0	11,7	10,8
As mg kg-1	18,3	16,4	18,1	14,3	13,0	12,0
B mg kg	5,7	5,7	6,4	4,1	4,3	3,8
Ba mg kg-1	29,1	25,3	29,2	18,5	17,1	14,9
Be mg kg-1	97,4	101,7	111,3	92,8	88,0	89,7
Ca mg kg-1	610,0	529,1	808,8	377,0	316,6	251,3
Cd mg kg-1	40,0	39,6	38,3	32,2	34,0	33,0
Co mg kg-1	5,9	6,7	7,0	12,9	14,0	15,6
Cr mg kg-1	7,6	7,6	7,5	6,5	6,0	5,9
Cu mg kg-1	58,0	45,2	56,7	46,5	47,7	36,5
Fe mg kg-1	1003,1	1012,5	1105,9	990,7	960,7	1010,9
K g kg-1	15,7	14,5	14,4	13,0	12,6	12,1
Mg g kg-1	4,2	3,9	4,4	3,7	3,5	3,1
Mn mg kg-1	550,7	517,2	566,2	444,4	422,1	382,1
Mo mg kg-1	2,9	2,6	2,5	2,5	2,4	2,2
Na g kg-1	8,2	7,9	7,8	6,4	6,1	6,3
P g kg-1	1,4	1,9	1,9	1,1	1,0	1,3
Pb mg kg-1	1,7	1,9	2,2	1,3	1,4	1,2
S g kg-1	1,6	1,8	2,2	1,0	1,2	0,9
Sb mg kg-1	45,0	135,1	418,2	28,1	156,7	299,4
Se mg kg-1	1,2	1,4	1,6	0,9	1,0	1,0
Si mg kg-1	261,4	265,2	303,2	230,4	215,4	225,6
Sn µg kg-1	806,6	994,4	940,2	315,3	480,6	322,1
Sr mg kg-1	103,1	104,2	113,1	123,6	138,1	120,8
Ti mg kg-1	108,9	106,0	107,9	100,8	98,8	99,4
V mg kg -1	1,1	1,3	1,4	1,3	1,5	1,6
Zn mg kg-1	158,0	154,7	149,2	134,0	140,0	134,7

2.2 Plant material, greenhouse trial conditions and harvesting

A randomized block scheme with three replicates, for both species was adopted, and a control (not treated) was provided (**Figure 1**).

Seed material of commercial varieties of *Hypericum perforatum* L. and *Melissa officinalis* L., specifically selected for the harvest of bioactive compounds, was purchased from seed production company Saflax©. The seeds were germinated in multicell trays and seedling establishment performed under hydroponic system in growing chamber conditions at 25 ◦C. Until transplanting plants were fertilized with 1/5 Hoagland Solution (Rajan et al. 2019). After 40 days, the seedlings were transplanted in green house at a recommended plant density of 5 plants/m2 (Catizzone et al. 2013). For each species and treatment, three leaf harvests were made for each year of cultivation (2023-2024) respectively, 30, 60, 90 and 120 days after transplant, to verify stress condition.

The aerial parts were harvested from each condition (NT, 100 and 300) within each replicate block. Aerial material was removed manually from the top 10 – 15 cm of the plant and 50 – 60 cm for *Melissa officinalis* L. and *Hypericum perforatum* L. respectively. Leaf biomass was determined by weighing the fresh leaves. Dry mass was calculated after drying the fresh leaves to a constant weight in a dryer 40 °C with relative humidity < 20%. After each leaf harvest, the plants were allowed to resume growth over next growth cycle (Marotti et al. 2022).

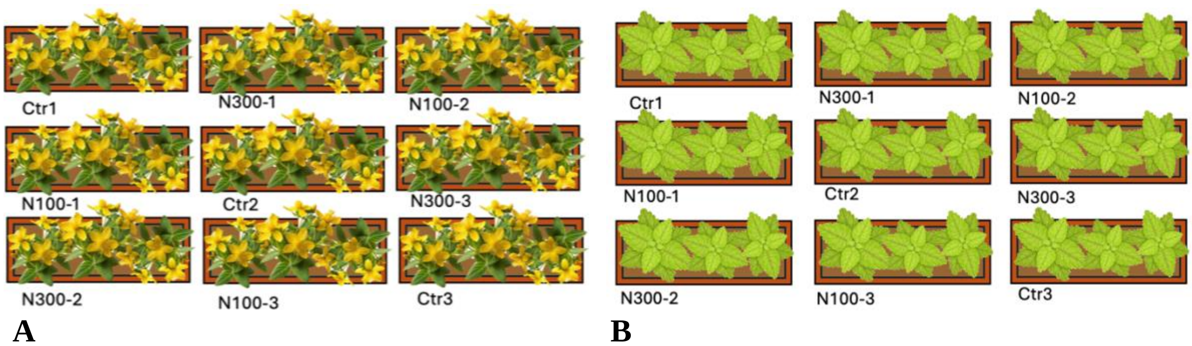


Figure 1. Representation of the randomized plot scheme adopted for *Hypericum perforatum* L. (**A**) and *Melissa officinalis* L. (**B**) trials under control environment condition for the two year-trials.

2.3 Essential oil production and Yield

Essential oil was extracted from fresh aerial parts for both examined species, for each replicate and condition. Fresh samples were harvested at the end of each crop cycle, 120 days after planting and during full-bloom stage as reported previously (Crockett et al. 2010), for *Melissa officinalis* L. and *Hypericum perforatum* L. respectively. Considering *Melissa officinalis* L. distillation was realized from the whole aerial parts, leaves and stems. For *Hypericum perforatum* L. a selection of inflorescences and leaves instead of the total aerial part was performed (Grafakou et al. 2021).

The essential oil was obtained through hydrodistillation process. Plant material was placed in a 2-liter flask with distilled, deionized water (600 ml for 200 g fresh material) and the essential oil was extracted by water distillation using a 12L distiller (AgritechStore). The distillation chamber joined the temperature required (100°C) in 20 minutes and the complete distillation cycle took 60 minutes. The solution obtained in the process was divided in aqueous extract (hydrolat) and essential oil with an essencier (Florentine vase). Both products were stored in dark glass bottles at 4°C. The essential oil content was determined on an oil volume to tissue weight (Charles and Simon, 1990). At the end of the extraction for each treatment the effect on essential oil yield were evaluated. Fresh material for both examined species were adopted.

2.4 GCA, GI and Chlorophyll content

The effects of metal contamination on growth parameters were assessed evaluating Growth index (GI), Green Cover Area (GCA) and Chlorophyll content. These parameters were recorded 30 days after the seedlings were planted up to 120 days at subsequent 30-day intervals. A completely randomized plot design was adopted; each species was replicated 3 times, each replication containing 5 plantlets. The growth index (GI) was used as a quantitative indicator when comparing plant sizes. As reported by Tuttolomondo et at. (2018), growth index was calculated using the following equation (H+W1+W2)/3 where: H is the plant height; W1 is the transversal diameter of the plant; W2 is the longitudinal diameter of the plant. Green cover area was measured using ImageJ software as previously reported Tong et al. 2024. Yield of total biomass and essential oil were purchased by harvesting fresh leaves at 120 days after transplant for each species and condition.

Leaf chlorophyll content was estimated with a SPAD-502 meter (Konica Minolta SpA). SPAD values were converted to chlorophyll content (μg cm−2) using the following equation: Chlorophyll content= (99×SPAD value)/(144−SPAD value) (Pietrini et al. 2015).

2.5 Free PROLINE content

Free Proline content of fresh leaves samples harvested in the early morning had been determined following Raziq et al. (2022) and Claussen (2005) modified methods. Briefly, vegetal tissues of both species were harvested in the morning and immediately grinded with liquid nitrogen. Samples were extracted with a solution of acid 5-sulphosalycilic dii-hydrate 3% (w/v) and stored at -18°C until the quantification.

For the quantification Nynidrin 2,5% solution was added to samples and tubes were incubated in water bath at 90°C for 1h. The obtained solution was filtered (0.45 µm) and quantified. Separation was achieved with a Waters e2695 Alliance HPLC System combined with a Waters ACQUITY QDa 2489 UV/Vis Mass Detector.

2.6 OAs (Organic Acids)

OAs (Organic Acids) as Citric acid, malic acid and oxalic acid were extracted from fresh leaf material for each time and condition as previously reported (Pietrini et al. 2015). OAs were extracted in a pre-chilled mortar and pestle with 5 % (v/v) phosphoric acid, centrifuged, and supernatants were set aside, filtered (0.2 µm) and injected into a HPLC system. Separation was achieved with a Waters e2695 Alliance HPLC System combined with a Waters ACQUITY QDa Mass Detector. The UV detector had been set up at 89, 133 and 190 nm wavelength for oxalic, malic and citric acid, respectively, and a Kinetex Evo EVO C18 (5 µm, 100 Å, 250 X 4,6 mm) (Phenomenex, Torrance, CA, USA) column was used at a temperature of 40 ◦C, and SIR negative mode. An isocratic mobile phase with 0.6 ml/min flux was adopted and composed of 30% solution A (MeOh), 10% solution B (CH3N 0.05%), and 60% solution C ($(NH_4)_2CO_3$ 25 mM). Data acquisition and instrument control were performed using the Empower 3 software.

2.7 Leaf quality analyses

For the analysis of functional quality parameters, the fresh leaf material was ground with liquid nitrogen to produce a fine powder and three replicates performed for each species, treatment and cut, respectively. The functional quality analyses included polyphenol and flavonoid content, anti-radical activity using the 1,1- diphenyl-2-picrylhydrazyl (DPPH) radical. Rosmarinic acid and Hypericin content were performed, for lemon balm and Hypericum respectively.

Part of the material collected at the end of the first crop cycle (120 days after transplant) was freeze dried and ground to powder and three replicates performed for each species and treatment, respectively. On the material metabolomic analysis were carried out.

2.7.1 Polyphenol and flavonoid content and antioxidant activity analyses

Polyphenols, comprising both free and bound constituents, were extracted as described previously (Dinelli et al., 2011). Free (FP) and bound polyphenols (BP) were then measured according to the Folin-Ciocalteau spectrophotometric (765 nm) method using gallic acid (GA) as a reference standard (Singleton et al., 1999). Likewise, the free (FF) and bound flavonoids (BF) were individually measured using a spectrophotometric (510 nm) colorimetric assay with catechin (CA) as a reference standard (Adom et al., 2003). From the free and bound polyphenol and flavonoids, respectively, the respective totals were calculated. The DPPH assay was performed by measuring the reduction (515 nm) of DPPH• to 1,1-diphenyl-2-picryl hydrazine (Floegel et al., 2011). The antioxidant activity in the free and bound fraction were summed and expressed as total DPPH.

2.7.2 Rosmarinic acid extraction and quantification in *Melissa officinalis* L.

Rosmarinic acid was extracted from fresh leaf material for each time and condition as previously reported (Sanchez-Medina et al. 2007). The extraction was made in ethanol 100% solution, and extracts had been diluted in water (HPLC grade) and filtered (0,25 µm filter Sarsted) before the injection. Separation was achieved with a Waters e2695 Alliance HPLC System combined with a Waters ACQUITY QDa Mass Detector. The UV detector at a 333 nm wavelength had been set up and a Kinetex Evo EVO C18 (5 µm, 100 Å, 250 X 4,6 mm) (Phenomenex, Torrance, CA, USA) column was used at a temperature of 40 ◦C. A gradient mobile phase was adopted and composed as reported below in **Table 4**. The monitored (M–H) were m/z and retention time (**Figure 2**) Data acquisition and instrument control were performed using the Empower 3 software.

Table 4. description of the flux gradient used for Rosmarinic Acid quantification in *Melissa officinalis* L. samples.

Time (min)	Flux (ml/min)	A (formic acid 0.1%)	B (Methanol)	C (Acetonitrile)
	0,6	70 %	25 %	5 %
10	0,7	70 %	25 %	5 %
18	0,8	60 %	30 %	10 %
22	0,8	70 %	25 %	5 %

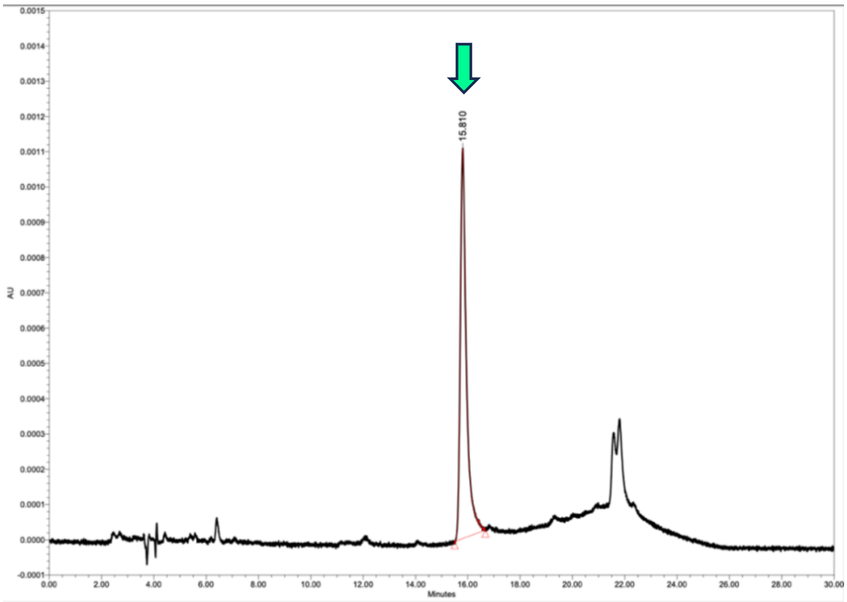


Figure 2. *Melissa officinalis* L. spectrum, Rosmarinic acid peak identification at 333 nm.

2.7.3 Hypericin content in *Hypericum perforatum* L.

Hypericin was extracted from fresh leaf material for each time and condition as previously reported (Zhang et al. 2020). The extraction was made in methanol/pyridine 99/1 (v/v). solution, and extracts had been diluted in methanol 100% and filtered (0,25 µm filter Sarstedt) before the injection. Separation was achieved with a Waters e2695 Alliance HPLC System combined with a Waters ACQUITY QDa Mass Detector. The UV detector at a 590 nm wavelength had been set up and a Kinetex Evo EVO C18 (5 µm, 100 Å, 250 X 4,6 mm) (Phenomenex, Torrance, CA, USA) column was used at a temperature of 40 ◦C. A gradient mobile phase was adopted and composed as reported below in **Table 5**. A total run time of 40 min (35 min run + 5 delay) had been set up. The monitored (M–H) were m/z and retention time (**Figure 3**) Data acquisition and instrument control were performed using the Empower 3 software. Data acquisition and instrument control were performed using the Empower 3 software.

Table 5. Description of the flux gradient used for Hypericin quantification in *Hypericum perforatum* L. samples.

Time (min)	Flux (ml/min)	A (HCOOH0.1%)	B	C (CH3CN)	D
0	0,8	15	0	85	0
5	0,9	15	0	85	0
26	1,2	5	0	95	0
30	0,8	15	0	85	0

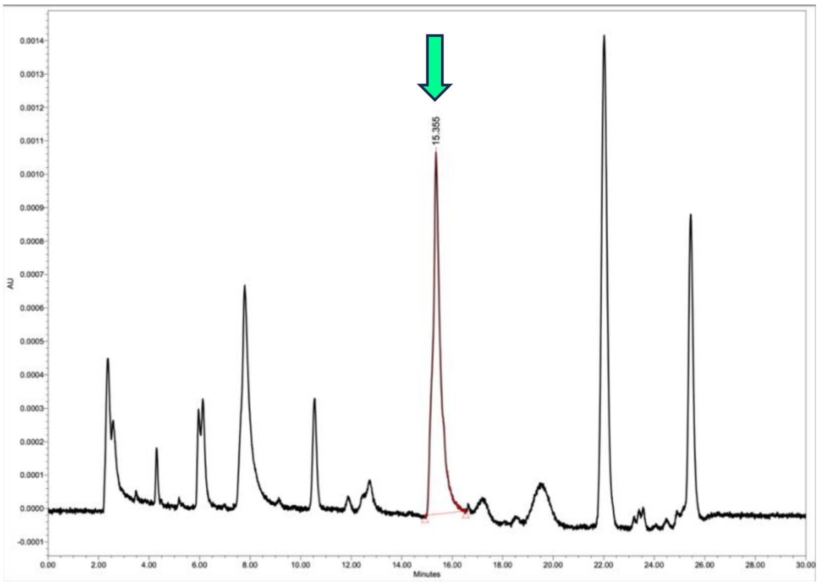


Figure 3. *Hypericum perforatum* L. spectra, with Hypericin peak at a wavelength of 590 nm.

2.8 Untargeted Metabolomic Analysis

2.8.1 Polar and semi-polar metabolite extraction

Metabolites were extracted from 10 mg of hypericum and melissa freeze-dried leaves samples. The metabolites extraction protocol was adapted from Routaboul et al., (2006). Each sample was extracted with 1 mL of Methanol/acetone/H2O/TCA (30/42/28/0.1 v/v/v/v) extraction buffer, conserved at 4°C. An internal standard (Apigenin) was added. The mixtures were placed in ultrasonic bath for 2 min, then shaken for 30 min at 4°C using ThermoMixer™ C (Eppendorf), and centrifuged for 10 minutes at 1000 rpm and 4°C. Supernatant of each sample was collected in new glace tubes and the same previous extraction was repeated on the pellet left. 1.8 mL of resulting supernatants was collected in new 2 mL Eppendorf and the metabolites extracts were dried down in a SpeedVac vacuum concentrator (o/n) and stored at -20°C until resuspension. The previously described extraction protocol was used for the untargeted metabolomic analysis.

2.8.2 UPLC-MS/MS analysis of specialized metabolites

Untargeted metabolomic data were acquired using a UHPLC system (Ultimate 3000 Thermo) coupled to quadrupole time of flight mass spectrometer (Q491 Tof Impact II Bruker Daltonics, Bremen, Germany). For chromatographic separation a Nucleoshell RP 18 plus reversed-phase column was used. Samples were injected in positive ionization mode (ESI+). The extracted samples were stored at -80°C until analysis. For untargeted metabolomics, data-dependent acquisition methods were used for mass spectrometer data in positive ESI modes.

2.8.3 UPLC-MS/MS data processing.

The .d data files previously obtained (Bruker Daltonics, Bremen, Germany) were converted to .mzXML format using the MSConvert software (ProteoWizard package 3.0; Chambers et al., 2012). mzXML data processing, mass detection, chromatogram building, deconvolution, samples alignment and data export were performed using MZmine 2.52 software (http://mzmine.github.io/) for both positive and negative data files. The ADAP chromatogram builder (Myers et al., 2017) method was used with a minimum group size of scan 3, a group intensity threshold of 1000, a minimum highest intensity of 1500 and m/z tolerance of 10 ppm. Deconvolution was performed with the ADAP wavelets algorithm and isotopic peak grouper algorithm was used based on m/z and RT (Retention Time). All the peaks were filtered using feature list row filter keeping only peaks with MS2 scan. The alignment of samples was performed based on m/z tolerance of 10 ppm, and RT. Metabolites accumulation was normalized according to the internal standard (Apigenin) and weight of leaves used for the extraction. *H. perforatum* L. and *M. officinalis* L. metabolic features research in the library with Mzmine was done, with identification module and 'custom database search' to begin the annotation with IJPB library, currently containing 120 annotations or experimental common features (RT and m/z) in positive mode.

2.8.4 Molecular networking of untargeted metabolomic data

Molecular networks were generated with MetGem software (https://metgem.github.io) using files obtained with MZmine2 analysis (Olivon et al., 2017). The molecular network was optimized for the ESI+ datasets, and different cosine similarity score thresholds were tested. ESI+ molecular networks were generated using cosine score thresholds of 0.8. Molecular networks were exported to Cytoscape software (Shannon et al., 2003; https://cytoscape.org/) to format the metabolic categories (**Figure 4**).

2.8.5 Metabolite annotation of untargeted metabolomic data

Metabolite annotation was performed in four consecutive steps. First, the obtained RT and m/z data of each feature were compared with our homemade library containing more than 150 standards or experimental common features (RT, m/z). Second, the ESI+ metabolomic data used for molecular network analyses were searched against the available MS2 spectral libraries (Massbank NA, GNPS Public Spectral Library, NIST14 Tandem, NIH Natural Product and MS-Dial), with absolute m/z tolerance of 0.02, 4 minimum matched peaks and minimal cosine score of 0.8. Third, not-annotated metabolites that belong to molecular network clusters containing annotated metabolites from steps 1 and 2 were assigned to the same chemical family. Finally, for metabolites that had no or unclear annotation, Sirius software (https://bio.informatik.uni-jena.de/software/sirius/) was used. Sirius is based on machine learning techniques that used available chemical structures and MS/MS data from chemical databanks to propose structures of unknown compounds. Raw data were normalized on the internal standard (Apigenin) and weight of plant tissues used for the extraction (**Figure 4**).

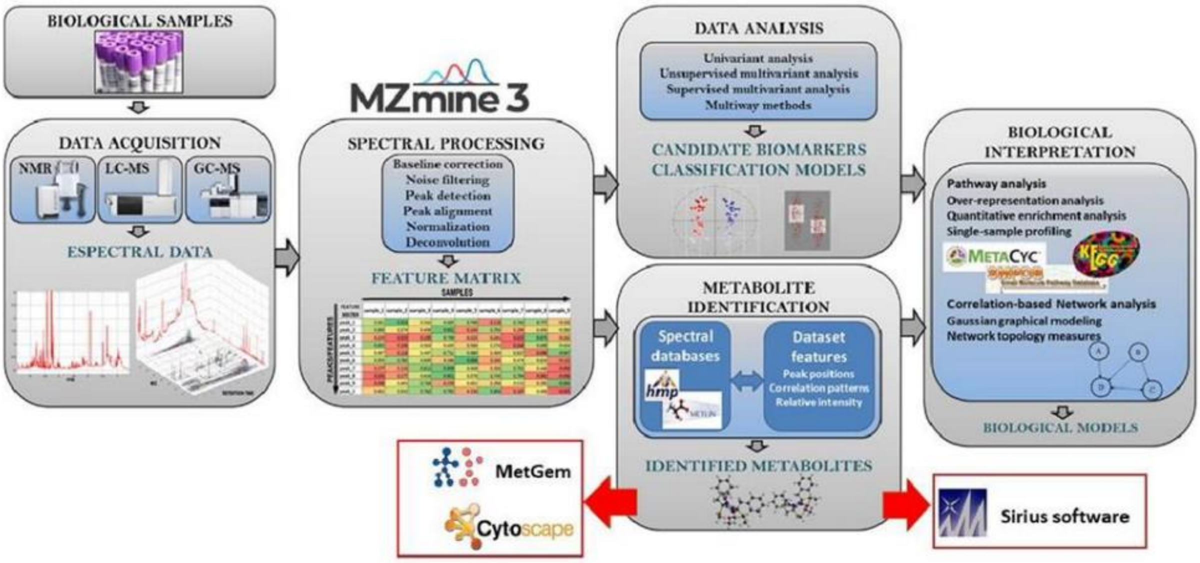


Figure 4. Schematic representation of data acquisition, processing and analysis using MZmine, MetGem, Cytoscape and Sirius software, as previously reported and described.

2.9 Mineralization of soil and plant material

The metal content was determined according to Vittori Antisari et al. (2013). Briefly, the soil (0.25 g) was treated with *aqua regia* (2 mL HNO_3 65% plus 6 mL HCl 37%, suprapur grade Carlo Erba) in a microwave oven (Start D 1200, Milestone, USA) and metal concentrations were determined by ICP-OES. The analysis of each sample was replicated three times and compared with analyses of the International Reference Materials (BCR 141) and laboratory internal standards (MO and ML), which was run after every 10 samples to check changes in sensitivity. Controls with only reagents were also determined.

Dry tissues of different organs of each MAP were finely grounded and digested using a nitric acid and oxygen peroxide solution in the microwave oven according to the United States Environmental Protection Agency – USEPA (2009) method, modify by Vittori Antisari et al. (2014). Approximately 0.25 g sub-sample of plant tissue was treated with 6 mL of concentrated suprapur nitric acid (Merck) plus 1.5 mL of hydrogen peroxide (Carlo Erba for electronic use). The mineralization was carried out in PTFE vessels in the microwave oven, and both the content of nutrients (Ca, Mg, K, Na, P, S) and the component metal (Ag, Co, Ce, Fe, Ni, Sn, Ti) in aboveground organs and roots, were quantified by ICP-OES. Blank and International Reference Materials (Olive leaves BCR-CRM 062) were analyzed to validate the method. In addition, standard solutions were analyzed every 10 samples for quality control/quality assurance purposes (Vittori Antisari et al. 2018).

2.9.1 Bioaccumulation and translocation factors.

To determine the clean-up potential of targeted plants bioaccumulation factor (BAF) was evaluated (McGrath and Zhao, 2003), with the following equation:

- BAF = $[Me]p / [Me]s$

  [Me]p = Metal concentration in plant tissue

  [Me]s = Metal concentration in soil

In more details, to assess the accumulation and movement of metals from the roots to the aboveground portions, the bioconcentration factor (BAF) and translocation factor (TF) were used for comparison (Ye et al., 2018 and Rizzi et al. 2004). Hyperaccumulators are plants with a BCF exceeding 1.0 (Cluis, 2004). The equations were reported below:

- TF soil to roots = $[Me]r / [Me]s$

  [Me]r= Metal concentration in root tissue (mg/kg)

  [Me]s= Metal concentration in media (mg/kg).

- TF roots to aboveground organs= $[Me]a / [Me]r$

  [Me]a = Metal concentration in aboveground organs (mg kg-1)

  [Me]r = Metal concentration in below ground organs (mg kg-1).

2.10 Statistical analysis

Statistical analyses were conducted using the Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA). A one-way analysis of variance (ANOVA) in conjunction with Tukey's honest significant difference was performed to compare the three growing locations. Significant differences between means were determined by least significant difference values for $p < 0.05$. Pearson's correlation coefficient (r) was calculated at significance level of $p < 0.01$.

Statistical analysis Heat map and Volcano plot analysis of Specialized Metabolites were carried out using the 'heatmap.2' function (GRPLOTS R package). Expression values used for the analysis were filtered based on median values.

The principal coordinate analysis (PCA) was used in the present study. PCA is an unsupervised clustering method and is a powerful tool for analysis of multivariate data, without requiring any knowledge of the dataset (Jambu, 1991). PCA was used to transform numerous correlated variables into a smaller number of uncorrelated variables called principal components (Tabachnick and Fidell, 2001). In the present study, the correlation method was preferred over covariance since PCA on the covariance matrix is not invariant to a component-wise change of scale (Bilodeau and Duchesne, 2002). With this method the original space for variable measurements was projected down onto two low-dimensional subspaces. One of these was case-related (12 for treatments and harvest time for each case study plant, Hypericum and lemon balm, respectively), the other was variable-related. The nine variables were as follows: total polyphenols (TP), total flavonoids (TF), DPPH antioxidant activities, Hypericin (HYP) for Hypericum, Rosmarinic Acis, for melissa (RA), oxalic acid (OA), malic acid (MA), citric acid (CA), chlorophyll (CHL) and proline (PRO). The variable-related subspace was analyzed (factor loading) to understand the correlation between the variables and factors (principal component).

3 Results and discussions

Recent works highlights the use of different types of MAPs plants for medicinal purposes over time around the world. As estimated by the World Health Organization (WHO), approximately 25% of modern medicine has been derived from plants being used in traditional medicine, while there is an estimate of 80% of the world's population using medicinal plants as the main form of health (Ahmad Khan et al. 2018; Asiminicesei et al. 2020)

The role of medicinal plants is not only limited to traditional medicine. Due to increasing demand of plant-based products in industries such as foods, pharmaceuticals, essential oils, cosmetics, and even ornaments, as well as environmental counterbalances to industrial and agriculture pollution, the economic value of these plants has been improved (Manan et al. 2015; Pruteanu et al. 2014).

MAPs are considered a good opportunity for phytoremediation, as they are grown primarily for processing and have the capacity to accumulate and/or eliminate essential and toxic trace metal elements from the soil, therefore reducing the associate risks for human health (Mishra and Chandra, 2022; Fattahi et al. 2019). TME accumulated by MAPs species are not transferred in the essential oils. Thus, the possibility of further industrial processing makes these species an economically interesting crop for farmers considering the phytoextraction technology (Angelova et al. 2015).

To expand on the earlier studies by including *Hypericum perforatum* L. and *Melissa officinalis* L. cultivation under controlled conditions with multiple Nickel concentrations and phenological stages (15, 90, 60 and 120 days after transplant) was evaluated to expand on the earlier studies. Biological and biochemical parameters measured were Chlorophyll index (SPAD), Growth index, Proline content and yield (Biomass and essential oils), and of specific interest, the contents of polyphenols and flavonoids, as well as untargeted metabolomic profile, antioxidant activities (DPPH), Rosmarinic acid, and Hypericin as main constituent of *M. officinalis* L. and *H. perforatum*, respectively.

Given that *H. perforatum* L. and *M. offcinalis* L. could be used for phytoremediation purposes (Misha and Chandra, 2022), the present study was aimed addressing the impact of moderate-high Nickel exposure (100 and 300 ppm, respectively) on plant physiology, ionomic, metabolome, bioactive compounds and essential oil yield. These responses and parameters were considered important towards prioritizing this crop for future research, development, and innovation for medicinal/herbalist products applied for remediation purposes.

3.1 Plant biomass and essential oils yield.

Yield (biomass) is one important determinant influencing the potential success of a MAP and phytoremediation operation. In this study, plant fresh biomass and essential oil yield were analyzed at 120 days after transplant for both Hypericum and Melissa species.

The recorded data on the effect of different nickel content on growth, herb and oil yield of *M. officinalis* L. and *H. perforatum* L. are given in **Table 6**.

Melissa officinalis L. average oil content (%) in fresh herb in control, N100 and N300 was recorded as 0.06, 0.07 and 0.08%, respectively (**Table 6**). The fresh herb yield was significantly impacted by treatments, unlike essential oil yield. The maximum fresh herb yield was recorded in Control and N300 treatments with 11190 and 11270 kg/ha, respectively.

Fresh biomass (kg/ha) levels obtained were comparable to previous data concerning the same harvest period but lower compared to biomass amount obtained 140 and 160 days after transplant (Singh et al. 2014). Previous research reported that harvesting before flower initiation and at flowering stages were found to be the best stages to harvest the plant to obtain the highest essential oil yield (Ayanoglu et al. 2005).

The effect of the Ni treatment impacted significantly herb and essential oil yield (fresh herb) for *Hypericum perforatum* L. (**Table 6**). The average oil content (%) in fresh herb in control, N100 and N300 was recorded as 0.13, 0.23, 0.25%, respectively. These results highlighted that Ni-treatment enhance Hypericum plant oil content. Essential oil yield had comparable values to previous data (Hevia et al. 2002; Chauhan et al. 2011; Schepetkin et al. 2020)

Soil nutrient level, temperature regimen, relative humidity, irradiance and photoperiod may play a specific role in yield and composition of the oil, in addition to the genetic diversity. Thus, in present study, the diversity in Ni concentration resulted in higher essential oil quantitative production. Indeed, it was observed that after TME exposure, the percentage of essential oil content in certain MAPs progressively increased, if their physiological activities were not severely compromised by heavy metals (Mishra and Chandra, 2022).

The maximum fresh herb yield was recorded in N300 treatment with 5154.0 kg/ha and the lowest for N100 (5002.7 kg/ha). Fresh biomass (kg/ha) levels obtained were comparable to previous results (Kizil et al. 2013; Azizi et al. 2001) but lower compared to data obtained by Hevia et al. (2002).

Nickel treatment had a greater impact on Hypericum essential oil yield and biomass compered to what has been observed for Melissa. These results strongly support a link between soil composition and the essential oil composition of MAPs, as previously suggested (Chauhan et al. 2011). Further studies should be carried out to better characterize essential oil composition of both Hypericum and lemon balm to evaluate any effects of Nickel on essential oil compounds.

Table 6. Green biomass and essential oil yield, as well as dry matter content for *Melissa officinalis* L. and *Hypericum perforatum* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) at the end of the crop cycle. Different letters within each column for each location represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, FW = fresh weight biomass, DM = dry matter and E.O = essential oil yield.

	Melissa officinalis L.			*Hypericum perforatum* L.		
	FW (kg/ha)	DM (%)	E.O (%)	FW (kg/ha)	DM (%)	E.O (%)
Control	11190.0 a	40.3 a	0.06 a	5099.0 ab	34.5 a	0.13 b
Nickel 100	10740.5 b	42.8 a	0.08 a	5002.7 b	35.2 a	0.23 a
Nickel 300	11270.5 a	41.8 a	0.07 a	5154.0 a	35.6 a	0.25 a
Mean	11070	41.6	0.07	1085.2	35.1	0.2
LSD 0.05	230.2	1	0.008	62.5	0.5	0.05
P	***	*ns*	*ns*	**	*ns*	***

3.2 Growth parameters, Proline and Chlorophyll.

In addition to biomass and essential oil yield, an investigation about the relationship between the Ni tolerance level and some physiological and biochemical responses was performed on *Hypericum perforatum* L. and *Melissa officinalis* L. exposed to environmentally relevant Ni concentrations in the soil. *Melissa officinalis* L. growth parameters as Green Cover Area (GCA) and Growth Index (GI), were not significantly impacted by the treatments, but only impacted by time of harvesting. Last cuts, T2 and T3 shown highest values in term of GI and GCA, respectively.

Similarly, chlorophyll content was not impacted by the treatments but only by the time, with higher chlorophyll content detected in T0 (33.08, in **Table 7**). The higher Ni concentration did not cause a considerable inhibition of plant growth and chlorophyll biosynthesis, suggesting good adaptation to high Ni concentration in the soil for this species. On the other hand, Proline content was impacted by both treatment and harvest. Proline highest content was detected in N300 treatment, with 17.42 mg kg-1; in accordance with medium values observed in literature

(Spormann et al. 2023; Zouari et al. 2018; Nawaz et al. 2010). In addition, a significant interaction ($p \leq 0.001$) between time and Treatment was observed, and proline content was impacted by Nickel content in T2 and T3 (**Figure 5**). Although enhancement of proline content in response to TME toxicity has been described by several authors (Alia and Pardha Saradhi 1991, Pandey and Sharma 2002), the physiological significance of this amino acid accumulation under metal stress may be involved in plants protection against injury caused by heavy metals (Matysik et al. 2002). To limit adverse Nickel effects on lemon balm, foliar application of SA (Salicilic Acid) mitigated the deleterious effects of Ni and decreased its transport to the shoots, without modification in proline content due to Nickel exposure (Soltani et al. 2016).

Table 7. Growth and stress parameters: green cover area, growth index, proline and chlorophyll content for *Melissa officinalis* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) for four consecutive time. Different letters within each column for each location represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, GCA = green cover area.

		PROLINE mg/kg		GCA %		Growth Index		Chlorophyll index	
Treatment	Control NT	10.89	c	79,09	a	33,96	a	25,52	a
	N100	14.32	b	79,77	a	34,48	a	24,52	a
	N300	17.42	a	78,64	a	34,72	a	24,67	a
Time	T0	6.91	c	72,28	c	15,16	d	33,08	a
	T1	13.12	b	81,22	b	23,96	c	19,81	d
	T2	9.89	c	77,21	b	50,33	a	21,79	c
	T3	19.62	a	85,97	a	48,11	b	24,93	b
	LSD 0.05 =	1.7		3.9		1.4		1.5	
	Treat	***		ns		ns		ns	
	Time	***		***		***		***	
	TxT	***		ns		ns		ns	

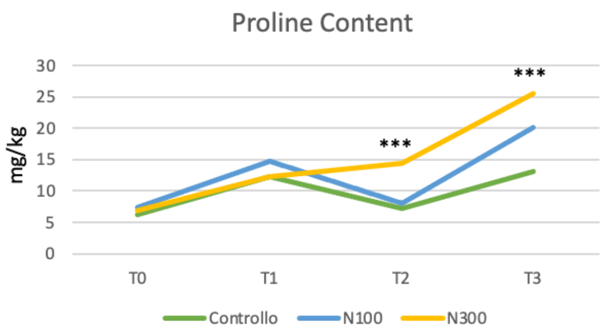


Figure 5. Interaction model of Proline in *Melissa officinalis* L. with different treatments (Control, 100 ppm of Nickel and 300 ppm of Nickel) over four harvests cut and phenological

phasis. The number of stars (***) in T2 and T3 represents significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively.

In contrast, *Hypericum perforatum* L. growth parameters as GCA and GI, Proline and Chlorophyll were significantly impacted by the treatments and the harvest time.

Growth parameters (GI and GCA) were higher for the N300 treatment, followed by N100 and the Control, and were highest at the last cut (T3) at 120 days after transplant, even if no interaction between treatment and harvest time was observed. Hypericum showed high adaptability to nickel even at high concentration for growing parameters (**Table 8**).

CI was strongly impacted by the harvest time and mildly influenced by the treatment, the highest chlorophyll amount was measured for the first and last harvest cut (37.6 and 40.1, respectively) and Control and N100 treatment (37.0).

The increase of Ni concentration did not cause a considerable inhibition of plant growth, demonstrating successful adaptation in term of growing parameters, even if it affects chlorophyll biosynthesis. The chlorophyll content in leaves of Hypericum plants were consistent with the tolerance response and the oxidative damage observed in plants because of the increasing Ni concentration in the growth solution as reported (Pietrini et al., 2015).

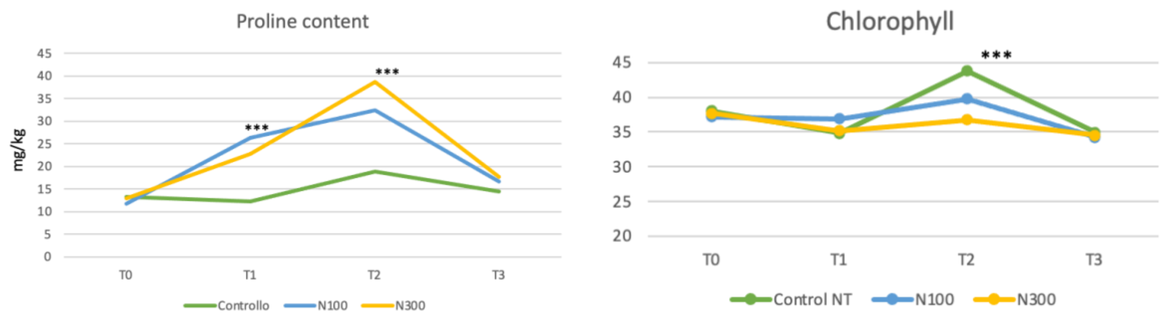
On the other hand, Proline content was significantly impacted by both factors (treatment and harvest time) and factors interaction. Proline highest content was detected in Nickel 300 treatment, with 17.42 mg/kg; in accordance with medium values observed in literature and already reported.

The positive interaction for Chlorophyll (* ; $p \leq 0.05$) and Proline (*** ; $p \leq 0.001$) between factors (harvest time and treatments) was showed in **Figure 6A** and **B**, respectively. Chlorophyll was impacted by Nickel content in third cut (T2), where it was significantly higher the control content (**Figure 6A**). Furthermore, Proline concentration was impacted by the treatment in central cuts (T1 and T2 respectively), where it was higher in N100 treatment for T1 and N300 treatment for T2. The trend in proline content, decreasing in the last cut, also coupled with an increase in the growth index may indicate a gradual adaptation of the plant to the nickel content in the soil.

Proline is well-known as an osmo-protectant that accumulates in response to various environmental stresses, particularly hydric (water) and osmotic stress. It plays a crucial role in cellular osmoregulation, helping to mitigate the effects of stress by stabilizing proteins and cellular structures, as well as scavenging reactive oxygen species (ROS). Previous data indicated that proline not only aids in osmotic adjustment but also detoxifies heavy metals by chelating them and acting as a reactive oxygen species scavenger (Atta et al., 2024). Indeed, enhanced proline accumulation might be positively correlated with improved plant tolerance to nickel stress, indicating a crucial role in the adaptation and resilience of plants under heavy metal exposure.

Table 8. Growth and stress parameters: green cover area, growth index, proline and chlorophyll content for *Hypericum perforatum* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) for four consecutive time. Different letters within each column for each location represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, GCA = green cover area.

		PROLINE mg/kg		GCA %		Growth Index		Chlorophyll index	
Treatment	Control NT	10.89	c	42,26	b	21,40	b	37,89	a
	N100	14.32	b	44,75	b	21,22	b	37,00	b
	N300	17.42	a	50,27	a	25,74	a	35,47	b
Time	T0	12.91	c	14,67	d	14,06	d	37,61	a
	T1	20.43	b	24,63	c	20,17	c	34,88	b
	T2	30.02	a	62,81	b	25,93	b	34,55	b
	T3	16.24	c	80,94	a	31,99	a	40,10	a
	LSD 0.05 =	1.7		4.1		3.2		2.2	
p	Treat	***		***		**		*	
	Time	***		***		***		***	
	TxT	***		ns		ns		*	



A B

Figure 6A. Interaction model of Proline in *Hypericum perforatum* L. with different treatments (Control, 100 ppm of Nickel and 300 ppm of Nickel) over four harvests cut and phenological phasis. The number of stars (***) in T1 and T2 represents significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. **Figure 6B**. Interaction model of chlorophyll in *Hypericum perforatum* L. with different treatments (Control, 100 ppm of Nickel and 300 ppm of Nickel) over four harvests cut and phenological phasis. The number of stars (***) in T2 represents significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively.

3.3 Organic acids

Organic acid analysis showed that the content of oxalic, malic and citric acids were modulated by Ni treatment in Hypericum leaves, while only malic acid accumulation was enhanced by Ni treatment in Melissa (**Table 9**). More specifically, citric acid was the most accumulated organic acid in Hypericum and its content in leaves was enhanced in Ni-exposed plants compared to the control, with the highest value occurring in plants treated with 300 ppm Ni. Thus, a positive trend in citric acid concentration in leaves and contamination exposure was observed.

Oxalic acid content decreased below the control level in plants treated with 100 ppm of Nickel in Hypericum.

Citric acid content showed the same trend as Oxalic acid in Melissa plants, with no difference recorded between nickel treated plants and control. In the literature, there is a large body of evidence indicating an involvement of organic acids in metal xylem loading, root-to-shoot transport and hyperaccumulation (Callahan et al. 2006), and OA metabolism has been reviewed as a key component of the metal tolerance mechanism (Lopez-Bucio et al. 2000). In particular, tolerance to Ni in both hyperaccumulating and non-hyperaccumulating plants has been associated with the metal complexation properties of OAs (Yang et al. 1997).

In Hypericum, the enhancement of organic acids level in aerial part of Ni-exposed plants compared to control plants may be associated with a notable tolerance responses expressed when exposed to the contaminant (**Table 9**). Thus, the increase in the citric acid content in leaves suggests that, at moderate Ni concentration, the absence of damage symptoms in growth parameters and photosynthetic activity (**Table 6, Table 8**) occurring in plants treated might be due, in part, to the complexing affinity of citric acid to Ni able to reduce free ion activity and thus toxicity. Citric acid exhibits a high affinity for Ni chelation, forming stable metal-ligand complexes that reduce the concentration of free Ni ions in the cellular compartments (Ehsan et al., 2014). This complexation likely mitigates Ni-induced toxicity by limiting its bioavailability and preventing excessive accumulation in physiologically sensitive compartments. As reported, the possible absence of detrimental effects on growth parameters and photosynthetic activity in Ni-treated plants may be, at least in part, attributed to the capacity of citric acid to modulate Ni speciation and reduce its toxic impact at the biochemical level (Yin et al., 2024).

A strong induction of oxalic and citric acid content in Ni hyperaccumulator plant, after Ni treatment was reported as well as a correlation between high oxalic and citric acid content and Ni tolerance (Saito et al. 2015). Otherwise, a minor metal-ligand role of malic acid in mitigating Ni stress was previously reported (Pietrini et al., 2015).

Thus, different trends of modification of OAs content occurring in Ni-treated plants belonging to different species, suggested that the role of oxalic, malic, or citric acid as metal ligand strictly depend on plant species characteristics. Many TME hyperaccumulator plants enhance metal uptake by exuding organic acids from their roots. This process increases metal bioavailability in the rhizosphere, facilitating uptake (Seregin et al., 2024).

Phenological stages (Harvest time) have a major impact on OAs content in both Melissa and Hypericum, particularly Oxalic and Malic acid that showed similar trends for these two species (**Table 9)**. Oxalic acid and Malic acid highest concentration was detected at 60 days and 120 days after the transplant, respectively, suggesting that the synthesis of these organic acids was probably influenced not only by nickel adaptability of the plant but also by the plant development, with low differences between species. On the other hand, citric acid trend was partially differentiated between the two species, since in Hypericum plants the highest concentration of this acid was recorded 60 and 120 days after treatments, otherwise in Melissa plants only 120 days after transplant (**Table 9**). In the present study, oxalic acid was synthetized by the plant in very early developmental stages (no later than 120 days after Nickel stress), otherwise malic and citric acid after long exposure to environmental stress; suggesting that differences in OAs biosynthesis may be linked to plants adaptability to Nickel stress conditions through different growth stages.

Table 9. Organic acids content in leaves material (dry weight): oxalic acid, malic acid, as well as citric acid for *Hypericum perforatum* L. and *Melissa officinalis* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) for four consecutive time. Different letters within each column for each location represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, OA = Oxalic acid, MA = Malic acid and CA = Citric acid.

		OA mg/100g				MA mg/100g				CA mg/100g			
		Hypericum		Melissa		Hypericum		Melissa		Hypericum		Melissa	
Treat.	NT	14.62	a	23.78	a	97.07	c	192.71	b	179.02	c	62.33	a
	N100	11.60	b	23.19	a	103.67	b	204.01	b	216.20	b	66.43	a
	N300	13.05	a	26.54	a	118.74	a	250.04	a	227.09	a	69.44	a
Harvest Time	T0	24.76	a	24.01	b	93.13	b	56.08	c	215.26	b	46.79	c
	T1	25.98	a	31.70	a	77.89	c	103.08	c	233.21	a	48.07	c
	T2	8.21	b	24.20	b	71.65	c	139.98	b	143.09	c	64.23	b
	T3	7.15	b	21.13	b	140.45	a	336.23	a	225.42	ab	79.20	a
	LSD 0.05	9.5		9.5		9.3		9.3		12.5		12.5	
P	*Treat*	*		ns		***		**		***		ns	
	HTime	***		***		***		**		***		***	
	TxHT	**ns**		**		**ns**		**ns**		**ns**		**ns**	

3.4 Targeted analyses of metabolic compounds

The cultivation of *Melissa officinalis* L. and *Hypericum perforatum* L. for medicinal purposes is a compromise between yield, and the content of bioactive compounds and essential oil in the harvested product (Grevsen et al., 2008).

The impact of TME on the accumulation of phenolic and other bioactive compounds, and essential oil yield is of main interest and needs to be further investigated (Adamczyk-Szabela et al. 2019; Adamczyk-Szabela et al. 2022; Zheljazkov et al. 2008; Adamczyk-Szabela et al. 2023). Although pharmacological activities of MAPs, as well as the influence of location and harvest cuts, have been extensively studied, limited data are available on the detailed phytochemical profiles of Melissa and Hypericum species, the impact of TME on the accumulation of these bioactive compounds (i.e. the specialized metabolites), and the cultivation of these species for phytoremediation purposes. Thus, in the present study the effect of Nickel on specialized metabolites accumulation and diversity was characterized.

Melissa officinalis L. free and total polyphenols (FP and TP, respectively) were strongly influenced by treatment and harvest time. The highest TP content was observed in plants subjected to 300ppm of Nickel contamination (2175.1 mgGAE/100g [FW]). Otherwise, for the bound components of polyphenols no statistical difference between the treatments was observed. Polyphenols are widely reputed in the literature to be induced under stress conditions (Di Virgilio et al. 2015). Indeed, the same trend was observed for Flavonoids (Free, Bound and Total Flavonoids), and antioxidant activity (DPPH) with higher concentration of molecules detected for the plants cultivated under Nickel stress conditions (300 ppm) (**Table 8**). The present results corroborated previous findings, showing higher levels of polyphenols and increased DPPH under stress conditions. The antioxidant activity of the plants characterized in these studies could be mainly attributed to phenolic compounds, regardless of the cultivation area or extraction method (Miraj et al., 2017). The recorded levels of phenolics and antioxidant activity where in the average range of values in literature, taking into account the crop age and the controlled environment conditions (Boneza et al. 2018; Petkova et al. 2017; Mabrouki et al., 2018; Atanasova et al. 2023).

The highest levels of rosmarinic acid (RA), the most abundant health-promoting metabolite in lemon balm, were recorded 120 days after transplant (T3) and for the N300 condition. While RA content of lemon balm stressed plants harvested in T3 and T2 was comparable to previous results (Atanasova et al. 2023), the remaining cuts were characterized by lower RA quantity in leaves (**Table 10).**

Unlike RA, for which the highest content was observed in T3, PT, FT and DPPH were more concentrated in T2 cut (**Table 10**).

Table 10. Polyphenols, flavonoids and rosmarinic acid content as well as antioxidant activity for *Melissa officinalis* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) for four consecutive harvest time. Different letters within each column for each location represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, FP = free polyphenols, FB = bound polyphenols, TP = total polyphenols, FF = free flavonoids, BF = bound flavonoids, TF = total flavonoids, DPPH = 1,1- diphenyl-2-picrylhydrazyl anti-radical activity, FW = fresh weight, GAE = gallic acid equivalent, CE = catechin equivalent and TE = Trolox equivalent

		Polyphenols mgGAE/100g FW						Flavonoids mgCE/100g FW						DPPH µmolTE/g FW		Rosmarinic acid mg/100g FW	
		FP		BP		TP		FF		FB		FT					
Treatment	Control	1149,51	c	530,36	ab	1679,87	c	966,3	c	285,38	a	1251,68	c	118,49	c	816,68	b
	N100	1618,68	b	467,83	b	2086,51	b	1346,9	b	300,66	a	1647,56	b	156,59	b	831,63	b
	N300	2164,65	a	578,35	a	2743	a	1854,33	a	320,73	a	2175,06	a	182,13	a	900,36	a
Harvest Time	T0	1461,49	bc	50,99	c	1512,48	b	1458,34	b	162,05	c	1620,39	b	173,06	a	575,91	c
	T1	1567,22	b	1397,1	a	2964,32	a	1307,44	b	479,37	a	1786,81	b	136,31	b	249,06	d
	T2	2189,67	a	176,35	b	2366,02	b	1974,39	a	101,5	c	2075,89	a	184,9	a	1122,22	b
	T3	1236,89	c	161,27	bc	1398,16	c	862,64	c	372,64	b	1235,28	c	119,60	c	1505,22	a
	LSD	208,4		99,5		204,2		233,4		113,3		259,6		16,9		72.8	
P	Treat.	***		ns		***		***		*		***		***		**	
	HT	***		***		***		***		***		***		***		***	
	TxHT	***		**		***		***		***		***		**		***	

Hypericum perforatum L. polyphenols (FP and TP) and flavonoids (FF and TF) were strongly influenced by treatments, and harvest time. The highest total phenolic content was observed in the condition Nickel 100 mg kg-1 and the lowest amount for control plants, with 1964.87 and 1436.07 mg/100g (FW), respectively. Otherwise, for the bound components of polyphenols no statistical difference between the treatments was observed. Similarly, flavonoids showed the highest content in N100 plants with 1372.5 mg/100g (FW). Antioxidant activity was weakly influenced by the treatment (**Table 11**). Total polyphenols, flavonoids as well as DPPH obtained results were in the range of previously reported values (Kalogeropoulos et al. 2010; öztürk et al., 2009)

As previously reported, *H. perforatum* medicinal properties have been associated with the phenolic composition. *H. perforatum* extracts, which contain manly polyphenolic compounds as flavonoids and phenolic acids, suggesting that are involved in important antioxidant properties DPPH (Silva et al. 2008). Among the numerous bioactive compounds synthesized in *H. perforatum* tissues, hypericin and pseudohypericin are the two major ones (Miller, 1998). The highest levels of Hypericin, were recorded 60 days after transplant (T1) and no significative difference between treatments was observed (**Table 11**). The peak of hypericin content in leaves coincide with vegetative stage and flower bud phases, as previously reported (Zobayed et al. 2006). Furthermore, Hypericin synthesis is strictly regulated by the plant and its environment (Zobayed *et al*., 2003; Zobayed *et al*., 2005; Mosaleeyanon *et al*., 2005). Major aspects of this regulation may involve transport, as well as accumulation in the dark glands located in different organs of the plant, including leaf, stem and flower tissues.

The content of hypericin varied based on different habitats and the stage of plant development also reported by Saddique et al. (2010), in particular, positive correlation between organic carbon and N content in soil and hypericin biosynthesis had been demonstrated (Saffariha et al. 2021). However, in present study no significative difference between soil conditions was found.

Table 11. Polyphenols, flavonoids and hypericin content as well as antioxidant activity for *Hypericum perforatum* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) for four consecutive harvest time. Different letters within each column for each treatment represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Ns = not significant, FP = free polyphenols, FB = bound polyphenols, TP = total polyphenols, FF = free flavonoids, BF = bound flavonoids, TF = total flavonoids, DPPH = 1,1- diphenyl-2-picrylhydrazyl anti-radical activity, FW = fresh weight, GAE = gallic acid equivalent, CE = catechin equivalent and TE = Trolox equivalent

		Polyphenols mgGAE/100g FW						Flavonoids mgCE/100g FW						DPPH µmolTE/g FW		Hypericin mg/kg FW	
		FP		BP		TP		FF		FB		FT					
Treatment	Control	1293,13	c	142,94	a	1436,07	c	947,9	c	55,28	a	1003,18	c	150,837	b	29,04	a
	N100	1858,75	a	106,12	a	1964,87	a	1.332	a	40,35	b	1372,483	a	166,286	ab	25,83	a
	N300	1617,88	b	132,78	a	1750,66	b	1154,69	b	51,38	ab	1206,07	b	173,478	a	33,32	a
Harvest Time	T0	681,14	c	46,53	c	727,67	d	474,87	c	19,75	c	494,62	c	90,789	c	40,90	a
	T1	818,9	c	208,18	a	1027,08	c	529,63	c	69,76	a	599,39	c	68,341	c	41,29	a
	T2	1928,67	b	101,5	b	2030,17	b	1631,97	a	42,19	b	1674,16	a	159,195	b	29,04	b
	T3	2325,11	a	99,08	b	2424,19	a	1496,47	b	44,82	b	1541,29	b	263,065	a	18,09	c
	LSD	251,4		46,5		250,5		162,5		16,2		161,7		33.6		9,3	
P	Treat.	***		ns		***		***		*		***		*		ns	
	HTime	***		***		***		***		***		***		***		***	
	TxHT	*		**		ns		**		**		*		ns		ns	

3.5 PCA metabolic compounds and physiological parameters

3.5.1 PCA *Hypericum perforatum* L.

To examine a possible grouping of the hypericum leaf samples for health promoting components and effects of treatments and harvest cuts, PCA was carried out (Fig. 6). According to the preliminary PCA, a communality value of ≥ 0.5 described all variables, thus they were all included in the test. The first two components (PC1 and PC2) explained the 66.87% of the variance (**Figure 7**). A distinctive separation for harvest time was evident. The quadrant with positive branch of PC1 and negative branch of PC2 was associated with oxalic (OA) and citric acid (CA), distinctive for first cut (T0) for all treatments, and T1 for control, inversely proline and phenolic content. T1 cuts for N300 and N100 treatments were placed on the positive branch PC1 and PC2 and associated with Chlorophyll and hypericin content and negatively associated with antioxidant activity DPPH and malic acid (MA), distinctive for T3 control and N300 treatments. Negative branch of PC1 and positive PC2 were associated with PT, FT and Proline content, representative of T2 harvest cut for all treatments (control, N100 and N300) and of T2 only the N100 treatment. TP and TF contents were detected in higher content in N100 treatment for T3 and T2, respectively. thus, the phenolic content is not strictly correlated to the highest contaminant concentration. In addition, the total hypericin content was not impacted by Nickel stress condition, as reported in **Table 11**. Between organic acids, oxalic acid was influenced mostly by the harvest cut, and it was higher in T0. In Hypericum, the enhancement of OAs level in aerial part of Ni-exposed plants compared to control plants may be associated with a notable tolerance responses expressed when exposed to the contaminant. Thus, OA and proline compounds may act as an auxiliary to the plant for stress management, preventing ROSS formation by consequently ranging to limitations in active principles with antioxidant activity in high Nickel occurrences.

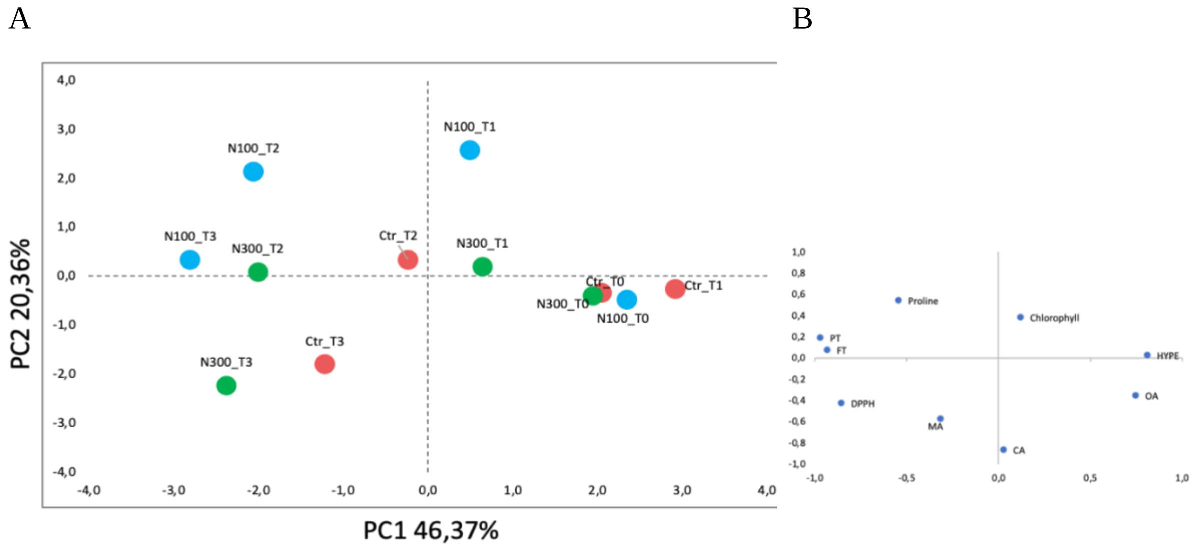


Figure 7. A - B. Principal component analysis based on *Hypericum perforatum* L. leaf bioactive and physiological markers contents. The scatter plots report the projection of cases (Hypericum samples) and variables (bioactive compounds, organic acids, proline and chlorophyll) on the first two principal components (PC1 and PC2). For treatments: (Ctr = control; N100 = Nickel 100 ppm and N300 = Nickel 300 ppm) and for harvest cut (T0 = 30 days after transplant; T1 = 60 days after transplant; T2 = 90 days after transplant; T3 = 120 days after transplant) (**Figure 7A**). TP = total polyphenols, TF = total flavonoids, DPPH = 1,1-diphenyl-2-picrylhydrazyl anti-radical activity, MA = malic acid, CA = citric acid, OA = oxalic acid, Proline, Chlorophyll, HYPE = hypericin (**Figure 7B**).

3.5.2 PCA *Melissa officinalis* L.

To examine a possible grouping of *Melissa officinalis* L. leaf samples for health promoting components and effects of treatments and harvest cuts, PCA was carried out (**Figure 8**). According to the preliminary PCA, a communality value of ≥ 0.5 described all variables, thus they were all included in the test. The first two components (PC1 and PC2) explained the 68.15% of the variance (Fig.7). A distinctive separation for harvest time was evident and the effects of single treatments was affected by the harvest time. The quadrant with positive branch of PC1 and negative branch of PC2 was associated with proline (PRO), total polyphenols (TP), rosmarinic acid (RA), malic (MA), distinctive for last two cuts (T2 and T3) for Nickel stressed plants with highest contaminant concentration, inversely oxalic acid and chlorophyll content. T1 cuts for all treatments (control, 100 and 300 nickel content) and N100 treatment T2 were placed on the negative branch PC1 and PC2 and associated with total flavonoids and antioxidant activity DPPH, negatively correlated with T3 for control and N100 treatments, placed in positive branch of PC1 and PC2. These cases were positively correlated to citric acid (CA). Phenolics and the major bioactive compound (RA) were strictly related to high Nickel level, thus a positive correlation between bioactive compounds biosynthesis and high contamination level for lemon balm may be hypnotized. Proline and malic acid showed to be involved in Nickel stress regulation for *Melissa officinalis* L., in case of high nickel concentration (300 ppm) after longer time exposure (more than 60 days). The enhancement of the other OAs (oxalic and citric) was not impacted by the content of the contaminant in soil, even if it was evident their action in short time exposure (before 60 days after the exposure). Oxalic and citric acids may act against the contamination in very early plant developmental stages, in order to avoid ROS formations. Furthermore, flavonoids as well as antioxidant activity DPPH were more representative for T1 harvest cut, suggesting that these molecules might be synthesized for plant protection from the earliest stages of development, regardless of the contaminant concentration in the soil.

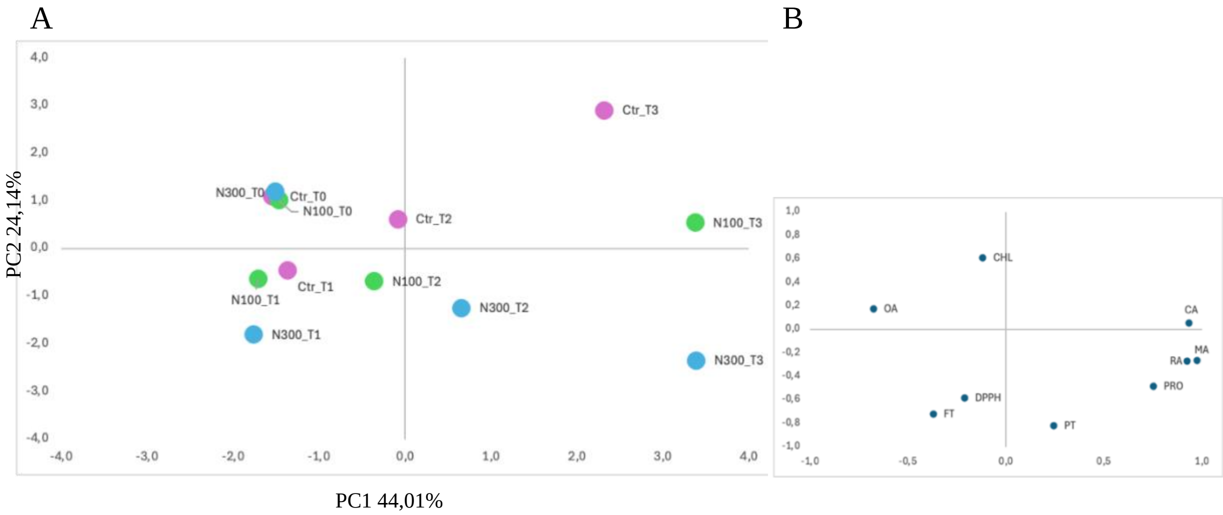


Figure 8. A - B. Principal component analysis based on *Melissa officinalis* L. leaf bioactive and physiological markers contents. The scatter plots report the projection of cases (Lomon balm samples) and variables (bioactive compounds, organic acids, proline and chlorophyll) on the first two principal components (PC1 and PC2). For treatments: (Ctr = control; N100 = Nickel 100 ppm and N300 = Nickel 300 ppm) and for harvest cut (T0 = 30 days after transplant; T1 = 60 days after transplant; T2 = 90 days after transplant; T3 = 120 days after transplant) (**Figure 8A**). TP = total polyphenols, TF = total flavonoids, DPPH = 1,1-diphenyl-2-picrylhydrazyl anti-radical activity, MA = malic acid, CA = citric acid, OA = oxalic acid, PRO = Proline, CHL = chlorophyll, RA = rosmarinic acid (**Figure 8B**).

3.6 Nickel and elements content in above ground material and roots

3.6.1 Total elements content in Above-Ground Materials

Nickel concentration as well as total element content in plants above ground material, was evaluated (**Table 12 A**; **Table 12 B**). The impact of Nickel on elements uptake for both species during the experimental period was analyzed. Ni has significantly affected the distribution of elements content in both examined MAPs. From analysis the difference between the two species was evident. Hypericum uptake of some elements as Ag, Ba, Ca, Cd, Co, Cu, Fe, K, Mn, S, Zn was statistically impacted by high Nickel content in soil, since all these elements were more concentrated in control plants tissues except for Cd, that was higher in N300 treatment (**Table 12 A**).

Otherwise, *Melissa officinalis* L. minerals uptake and translocation were less affected by high Nickel concentrations in soil if compared to Hypericum. Only Na and B uptake were compromised by Nickel 100 and 300 ppm conditions. Meanwhile Al, Ca, K, Li, Mg, Sr, Ti and V uptake and translocation to above ground organs was positively impacted by medium-high nickel level as reported in **Table 12 B**. This behavior suggested *Melissa officinalis* L. ability in mitigate Nickel impact on physiological mechanisms.

As previously reported in **Table 6**, the FW and total biomass for both MAPs were influenced by Nickel content in soil, with significantly higher yield values in Nickel 300 and 100 conditions for Hypericum and Melissa, respectively.

This result suggested that the increase in biomass observed under Nickel conditions might have influenced the apparent concentration of other elements observed in plants tissues. Specifically, the decrease in elemental concentrations at higher Ni levels might not necessarily indicate a reduced uptake but could be attributed to a dilution effect due to enhanced plant growth, as previously reported (Chen et al., 2010, Yoon et al., 2006).

Consequently, the apparent concentration of elements in above ground portions might be linked to biomass production and/or biomass dilution. For this reason, the absolute uptake based on obtained biomass was calculated on above-ground material results for both the examined species (**Table 13 A and B**).

In Hypericum differences were confirmed for the previously cited elements (**Table 13 A**) in terms of absolute uptake, except for Ba, K and V. Thus, it might be assumed that in the case study of *Hypericum perforatum* L. Nickel negatively affected Fe, Ag, Ca, Co, Mn, S, V, Zn translocation to above ground tissues, otherwise it positively impacted Cd, with changes in nutrient dynamics. In the case of *Melissa officinalis* L. absolute uptake results confirmed the Nickel effect on minerals uptake. Indeed, Al, B, Ca, K, Li, Mg, Sr, Ti and V absolute uptake for Melissa was statistically impacted by Nickel content (**Table 13B**). It might be assumed that Nickel 100 mg kg-1 concentration in soil positively affected these nutrients uptake, with changes in nutrient dynamics in Melissa. For *Melissa officinalis* L. nickel positively influenced biomass accumulation (**Table 6**) without significantly impairing nutrient uptake (**Table 12B and 13B**). Previous research shown that low to moderate nickel concentrations as 100 mg kg-1 condition can stimulate plant in growth and metabolic activities (Seregin & Kozhevnikova, 2006). Otherwise Hypericum positive effects on yield were not observed for nutrient dynamics as well. Nickel was reported to induce changes in nutrient dynamics (Amjad et al. 2019), affected the deficiency of Fe and Zn and hindered the uptake of other heavy metals such as Cd, Co, Cr, and Pb (Myśliwa-Kurdziel et al. 2004). Reported results might be observed in Hypericum lower translocation in Ni stressed plants, compared to control and higher absolute uptake levels for Cd. High concentration of Ni causes deficiency of these nutrients in plants by hindering their sorption, uptake, and translocation (Ahmad et al., 2010), and Ni toxicity significantly decreased the nutrient concentrations in both shoot and root (Ain et al. 2016; Amjad et al., 2019).

Further, Ni is reported to induce indirect damaging effects on photosynthesis by altering the uptake processes of several mineral nutrients, such as K, Mg, Cu, Fe and Zn (Palacios et al. 1998; Dietz et al. 2000), corroborating obtained results of chlorophyll content (**Fig. 6B**) in *Hypericum perforatum* L. As mentioned in chapter 3.2, the total chlorophyll content was affected by the contaminant only in the case study of *H. perforatum* L., but not for *M. officinalis* L; suggesting that plants protection mechanisms are mainly influenced by plant species, metal element, bioavailability and concentrations (Clemens 2006).

While excessive Ni exposure is generally toxic, plants may develop adaptive mechanisms to avoid metal stress, potentially leading to enhanced growth under certain conditions (Gajewska & Skłodowska, 2007). This could involve modifications in nutrient transport, hormonal balance, or antioxidant enzyme activity, all of which could contribute to increased biomass despite high Ni exposure.

Table 12 A and B. Elements content in above ground organs of A) *Hypericum perforatum* L. and B) *Melissa officinalis* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) at final harvest time (120 dat). Different letters within each column for each treatment represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Mineral are expressed in g/kg or mg/kg or μm/kg based on dry leaves material. AGO = Above ground organs; NS = No significant.

Above ground organs	CTR	Ni 100	Ni 300	mean	LSD	*p*
Ag μg kg-1	71,3 ^{A}	57,9 ^{AB}	39,9 ^{B}	56,4	12,9	*
Al mg kg-1	81,20	41,21	37,84	53,42	19,7	ns
As mg kg-1	1,10	0,89	0,92	0,97	0,1	ns
B mg kg-1	66,7	55,1	49,7	57,13	7,1	ns
Ba mg kg-1	8,17 ^{A}	7,08 ^{AB}	5,81 ^{B}	7,02	1,0	*
Ca g kg-1	6,12 ^{A}	5,35 ^{AB}	4,81 ^{B}	5,43	0,5	*
Cd μg kg-1	311,5 ^{AB}	294,4 ^{B}	385,5 ^{A}	330,5	39,5	**
Cl g kg-1	12,0	12,0	12,1	12,0	0,0	ns
Co μg kg-1	226,2 ^{A}	140,2 ^{B}	137,9 ^{B}	168,1	41,1	**
Cr μg kg-1	778,30	716,91	855,73	783,65	56,8	ns
Cu mg kg-1	10,4 ^{A}	9,94 ^{A}	7,98 ^{B}	9,43	1,0	**
Fe mg kg-1	135,8 ^{A}	70,0 ^{B}	64,7 ^{B}	90,13	32,3	**
K g kg-1	9,69	9,30	9,31	9,43	0,2	ns
Li μg kg-1	266,5 ^{A}	97,3 ^{B}	214,1 ^{AB}	192,7	70,7	**
Mg g kg-1	1,97	1,84	1,86	1,89	0,1	ns
Mn mg kg-1	67,4 ^{A}	46,4 ^{B}	38,6 ^{C}	50,8	12,2	**
Mo μg kg-1	852,2 ^{A}	403,8 ^{B}	330,4 ^{B}	528,8	230,6	**
Na mg kg-1	168,5	106,0	132,6	135,7	25,6	ns
P g kg-1	3,40	3,40	3,17	3,32	0,1	ns
Pb mg kg-1	1,27	1,18	1,09	1,18	0,1	ns
S g kg-1	2,21	2,11	1,83	2,05	0,2	ns
Sb μg kg-1	886,5	926,0	883,7	898,7	19,3	ns
Se μg kg-1	855,4	862,0	841,8	853,1	8,4	ns
Si mg kg-1	47,5	33,9	34,7	38,7	6,2	ns
Sr μg kg-1	234,5	215,5	188,9	212,9	18,7	ns
Ti μg kg-1	920,5	1024,00	902,4	949,0	53,6	ns
V μg kg-1	225,7 ^{A}	139,3 ^{B}	122,2 ^{B}	162,4	45,3	**
Zn mg kg-1	41,2 ^{A}	37,0 ^{A}	28,1 ^{B}	35,5	5,5	**

A

Above ground organs	CTR	Ni 100	Ni 300	mean	LSD	*p*
Ag μg kg-1	49,8	45,2	33,0	42,7	7,1	ns
Al mg kg-1	63,9 ^{B}	100,7 ^{A}	95,1 ^{A}	86,6	16,2	**
As mg kg-1	1,1	1,0	1,1	1,08	0,1	ns
B mg kg-1	42,3	40,2	36,4	39,62	2,5	*
Ba mg kg-1	28,0	34,3	32,9	31,7	2,7	ns
Ca g kg-1	11,0	12,2	13,5	12,2	1,0	***
Cd μg kg-1	235,4	313,4	249,2	266,0	34,0	ns
Cl g kg-1	11,29	9,07	8,57	9,64	1,2	ns
Co μg kg-1	89,17	61,68	69,78	73,54	11,5	ns
Cr mg kg-1	0,89	1,07	1,16	1,04	0,1	ns
Cu mg kg-1	9,48	10,01	9,96	9,82	0,2	ns
Fe mg kg-1	89,5	123,3	119,7	110,8	15,2	ns
K g kg-1	9,92 ^{B}	10,3 ^{A}	10,0 ^{A}	10,1	0,2	***
Li mg kg-1	1,62 ^{B}	1,83 ^{A}	1,66 ^{B}	1,70	0,1	**
Mg g kg-1	3,72	3,73	3,86	3,77	0,1	**
Mn mg kg-1	34,4	31,1	33,6	33,0	1,4	ns
Mo μg kg-1	110,5	127,9	116,9	118,5	7,2	ns
Na mg kg-1	1815 ^{A}	770 ^{C}	1196 ^{B}	1260	429,2	***
P g kg-1	2,87	3,03	3,14	3,01	0,1	ns
Pb mg kg-1	1,59	1,30	1,31	1,40	0,1	ns
S mg kg-1	309,3	372,4	341,4	341	25,8	ns
Sb μg kg-1	814,1	913,6	945,9	891,2	56,1	ns
Se μg kg-1	997,9 ^{A}	1046,9 ^{A}	921,2 ^{B}	988,7	51,8	**
Si mg kg-1	46,77	44,24	45,11	45,4	1,0	ns
Sr μg kg-1	42,88	46,32	51,65	46,95	3,6	***
Ti mg kg-1	1,21 ^{B}	1,95 ^{A}	1,89 ^{A}	1,68	0,3	*
V μg kg-1	224,8 ^{A}	217,6 ^{A}	156,3 ^{B}	199,6	30,7	**
Zn mg kg-1	31,78	32,19	27,51	30,5	2,1	ns

B

Table 13 A and B. Elements absolute uptake considering total biomass (kg/ha) in above ground organs of A) *Hypericum perforatum* L. and B) *Melissa officinalis* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) at final harvest time (120 dat). Different letters within each column for each treatment represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Mineral are expressed in g/kg or mg/kg or µm/kg based on dry leaves material. AGO = Above ground organs; NS = No significant.

A - *Hypericum perforatum* L. absolute uptake

	Ctr	N100	N300	Mean	LSD	*p*
Ag mg	363,6 A	289,5 AB	205,8 B	286,3	64,5	*
Al g	414,0	206,1	195,0	271,7	100,7	ns
As g	5,6	4,5	4,7	4,9	0,5	ns
B mg	340,0	275,4	255,9	290,4	35,9	ns
Ba g	41,7	35,4	29,9	35,7	4,8	ns
Ca kg	31,2 A	26,8 AB	24,8 B	27,6	2,7	*
Cd mg	1588,4 B	1472,7 B	1987,0 A	1682,7	220,3	**
Cl kg	61,1	60,0	61,9	61,0	0,8	ns
Co mg	1153,5 A	701,1 B	710,8 B	855,2	211,0	**
Cr mg	3968,6	3586,0	4410,4	3988,3	336,9	ns
Cu g	52,9	49,7	41,1	47,9	5,0	ns
Fe g	692,2 B	350,0 B	333,3 B	458,5	165,4	**
K kg	49,4	46,5	48,0	48,0	1,2	ns
Li mg	1359,0	487,1	1103,8	983,3	366,0	ns
Mg kg	10,1	9,2	9,6	9,6	0,4	ns
Mn g	344,0 A	232,2 B	199,2 B	258,5	62,0	**
Mo g	4345,3	2019,6	1702,9	2689,2	1178,1	ns
Na g	859,3	530,1	683,2	690,8	134,5	ns
P kg	17,3	17,0	16,3	16,9	0,4	ns
Pb g	6,5	5,9	5,6	6,0	0,4	ns
S kg	11,3 A	10,6 B	9,4 C	10,4	0,8	***
Sb mg	4520,0	4632,0	4554,4	4568,8	46,8	ns
Se mg	4361,5	4311,7	4338,6	4337,3	20,3	ns
Si g	242,3	169,5	178,8	196,9	32,3	ns
Sr g	1195,6	1078,0	973,5	1082,3	90,7	ns
Ti mg	4693,6	5122,0	4651,1	4822,2	212,7	ns
V mg	1150,8	696,8	629,7	825,8	231,5	ns
Zn g	210,2 A	185,3 AB	145,1 B	180,2	26,8	**

B- *Melissa officinalis* L. absolute uptake

	Ctr	N100	N300	Mean	LSD	*p*
Ag mg	557,5	485,7	372,7	472,0	76,1	ns
Al g	715,6 B	1081,8 A	1071,8 A	956,4	170,3	*
As g	12,8	10,7	12,5	12,0	0,9	ns
B mg	473,3 A	431,7 AB	409,9 B	438,3	26,3	**
Ba g	313,8	368,1	370,7	350,9	26,2	ns
Ca kg	123,2 B	130,7 AB	152,1 A	135,3	12,2	***
Cd mg	2634,3	3366,2	2809,0	2936,5	312,1	ns
Cl kg	126,4	97,4	96,6	106,8	13,9	ns
Co mg	997,8	662,4	786,4	815,6	138,4	ns
Cr mg	10,0	11,5	13,1	11,5	1,3	ns
Cu g	106,1	107,5	112,2	108,6	2,6	ns
Fe g	1001,2	1323,9	1349,3	1224,8	158,4	ns
K kg	111,0 B	110,5 C	113,1 A	111,6	1,1	**
Li mg	18,1	19,6	18,8	18,8	0,6	*
Mg kg	41,6	40,1	43,5	41,7	1,4	**
Mn g	384,5	334,2	378,2	365,6	22,4	ns
Mo g	1237,0	1373,2	1317,9	1309,4	56,0	ns
Na g	20315,8 A	8271,9 B	13483,2 AB	14023,7	4931,7	***
P kg	32,1	32,6	35,3	33,3	1,4	ns
Pb g	17,8	14,0	14,8	15,5	1,6	ns
S kg	3460,5	3999,0	3847,4	3769,0	226,8	ns
Sb mg	9109,3	9811,9	10660,6	9860,6	634,2	ns
Se mg	11167,0 B	11244,1 A	10381,5 C	10930,9	389,7	**
Si g	523,3	475,2	508,4	502,3	20,1	ns
Sr g	479,8 B	497,4 AB	582,1 A	519,8	44,7	***
Ti mg	13,6 B	21,0 A	21,3 A	18,6	3,6	*
V mg	1749,4 B	2414,3 A	2452,0 A	2205,2	322,7	**
Zn g	355,6	345,7	310,0	337,1	19,6	ns

3.6.2 Total elements content in Roots

Nickel concentration as well as total element content in plants roots, were evaluated (**Table 14 A**; **Table 14 B**). The impact of Nickel treatment on elements uptake for both species during the experimental period was analyzed. Reported results express the apparent concentration, rather than absolute elements uptake, since for roots the total biomass was not evaluated. In *Hypericum perforatum* L. Cd and Mn content in roots was impacted by Ni treatment (**Table 14 A**). In particular, Cd content was higher in N300 condition, despite Mn content was statistically higher in Control condition, as reported for above ground organs. In Hypericum Cd and Mn might have an higher affinity for nickel-influenced transporters as previously reported for other hyperaccumulator species (Ahmad and Ashraf, 2011).

Otherwise, Ni did not significantly affect the distribution of elements content in *Melissa officinalis* L. roots as reported (**Table 14 B**).

Results underlined the different behavior of the two species even if for both Nickel impact was more evident in above ground tissues than in roots. In particular, Melissa might be more effective in mitigating the impact of nickel, while St. John's Wort might have more sensitive transporters or higher specificity for those elements involved in altered translation for both above ground organs and roots.

Table 14 A – B. Elements content in roots of A) *Hypericum perforatum* L. and B) *Melissa officinalis* L. within each treatment (Control, Nickel 100 ppm and 300 ppm) at final harvest time (120 dat). Different letters within each column for each treatment represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Elements are expressed in g/kg or mg/kg or µm/kg based on dry leaves material.

A - *Hypericum perforatum* L.

Elements	CTR	Ni 100	Ni 300	LSD	Mean	*p*
Ag µg kg-1	43,8	29,0	58,9	15,0	43,9	ns
Al mg kg-1	88,8	70,2	99,2	14,7	86,1	ns
As mg kg-1	3,5	4,1	4,4	0,4	4,0	ns
B mg kg-1	21,0	21,1	24,9	2,2	22,3	ns
Ba mg kg-1	59,4	49,1	55,9	5,2	54,8	ns
Ca g kg-1	28,8	28,4	33,8	3,0	30,3	ns
Cd µg kg-1	989,0 B	821,9 B	1115,7 A	147,4	975,6	**
Cl g kg-1	8,59	7,41	6,37	1,1	7,5	ns
Co mg kg-1	5,79	4,71	6,32	0,8	5,6	ns
Cr mg kg-1	4,9	4,7	5,2	0,3	4,9	ns
Cu mg kg-1	41,7	34,9	41,5	3,9	39,4	ns
Fe g kg-1	10,6	9,4	12,1	1,3	10,7	ns
K g kg-1	4,9	4,0	4,5	0,5	4,4	ns
Li mg kg-1	20,7	17,6	23,9	3,2	20,7	ns
Mg g kg-1	4,3	3,7	4,7	0,5	4,2	ns
Mn mg kg-1	351,5 B	390,8 AB	510,3 A	82,7	417,5	*
Mo µg kg-1	557,5	400,0	485,8	78,8	481,1	ns
Na g kg-1	2,8	1,6	2,2	0,6	2,2	ns
P mg kg-1	941,3	692,1	796,2	125,1	809,9	ns
Pb mg kg-1	10,8	11,1	11,7	0,5	11,2	ns
S g kg-1	1,0	0,9	1,0	0,1	1,0	ns
Sb mg kg-1	1,4	1,4	1,4	0,0	1,4	ns
Se mg kg-1	1,7	1,4	2,1	0,4	1,7	ns
Si mg kg-1	35,7	73,4	44,2	19,8	51,1	ns
Sr mg kg-1	136,6	115,8	141,7	13,7	131,4	ns
Ti mg kg-1	26,1	47,7	27,9	12,0	33,9	ns
V mg kg-1	22,3	17,7	25,8	4,1	21,9	ns
Zn mg kg-1	46,8	44,4	56,3	6,3	49,2	ns

B – Melissa officinalis L.

Elements	CTR	Ni 100	Ni 300	LSD	Mean	*p*
Ag µg kg-1	25,8	35,2	48,7	11,5	36,6	ns
Al mg kg-1	7,08	8,59	6,31	1,2	7,3	ns
As mg kg-1	2,5	3,4	2,4	0,6	2,8	ns
B mg kg-1	20,3	20,7	19,7	0,5	20,2	ns
Ba mg kg-1	42,8	49,4	35,5	7,0	42,5	ns
Ca g kg-1	22,3	28,5	21,5	3,8	24,1	ns
Cd µg kg-1	761,0	972,1	789,0	114,7	840,7	ns
Cl g kg-1	8,0	7,9	9,4	0,8	8,5	ns
Co mg kg-1	4,7	5,7	3,9	0,9	4,8	ns
Cr mg kg-1	7,7	9,2	4,2	2,6	7,0	ns
Cu mg kg-1	38,7	36,8	36,0	1,4	37,2	ns
Fe g kg-1	8,2	10,4	7,5	1,5	8,7	ns
K g kg-1	8,3	7,3	8,2	0,5	7,9	ns
Li mg kg-1	16,4	20,7	15,8	2,7	17,6	ns
Mg g kg-1	4,3	4,7	4,2	0,3	4,4	ns
Mn mg kg-1	246,4	311,3	217,3	48,1	258,3	ns
Mo µg kg-1	316,3	296,9	281,3	17,5	298,1	ns
Na g kg-1	1,8	1,9	2,6	0,4	2,1	ns
P g kg-1	1,5	1,2	1,7	0,2	1,5	ns
Pb mg kg-1	10,3	12,6	8,4	2,1	10,4	ns
S mg kg-1	769,6	887,7	803,8	60,7	820,4	ns
Sb mg kg-1	1,3	1,8	1,3	0,3	1,4	ns
Se mg kg-1	1,3	1,5	1,3	0,1	1,4	ns
Si mg kg-1	28,1	31,0	33,4	2,7	30,8	ns
Sr mg kg-1	92,7	120,5	99,1	14,5	104,1	ns
Ti mg kg-1	48,8	49,2	23,0	15,0	40,3	ns
V mg kg-1	17,7	21,8	16,4	2,8	18,7	ns
Zn mg kg-1	45,7	50,2	43,8	3,3	46,6	ns

3.6.3 Nickel content : BAF and TF

Nickel content in aerial parts, roots and soil was analyzed 60 and 120 days after transplant, respectively, for both the tested species (**Figure 9, Figure 10**). For both species, the differences of each treatment (Ctr, N100 and N300) within Roots, Above ground material and soil, respectively, and of harvests cuts (T60 and T120) within each treatment (Ctr, N100 and N300) were evaluated.

Hypericum perforatum L. roots showed higher Nickel concentrations in N100 and N300, compared to control. Highest values were detected in the last harvest cut and were 309 and 122 mg/kg for N300 and N100 treatment, respectively (**Figure 9A**), assuming a total Ni uptake in roots. The same trend was observed in leaves for control and Nickel 300 treatment, otherwise in Nickel 100 treatment the highest accumulation at 60 days (14.56 mg/kg) was reported (**Figure 9B**). The different behavior depending on Nickel soil content might be linked to the difference in concentration of Nickel in soil. In the first 60 days metal excess might induce physiological mechanisms such as saturation of transporters (reducing metal uptake from roots), metal exlusions mechanisms and induction of stress response. Otherwise, at Nickel 100 mg kg-1 concentration the plant still uptake efficiently, leading to higher accumulation in the above ground tissues in the first 60 days (Mohammad et al. 2011). It was evident that the uptake and translocation were proportional to Ni content in soil, since the concentration of metal in Ni treated plants was in line with the contaminant presence in soil and significantly higher than control.

Concerning metal availability in soil, at the end of the crop cycle, a complete soil restoration with nickel level comparable to control was observed (**Figure 9C**). Obtained final nickel content in each treated condition was 60.2 ± 3 mg/kg. This positive result is mainly due to the addition of Ni (NO3)2 6H2O to experimental soil, since it is characterized by high mobility and bioavailability.

Melissa officinalis L. Nickel uptake in roots was not impacted by harvest time for the control as well as the lowest Ni treated soil (N100) (**Figure 10A**). Thus, Ni absorption was immediately achieved within the first cut considering 100 mg kg-1 Ni (NO3)2 6H2O concentration. Indeed, at N100 the concentration might be within a range that allowed the plant uptake efficacely. Further for N300 condition roots metal uptake was higher in the first cut (60 days after transplant), with 254.5 mg kg-1 detected. However, 120 days after treatment the content of Ni in roots decreased, suggesting the transport to above grounds organs, or senescence mechanisms in roots. This could be supported by the costant decrease of Ni in soil (**Figure 10C**).

In above ground material, no effect of time was observed for both control and Ni 300 treatments, although metal content in Ni 100 treatment has been hardly impacted with higher concentration of metal 120 days after transplant (8.1 mg/kg of Nickel) (**Figure 10B**).

Nickel content in soil for N100 is not impacted by time, whereas for N300 differences between 60 and 120 days after transplant (156,2 and 55,7 mg/kg, respectively) were observed. Suggesting that in the case of low Nickel concentrations, the first effects of *Melissa officinalis* L. cultivation may be evident in the time prior to 60 days after transplanting (**Figure 10C**). In contrast, in the case of higher concentrations of nickel in the soil, a longer time may be needed to observe effects on the soil in term of contaminant concentration. In fact, in N300 the first results were obtained at 120 days after transplanting.

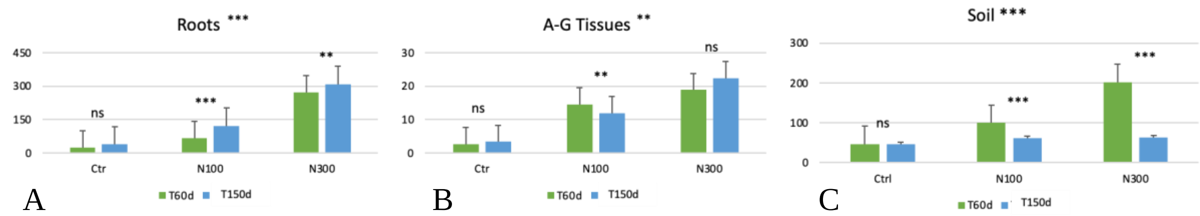


Figure 9. Graphical representation (histogram) of Nickel content in *Hypericum perforatum* L. with different treatments (Control, 100 ppm of Nickel and 300 ppm of Nickel) over two harvests cuts (T60d, T120d). The number of stars for each treatment represents significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. The difference of treatments within Roots, Above ground material and soil, respectively, and the differences within each treatment (Ctr, N100 and N300) concerning harvests cuts were evaluated. **Figure A, B** and **C** represent Roots, leaves and soil Nickel content, respectively.

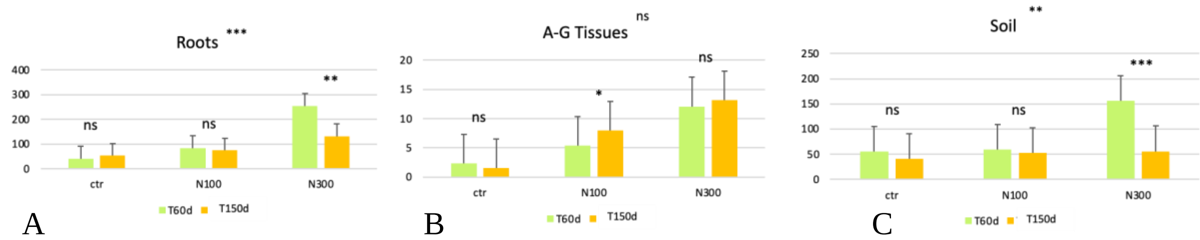


Figure 10. Graphical representation (histogram) of Nickel content in *Melissa officinalis* L. with different treatments (Control, 100 ppm of Nickel and 300 ppm of Nickel) over two harvests cuts. The number of stars for each treatment represents significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Fig. A, B and C represent Roots, leaves and soil Nickel content, respectively. The number of stars for each graph represents significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. The difference of treatments within Roots, Above ground material and soil, respectively, and the differences within each treatment (Ctr, N100 and N300) concerning harvests cuts were evaluated.

Plans growing in the presence of toxic elements might be categorized as “tolerant” and “hyperaccumulator”. A tolerant species is one that can grow on soil with concentrations of a particular element that are toxic to most other plants (Assuncao et al., 2001; Bert et al., 2003), therefore both examined species *Hypericum perforatum* L. and *Melissa officinalis* L. grown in contaminated soil were tolerant to the targeted heavy metal, as demonstrated by growth parameters results. Considering the hyperaccumulator definition of Baker and Brooks (1989), both analyzed plant species were hyperaccumulator for Ni. Bioaccumulation factor (BAF) and translocation factor (TF) for both analyzed species had been evaluated.

Both analyzed species had higher nickel content in roots than shoots, leading to low translocation factor (TF) from roots to above ground organs (**Table 15**). Higher Ni content in roots of a metal accumulator plant species is mainly dependent on the two main factors sequestration and/or translocation. The metal translocation process in plant species is a crucial factor in determining the metal distribution in different plant tissues (Page and Feller, 2015). Several factors including anatomical, biochemical and physiological factors (Antoniadis et al., 2017) may contribute to heavy metal accumulation and distribution in the upper vegetative parts. The tolerance of plants to increasing levels of toxic elements may result from exclusion or metabolic tolerance of these elements. Moreover, metal confinement in the roots is known to be a primary defense process in order to protect the photosynthetic apparatus in the leaves (Draźkiewicz and Baszyński 2005; Pietrini et al. 2005), allowing plants to differently withstand the metal presence in the environment.

BAF in N100 and N300 for both crop is higher than 1, the fixed threshold to indicate hyperaccumulating plants. Otherwise, at the end of crop cycle, where mean Nickel content in soil was comparable to the one of control, BAF values became higher (**Table 15**).

Hypericum perforatum L. showed higher BAF than *Melissa officinalis* L. at the end of crop cycle (120 days), but similar range were reached in BAF mean values. BAF values obtained for both crops were strictly related to metal content in soil (100, 300 or control).

In the present study, an effect in BAF was evident in relation to the metal enrichment in the soil, due more to the increasing bioavailability of Ni compared to control. However, the effect of the two different Ni concentrations impacted differently the respective TF and BAF values, for the two MAPs.

As reported, BAF mainly depends on both soil properties and plants characteristics, and it is mainly linked to the "availability concept" linked to both soil and plant (Farabegoli et al., 2009). Furter, roots compartmentalization also resulted in low levels of Nickel in the aerial parts, not affecting the quality of the final product, since maximum detected content of nickel ranged 13 and 22 mg/kg for lemon balm and hypericum, respectively. Thus, both species shown good adaptation to the metal with optimal BAF values and due to low TFs, that allowed the protection of above ground portions from metal damage.

Table 15. Translocation factor from soil to roots and roots to above ground organs, as well as Bioaccumulation factors (mean and 120 days after transplant), for *Hypericum perforatum* L.

and *Melissa officinalis* L. within each treatment (Control, Nickel 100 ppm and 300 ppm). Different letters within each column for each treatment represent significant different values ($p \leq 0.05$, Tukey's least significant difference test). The number of stars represent significant differences at the 0.05 (*), 0.01 (**), and 0.001 (***) probability level, respectively. Mineral are expressed in ppm or ppb based on dry leaves material.

	TF soil-roots		TF root-agm		BAF		BAF (120d)	
	HP	MO	HP	MO	HP	MO	HP	MO
CTR	0,849 b	0,945 b	0,086 b	0,044 b	0,905 b	0,993 a	0,765 c	1,030 b
N100	1,393 a	1,022 b	0,119 a	0,097 a	1,559 a	1,117 a	2,587 b	1,398 b
N300	1,688 a	1,410 a	0,072 b	0,052 b	1,704 a	1,141 a	5,264 a	3,321 a
p	**	*	*	*	**	*	***	***

3.7 Untargeted metabolomics to analyse specialized metabolite diversity

Specialized metabolite landscape was analyzed in Melissa and Hypericum species in cultivated in soil contaminated by 300 ppm of Nickel or in non-contaminated soil. In particular LC-MS/MS untargeted metabolomic analyses allowed the detection and characterization of polar and semi-polar metabolites, including phenylpropanoids, alkaloids and N-containing compounds and terpenoids. These analyses allowed the detection of 601 and 505 ions (or metabolic features) for *Melissa officinalis* L. and *Hypericum perforatum* L., respectively. Metabolic features were categorized into metabolic families: Amino acids and derivatives, Alkaloids, Cinnamic acids, Flavonoids, Lipids and FAs, Organic acids, Phenolic acids, Polyketides, Terpenoids and Carbohydrates. For *Melissa officinalis* L. the families were detected with the reported percentage: Amino acids and derivatives 7%, Alkaloids 3%, Cinnamic acids 27%, Flavonoids 4%, Lipids and FAs 13%, Phenolic acids 1%, Polyketides 1%, Terpenoids 20% and Carbohydrates 20%. Otherwise, for *Hypericum perforatum* L. percentages were detected as follow: Amino acids and derivatives 9%, Alkaloids 3%, Cinnamic acids 24%, Flavonoids 13%, Lipids and FAs 12%, Phenolic acids 1%, Polyketides 2%, Terpenoids 12% and Carbohydrates 2%. Thus, among all detected features, 143 and 109 had not been still annotated for lemon balm and hypericum, respectively. The higher Ni content in soil and consequent uptake of the metal in roots activated metabolic response in both analyzed MAPs. The adaptability response was reflected in the activation of metabolic pathways involved in metal and ROS detoxification (**Figure 11**). Identified candidate molecules involved in these processes for hypericum were molecules belonging to Terpenoids, FA (fatty acids), OA (Organic acids), Alkaloids and Cinnamic acids (Ion et al.,2022; Yılmazoğlu et al., 2023;

Sekeroglu et al. 2018). In the case study of *M. officinalis* L., the families involved in detoxification process were Phenolic acids, FA, Terpenoids and Cinnamic acids (Petrisor et al., 2022; Miraj et al., 2017) (**Figure 11**).

Lemon balm was characterized by higher Terpenoides and Carbohydrates compared to hypericum, otherwise a higher flavonoids percentage was detected for hypericum.

Statistical analysis of specialized metabolites allowed the identification of metabolic features whose accumulation was enhanced or repressed by Ni treatment (Volcano plot and Heatmap *p*-value of 0.001). As for *Hypericum perforatum* L., 15 (left side) and 14 (right side) metabolic features were enhanced and repressed by Nickel stress, respectively (**Figure 12**). More accumulated metabolites upon Ni stress were linked to Cinnamic acid, Terpenoid and Flavonoid families. As for *Melissa officinalis* L., 35 (left side) and 30 (right side) metabolic features were enhanced and repressed by Nickel stress, respectively (**Figure 13**). More accumulated metabolites upon Ni stress were mainly linked to Cinnamic acid, Terpenoid, Flavonoid and amino acid and derivatives families.

The metabolic response to stress observed in LC/MSMS analysis corroborated previous showed results on phenolics, proline and chlorophyll analysis (**Paragraphs 3.2, 3.3 and 3.4**).

Metabolomic profile results suggested two different strategies adopted by *H. perforatum* L. and *M. officinalis* L. to Ni, activating different metabolic pathways. Comparative metabolomics analyses of Ni-stress response highlighted several metabolites involved in diverse metabolic pathways. Several studies further confirmed results obtained (Villiers et al., 2011; Feng et al. 2021; Dubey et al., 2010)

Once entered the plants, Ni induce reactive oxygen species (ROS) formation, and the plants respond to ROS by stimulates the production of antioxidants and antioxidant enzymes as well as the production of various primary and secondary metabolites like flavonoids and phenolic compounds. Thus, Ni led to huge changes in the metabolome pattern of plants to contrast the ROS imposed by metal concentration in plant tissues.

Figure 11. Expression of metabolic class in *Hypericum perforatum* L. and *Melissa officinalis* L.. Heat map showing the metabolites expression of each metabolic family. The expression values were calculated as a Log value related to the sample showing the highest expression value for each metabolite (3 and -3 for red and blue colors, respectively) within the two MAPs species (control (Ctr) and Nickel (stress)). Metabolic class (METABOCLASS) names are also reported.

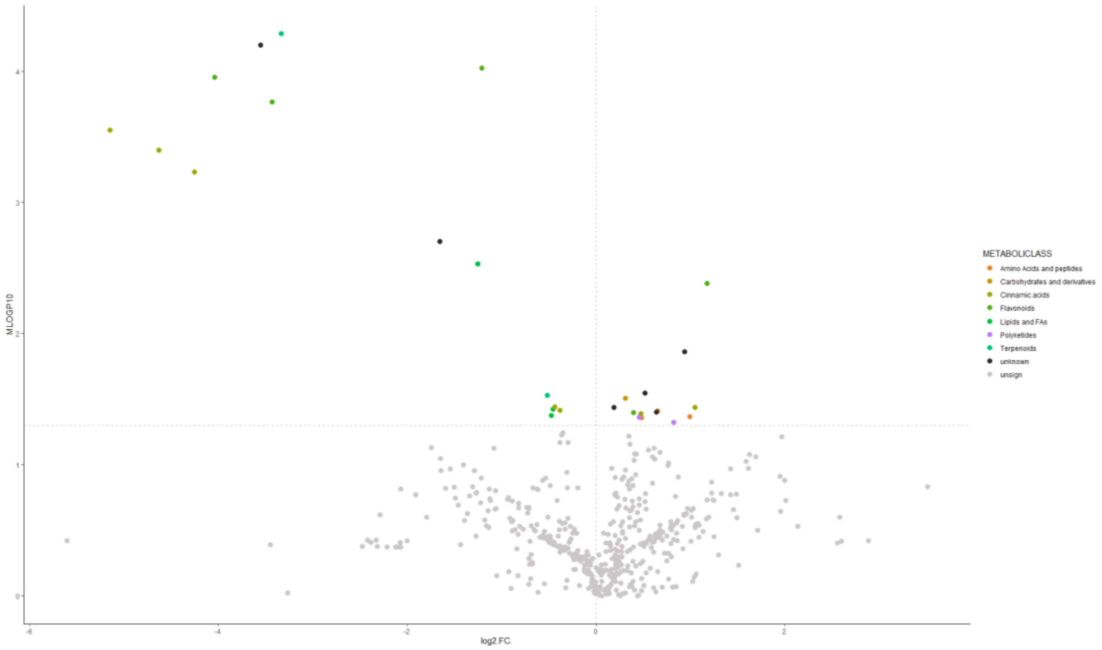


Figure 12. Volcano plot incorporating metabolic class and their up and down regulation in *Hypericum perforatum* L. Starting at upper-left-hand corner, volcano plots summarize the incidence of Ni on metabolites biosynthesis (up regulated in Ni than Ctr). Only events that are statistically significant (above the dashed red line) are displayed. The dashed red line, drawn at 1.25, is equivalent to a *p*-value of 0.001.

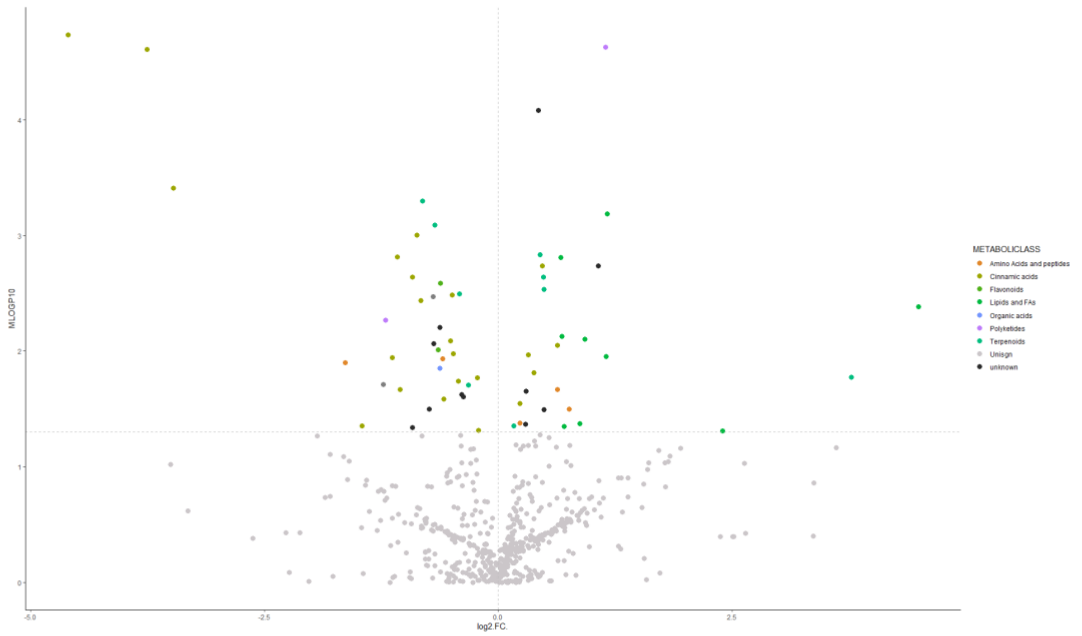


Figure 13. Volcano plot incorporating metabolic class and their up and down regulation in *Melissa officinalis* L. Starting at upper-left-hand corner, volcano plots summarize the incidence of Ni on metabolites biosynthesis (up regulated in Ni than Ctr). Only events that are statistically significant (above the dashed red line) are displayed. The dashed red line, drawn at 1.25, is equivalent to a *p*-value of 0.001.

4 CONCLUSIONS

The growing ecological load caused by industrial, agricultural, and human activities, municipal sources, energy sources, and heavy metal and metalloid contamination of soil and water is a global concern. Heavy metal pollution is gaining more and more importance in the last decades. According to a recent report, soil contamination, pollution, and deterioration affect nearly 20% of the total land surface or over 100 million acres. The most polluted places are those that are industrialized, posing the greatest threat to the environment and human health.

In 2002, the European Union predicted that partially cleaning up its hazardous sites would cost more than 100 billion dollars (Saier and Trevors 2010). Physical or chemical soil decontamination are time-consuming and expensive, and the now available procedures and technologies are insufficient for the demand. Thus, phytoremediation is gaining interest as a strong, alternative, environmentally friendly, and practical approach for remediation, decontamination, and industrial waste/environmental waste stabilization. The use of edible plants as phytoremediation crops is not feasible, as heavy metals may enter the food chain. As a result, non-edible crops like MAPs might be suggested to be potential sources of long-term phytoremediation.

The potential for the cultivation of the two MAPs *Hypericum perforatum* L. and *Melissa officinalis* L. for phytoremediation purposes in Nickel contaminated soil had been evaluated; focusing on the physiological mechanisms and responses of the targeted MAPs to two contamination level (moderate and high) as well as their clean up capacity.

The present study showed that both MAPs cultivation in Nickel contaminated soil with 300 and 100 ppm was feasible. Satisfactory biomass and essential oil yields in combination with high phenolics (TF and TP) and bioactive compounds (rosmarinic acid and hypericin, for lemon balm and hypericum respectively) in both moderate and high Ni contamination was observed. Hypericum essential oil production (%) progressively increased after Nickel exposure, compared to control plants, otherwise any differences in *Melissa officinalis* L. essential oil production were not detected.

A different capability of Ni tolerance and accumulation was observed for the two targeted MAPs, depending on the phenological phase (harvest cut) and Ni concentration of soil.

Results indicate that, up to 300 mg/kg nickel content both Hypericum and lemon balm could maintain adequate physiological functions, showing Nickel tolerance even at elevate metal concentration in their organs. As reported (Seregin and Kozhevnikova 2006), the restriction of metal transport from roots to shoots, as a mechanism of detoxification, is overcame when Ni accumulation exceeds a threshold. Accordingly, an increase in proline content was detected in Nickel stressed plants compared with the control (from 60 days after transplant). Otherwise, chlorophyll content was negatively impacted only for Hypericum in the N300 treatment 90 days after transplant. This was mainly due to Nickel-induced changes in nutrient dynamics (by altering the uptake processes of several minerals), leading to damage of chlorophyll biosynthesis. Further, modifications in the concentration of Organic acids in Nickel treated plants were observed. Respectively, in hypericum all analyzed OAs (oxalic, citric, malic acid) were impacted by metal concentration in the medium, otherwise in lemon balm modifications only in malic acid were observed. Thus, Lemon balm (*Melissa officinalis* L.) demonstrated a higher efficiency in mitigating the physiological impact of nickel (Ni), whereas St. John's Wort (*Hypericum perforatum* L.) exhibited a more pronounced physiological response to Ni exposure. However, despite employing distinct mechanisms, both species displayed effective adaptation even under high Ni concentrations. This was further supported by BAF and TFs level. Indeed, optimal BAF levels combined with low TF (roots to above ground parts) were obtained for both MAPs. Metal confinement in the roots is a primary defense process to protect the photosynthetic apparatus in the leaves and reduce ROS formation, allowing plants to tolerate the metal presence in the environment.

The low TF is also favorable for the use of the aerial part of the two MAPs, as it allowed to obtain a final product with moderate Nickel content in plant tissues.

Indeed, the danger in the final product consumption of the plant components is crucial, and using MAPs for phytoremediation allows to obtain a safer, cheaper, and far more eco-friendly solution. Accordingly, growing *H. perforatum* L. and *M. offcinalis* L. on Ni (Ni (NO3)2 6H2O) polluted soils for phytoremediation purposes, facilitate restoring the soil and provide economic benefits, since these MAPs showed good Ni-cleaning up capacities in high Ni bioavailability conditions, low TF from roots to above ground organs, as well as biomass and essential oil yield.

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FINAL CONCLUSIONS

The growing ecological load caused by industrial, agricultural, and human activities, municipal sources, energy sources, heavy metal and Nitrate contamination of soil and water is a global concern. Soil pollution is gaining more and more importance in the last decades, since many contaminants may accumulate in living organisms, causing implications for plants, animals, and human health. Healthy soils are crucial for food, biomass, fiber, and medical production, retaining and filtering water. Thus, pollution affects their fertility, carbon and nutrient cycles (EEA, 2022)

And as a result, food security. From an environmental perspective, the contamination of soils and water is one of the main problems caused by the incorrect disposal of solid and liquid residues from agriculture, industry, and domestic activities. Currently, it is estimated that 2.8 million potentially contaminated sites exist in the EU. A large proportion of these are legacy sites (so-called brownfield sites), often with unknown ownership.

Soil decontamination existing physic and chemical procedures are still inadequate and too expensive to achieve the goal. Thus, bioremediation has gained a lot of importance recently as an alternate technology for removal of elemental pollutants in soil and water, which require effective methods of decontamination. Phytoremediation: the use of plants to remove, contain or render harmless environmental pollutants, may offer an efficient, easy to use, cost-effective, as well as environmentally nondestructive remediation method.

Among green technologies addressed to metal and nitrate pollution, phytoremediation has received increasing attention from the discovery of hyperaccumulator plants, which are able to concentrate high levels of specific metals in the above-ground harvestable biomass (Vamerali et al. 2010).

The critical part of phytoremediation is the selection of phytoremediation species allowing the removal of contaminants from soils. Phytoremediation potential owing to the biosynthesis of secondary metabolites in addition to distinctive morphological characteristics of medicinal and aromatic plants (MAPs) make them a valid alternative for effective phytoremediation.

Thus, MAPs offer huge potential to be grown on contaminated sites to recover soil health, in addition to oil and metabolites production, to address the rising demand for pharmaceuticals, essential oils, and bioenergy.

In order to address the challenge of remediation and management of the increasing number of areas considered as contaminated sites, the use of MAPs for phytoremediation purposes may be a considered as a commercial opportunity for phytoremediation (Pandey and Souza-Alonso 2019). The remediation of contaminated sites with appropriate and economically valuable crops may become a dynamic strategy to provide additional benefits.

The aim of the present study was to comparatively evaluate two mainly soil contaminants Nitrate and Nickel. Thus, different MAPs had been tested, *Urtica dioica* L., and *Melissa officinalis* L. and *Hypericum perforatum* L. for nitrate and nickel respectively.

MAPs had been evaluated for their agronomic adaptability to contaminants, growth, yield, uptake capacity and physiological response in term of secondary metabolites biosynthesis.

Firstly, nitrate uptake of *Urtica dioica* L. grown in two different areas located in Nitrate Vulnerable Zones of Emilia Romagna Region had been evaluated. The study showed that stinging nettle cultivation was feasible in NVZs, due to the high amount of leaf bioactive components and decrease of Nitrate impact in soil. Satisfactory yields in combination with high levels of polyphenols and flavonoids, as well as DDPH and FRAP, and Folic Acid were obtainable in July, otherwise the highest Nitrate level was obtained in September, as well as ascorbate and highest yields. This trend suggests that two main harvest seasons might be feasible in Emilia Romagna Region: the fist in in July for bioactive compounds production, and the second in September for fiber or bioenergy production.

Satisfactory reduction on total Nitrate content in soil after two years of cultivation in both locations was obtained. Despite that detected nitrate levels for every cut, year and location were lower than the fixed threshold (EFSA, 2008) for leafy vegetables. Multiple harvest cuts through the growing season allowed to maintain contaminant levels in above ground organs lower than thresholds. This management, allowed to obtain a marketable product with high content in bioactive compounds in NVZs. Thus, the cultivation of Nettle in these marginal areas does not affect the potential application of the plant for nutraceutical and cosmetic purposes and no additional fertilization are needed or recommended to achieve a desirable quality and chemical composition. In addition, nettle cultivation in these marginal areas might be improved with good agronomic practices (as weed removal and uniform supply of water) and inclusion in crop rotation systems, to obtain desirable biomass yields.

In conclusion, NVZs cultivated nettle is a valuable source of bioactive compounds for pharmaceutical, agro-food and cosmetic industries, thanks to it is richness in minerals (iron, selenium, potassium), Vitamin C, Folate, phenols and high antioxidant capacity.

The second research topic was focused on the evaluation of phytoremediation potential of two targeted MAPs *Hypericum perforatum* L. and *Melissa officinalis* L. under control environment.

The potential for the cultivation of MAPs under two Ni-contamination level (moderate and high, 100 and 300 ppm, respectively), focusing on their physiological mechanisms and responses, had been evaluated. Satisfactory biomass and essential oil yields in combination with high phenolics (TF and TP) and bioactive compounds (rosmarinic acid and hypericin, for lemon balm and hypericum respectively) in both moderate and high Ni contamination were observed. Nickel content in the soil was significantly lowered after cultivation cycle in both *Hypericum perforatum* L. and *Melissa officinalis* L. trials. Acceptable BAF values and negative TF (from roots to above grounds organs) were also detected.
Although differences in physiological responses and mechanisms, as well as Ni tolerance and accumulation between the two examined species were noticed. Accordingly, modifications in stressed plants for minerals uptake, chlorophyll and organic acids content were more evident in hypericum than in lemon balm. Moreover, LC/MSMS results clearly demonstrated different metabolic responses of the two MAPs, highlighting distinctive up and down regulation of metabolic families in response to Ni stress.
Despite the different responses, both Hypericum and lemon balm could maintain adequate physiological functions even at elevate metal concentration thanks to the restriction in metal transport from roots to shoots (low values of TF). Metal confinement in the roots may be considered an efficient detoxification process to protect the photosynthetic apparatus in the leaves and reduce ROS formation, allowing plants to tolerate the metal presence in the environment. Effective soil decontamination combined with low TF and satisfactory yields in bioactive compounds, essential oils and biomass makes the investigated MAPs suitable for a phytoremediation process. Accordingly, growing *H. perforatum* L. and *M. offcinalis* L. on Ni polluted soils for phytoremediation purposes, facilitate restoring the soil and provide economic benefits. These results should be corroborated with further trials, in order to evaluate *H. perforatum* L. and *M. offcinalis* L. open field remediation capacity as well as contaminant effects on metabolites biosynthesis, essential oil production and biomass yield.

The three examinate MAPs showed satisfactory yields in combination with high levels of demanded bioactive compounds. Furthermore, phytoremediation capacity and a positive physiological response to contaminant of the species was demonstrated.
This research highlights that medicinal and aromatic plants (MAPs) are suggested to be potential sources of long-term phytoremediation. MAPs are essential oils and bioactive compounds producing plants, with possible applications in agro-food, pharmaceutical and cosmetic industries.

Furthermore, the absence of high contaminant accumulation in targeted organs and consequent entry in the food chain was demonstrated. The last century has witnessed an increase in the demand for essential oils and bioactive molecules from MAPs, for the cosmetic and pharmaceutical industry.

Hence, innovative remediation approaches with appropriate and economically valuable crops like MAPs could be a dynamic strategy to provide additional benefits. Thus, the cultivation of MAPs in marginal areas proposes a novel approach for phytoremediation, and a commercial opportunity thanks to high value products obtained from bioactive compounds and essential oils production.