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Alternatywny ogród metali ciężkich – ocena perspektyw wykorzystania nowych bioindykatorów zanieczyszczenia powietrza

Alternative heavy metals garden – evaluation of perspectives to use new bioindicators for air pollution

Doctoral dissertation in the field of engineering and technology
in the discipline of environmental engineering, mining and energy

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List of abbreviations used

APOX – Ascorbate peroxidase

BCF – Bioconcentration factor

CAT – Catalase

C_i – Intercellular CO_2 concentration

G_s – Stomatal conductance

HMs – Heavy metals

H_2O_2 – Hydrogen peroxide

MDA – Malondialdehyde

MSI – Cell membrane stability index

P_N – Net photosynthesis rate

RWC – Relative water content

TCF – Translocation factor

Streszczenie

Zanieczyszczenie metalami ciężkimi stwarza poważne ryzyko dla ekosystemów i zdrowia ludzkiego, wymaga więc skutecznych strategii monitorowania. W związku z tym, głównym celem badań było poszukiwanie odpowiednich bioindykatorów zanieczyszczeń metalami ciężkimi (takimi jak Cd, Pb, Cu, Zn, Ni), wśród szeroko rozpowszechnionych pospolitych chwastów - szarłatu szorstkiego (*Amaranthus retroflexus* L.), koniczyny łąkowej (*Trifolium pratense* L.), szczawiu zwyczajnego (*Rumex acetosa* L.), babki lancetowatej (*Plantago lanceolata* L.) oraz malwy różowej (*Alcea rosea* L.). W związku z cyrkulacją metali ciężkich w środowisku oraz istnieniem dwóch źródeł ich poboru przez rośliny, zaplanowane eksperymenty miały na celu porównawczą ocenę zdolności badanych roślin do akumulacji pochodzących z różnych źródeł metali ciężkich oraz poziomu ich reakcji fizjologicznej na stres. W pierwszym z nich, w celu oceny zdolności poszczególnych chwastów do akumulacji metali ciężkich, głównie związanych z zanieczyszczeniem powietrza, wybrane rośliny eksponowano na stanowiskach miejskich, w pobliżu referencyjnych stacji pomiarowych. Wyniki wykazały zróżnicowany, choć ogólnie wysoki potencjał do akumulacji poszczególnych metali ciężkich oraz adekwatny poziom odpowiedzi fizjologicznej oraz aktywności systemu obronnego w reakcji na stres. Zarówno w korzeniach jak i liściach wykryto podwyższoną zawartość H_2O_2, podwyższony poziom markerów enzymatycznych katalazy (CAT) i peroksydazy askorbinianowej (APOX) oraz markera peroksydacji lipidów błonowych - aldehydu malonowego (MDA). Istotne było również wykrycie obniżenia wartości parametrów aktywności fotosyntetycznej, takich jak intensywność fotosyntezy netto (P_N), przewodność szparkowa (g_s) i międzykomórkowe stężenie CO_2 (C_i). W związku z przemieszczaniem się metali ciężkich z powietrza do gleby, drugi eksperyment, przeprowadzony w warunkach kontrolowanych miał na celu ocenę stopnia akumulacji jonów Cd, Pb, Zn i Ni z roztworu glebowego u testowanych w pierwszym doświadczeniu roślin. *Trifolium pratense* okazało się wskaźnikiem, wykazującym stałą skuteczność w kumulacji Zn, Ni i Cd, natomiast *Plantago lanceolata* skażenia cynkiem (Zn) i kadmem (Cd) z adekwatnym wzrostem w liściach i korzeniach aktywności markerów stresu u obu gatunków. W trzecim badaniu bioindykacyjnym *in situ,* oceniono skuteczność *Trifolium pratense* jako bioindykatora zanieczyszczeń w różnych obszarach aglomeracji miasta, a co za tym idzie w zróżnicowanych pod względem zanieczyszczenia powietrza i pod wzgędem edaficznym siedliskach. Wykryte bardzo wysokie poziomy biokoncentracji (BCF) i translokacji (TF) badanych pierwiastków w roślinach, adekwatne do poziomu skażenia, sugerują duży potencjał koniczyny łąkowej jako bioindykatora skażenia metalami ciężkimi, na obszarach miejskich o różnym sposobie użytkowania gruntów. Podsumowując wyniki przeprowadzonych doświadczeń, *Trifolium pratense* i *Plantago lanceolata* są zalecane do monitorowania i oceny środowiska, dostarczając informacji na temat poziomu zanieczyszczenia metalami ciężkimi w różnych ekosystemów.

Słowa kluczowe: metale ciężkie, zanieczyszczenia, bioindykatory, chwasty, stres fizjologiczny

Abstract

Heavy metal pollution poses serious risks to ecosystems and human health and requires effective monitoring strategies. Therefore, the search for appropriate bioindicators of heavy metal pollution (such as Cd, Pb, Cu, Zn, Ni) among widespread common weeds—rough amaranth (*Amaranthus retroflexus* L.), meadow clover (*Trifolium pratense* L.), common sorrel (*Rumex acetosa* L.), narrowleaf plantain (*Plantago lanceolata* L.), and pink mallow (*Alcea rosea* L.)—was the main aim of the research. Due to the circulation of heavy metals in the environment and the existence of two sources of their uptake by plants, the planned experiments aimed to the comparative assessment of selected species ability to accumulate heavy metals from various sources and tested their physiological response to stress. In the first experiment, to assess the ability of individual weeds to accumulate heavy metals mainly related to air pollution, selected plants were exposed at urban sites near reference measurement stations. The results showed a varied but generally high potential for the accumulation of individual heavy metals and an adequate level of physiological response and activity of the defense system in response to stress. Increased H_2O_2 content, increased levels of enzyme markers catalase (CAT) and ascorbate peroxidase (APOX), and a marker of membrane lipid peroxidation—malonaldehyde (MDA) were detected in both roots and leaves. There was also a significant reduction in the values of photosynthetic activity parameters, such as net photosynthetic intensity (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i). Due to the movement of heavy metals from the air into the soil, the second experiment was conducted under controlled conditions. Its aim was to assess the accumulation of Cd, Pb, Zn, and Ni ions from the soil solution in the plants tested in the first experiment. *T. pratense* showed to be an indicator showing consistent effectiveness in the accumulation of Zn, Ni, and Cd ions, *P. lanceolata* proved to be an indicator of Zn and Cd contamination, with an adequate increase in the activity of stress markers in the leaves and roots of both species. In the third on-site bioindication investigation, the effectiveness of *T. pratense* as a bioindicator of pollution was assessed in various areas of the city agglomeration, and therefore in habitats diversified in terms of air pollution and edaphic aspects. The detected very high levels of bioconcentration (BCF) and translocation (TF) of the tested elements in plants, adequate to the level of contamination, suggest a high potential of meadow clover as a bioindicator of heavy metal contamination in urban areas with different land uses. Summarizing the results of the conducted experiments, *T. pratense* and *P. lanceolata* are recommended for environmental monitoring and assessment, providing information on the level of heavy metal pollution in various ecosystems.

Keywords: heavy metal pollution, bioindicators, weed species, physiological stress responses

1. Introduction

Heavy metals are elements with both natural and anthropogenic sources (Vareda *et* al., 2019). Anthropogenic activities, such as urban runoff, agriculture, and industrial processes, significantly contribute to the presence of heavy metals in the environment (Morais *et* al., 2012; Popoola *et* al., 2018). Due to their toxic nature and potential to harm living organisms and the environment, they are often referred to as "silent killers" (Orji *et* al., 2018). Elevated levels of these metals, particularly Pb, Cd, Cr, Cu, Ni, Zn, and other trace metals, have been detected across various regions, presenting substantial hazards to both local ecosystems and human health (Wang *et* al., 2005; Alomary and Belhadj, 2007; Nazir *et* al., 2015). The exact proportion of anthropogenic to natural contributions can vary depending on the specific environmental context and geographical location (Rasmussen, 1998; Haider, 2010). Moreover, the enduring presence of heavy metals in the environment poses a critical challenge, as their non-biodegradable nature means they persist and accumulate over time (Ghori *et* al., 2019). This persistent accumulation not only presents a significant challenge but also directly contributes to numerous negative consequences (Pujari and Kapoor, 2021). They pose a substantial environmental risk due to the severe consequences associated with their presence and dispersion through soil, water, and air (Masindi and Muedi, 2018; Cunningham *et* al., 2022). Thus, heavy metals can cause soil and water pollution, leading to a decline in plant and animal populations and altering the dynamics of the food chain (Pujari and Kapoor, 2021). In response to heavy metal pollution, plants may exhibit various adaptive mechanisms, such as the accumulation of heavy metals in their tissues, changes in root morphology, and activation of detoxification pathways. However, prolonged exposure to high levels of heavy metals can adversely affect plant growth, development, and overall ecosystem functioning (Azmat *et* al., 2009; Sharma and Chakraverty, 2013; Viehweger, 2014). Therefore, there has been a significant increase in attention towards the issue of heavy metals (Masindi and Muedi, 2018). Monitoring of environmental pollution levels is crucial. While dispersion modelling and field measurements of emissions offer insights into pollution sources, they have limitations when assessing the biological effects of pollutants and are typically confined to small geographic areas (Wolterbeek, 2002).

To address these limitations, the use of various plants as bioindicators has emerged as a valuable additional approach to traditional investigation methods (Pellegrini *et* al., 2014). Bioindicators are valuable tools because they are helpful, objective, straightforward, and reproducible. They can be used at various scales, from the cellular to the environmental level, to assess changes within specific biological communities (Parmar *et* al., 2016). Thus, they play a crucial role in evaluating environmental health and detecting both positive and negative environmental changes, with implications for human society (Khatri and Tyagi, 2015; Parmar *et* al., 2016). This role is particularly crucial in urban areas, where the use of plants as bioindicators has revealed the presence of heavy metals such as Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn, and Co (Martínez-Pérez *et* al., 2021; Cortés-Eslava *et* al., 2023; Abrica-González *et* al., 2023). Urban environments, characterized by dense human activity and industrialization, serve as focal points of heavy metal contamination, making the use of bioindicators imperative for monitoring and assessing environmental quality and public health in these areas (Li *et* al., 2023; Ahamad *et* al., 2024; Edo *et* al., 2024). However, the ability to find plants that can endure challenging environmental conditions and contaminants in practically any urban landscape remains a challenge (Abrica-González *et* al., 2023). Over the years, bioindicators have encompassed a variety of plant types, including trees, ornamental plants, crops, and non-vascular plants (Sawidis *et* al., 2011). The accelerated growth rate of certain herbaceous plants further expedites the evaluation of heavy metal exposure (Påhlsson, 1989; Riaz *et* al., 2021). Therefore, numerous studies conducted worldwide over the years have used common herbaceous species in different environments, such as *Lactuca sativa* L., *Ambrosia artemisiifolia* L., *Taraxacum officinale* L., *Portulaca oleracea* L., *Carduus nutans* L., *Plantago major* L., *Urtica dioica* L., and others, as bioindicators in urban areas (e.g., Diatta *et* al., 2003; Malizia *et* al., 2012; Hassan *et* al., 2013; Radulescu *et* al., 2013; Borowiak *et* al., 2018; Lisiak-Zielińska *et* al., 2021; Pietrelli *et* al., 2022). These plants have proven effective in detecting pollutants in various settings. In this context, it's noteworthy that some of these species were also used as ozone bioindicators. Thus, in the realm of environmental research, the concept of 'ozone gardens' has gained notable traction as a means of assessing air quality through plant responses. However, similar gardens for assessing heavy metal pollution are absent from scholarly discourse.

This study introduces the concept of an 'alternative heavy metal garden,' using plants to evaluate air pollution and improve our understanding of environmental issues and solutions. Moreover, numerous studies worldwide have explored the use of common herbaceous species as bioindicators in urban or rural areas, there remains a notable gap in the literature regarding the direct exposure of plants to heavy metals and their physiological response, particularly through direct contact with heavy metals. This is where the aim of the study lies, the main aim of this research was to investigate the potential of five commonly encountered weed species - *Trifolium pratense* L., *Rumex acetosa* L., *Alcea rosea* L., *Amaranthus retroflexus* L., and *Plantago lanceolata* L. - as bioindicators of pollution by heavy metals. Additionally, the well-known heavy metal accumulator *Lolium multiflorum* var. Ponto (Klumpp *et* al., 2009) served as a reference. The study aimed to identify which weed species demonstrated the highest efficacy as bioindicators and to evaluate their physiological responses when exposed to heavy metals both in the air and through direct contact, specifically via irrigation with heavy metal solutions. Furthermore, this research aimed to address a critical gap in the existing literature concerning the limited exploration of the complex physiological responses exhibited by these weed species when confronted with heavy metals. By systematically investigating the physiological responses of weed species to various levels of heavy metal pollution, the goal was to elucidate the mechanisms underlying their potential as bioindicators. Ultimately, this study aimed to identify the most suitable bioindicator species and provide valuable insights into their physiological adaptations to heavy metal exposure. To observe the practical application of *Trifolium pratense* as a bioindicator in different types of land use, third research was conducted. In this experiment, *T. pratense* were collected from different urban environments. The objective was to assess the heavy metal accumulation in *T. pratense* across these diverse land uses. The collected plants were analyzed for their bioconcentration and translocation factors, specifically focusing on heavy metals such as Cd, Cu, Cr, Ni, and Pb. This experiment aimed to determine how effectively *T. pratense* can reflect the levels and sources of heavy metal pollution in different urban settings, thereby evaluating its potential as a reliable bioindicator for environmental monitoring.

2. Research hypotheses, aim and detailed aims of the study

Research hypotheses:

1. Hypothesis 1: *Amaranthus retroflexus* L., *Trifolium pratense* L., *Rumex acetosa* L., and *Plantago lanceolata* L. can be considered unique bioindicators of many heavy metals simultaneously occurring in the environment (air and soil).
2. Hypothesis 2: The physiological responses to stress of selected species (*Amaranthus retroflexus* L., *Plantago lanceolata* L., *Rumex acetosa* L., and *Trifolium pratense* L.) were adequate to the level of heavy metals accumulation in plant organs.
3. Hypothesis 3: Selected weeds are useful to detect the level of heavy metal contamination in a diverse urban environment and have the potential to accumulate HMs in plant organs depending on soil content.

Aim of the study:

This study introduces the concept of an 'alternative heavy metal garden,' using plants to evaluate air pollution levels and improve our understanding of environmental issues and solutions. Similar to how ozone gardens are used for knowledge promotion and education, heavy metal gardens could also serve this purpose. With positive results and further adjustments, these gardens could not only monitor environmental pollution but also educate the public about the impacts of heavy metals and the importance of environmental stewardship. Thus, the main aim of the doctoral dissertation was to explore the heavy metal bioindicator potential of various plant species. This investigation focused on assessing the effectiveness of widespread weed species such as *T. pratense* L., *R. acetosa* L., *A.retroflexus* L., *A. rosea* L., and *P. lanceolata* L. in detecting and monitoring heavy metal contamination. Additionally, the study aimed to delve into the physiological responses of these selected plant species under the influence of heavy metal pollutants. By analyzing these physiological responses, the research sought to uncover the mechanisms underlying the plants' tolerance and accumulation of heavy metals.

Moreover, the assessment considered various factors, including the plants' bioaccumulation capacity, translocation efficiency, and overall suitability for biomonitoring applications. By harnessing the bioindicator potential of diverse plant species, the study sought to enhance the reliability and comprehensiveness of environmental monitoring efforts, particularly in the context of heavy metal contamination.

The detailed aims included:

1. Evaluation of heavy metal accumulation: assessment the ability of selected plant species, including *T. pratense* L., *R. acetosa* L., *A. retroflexus* L., *P. lanceolata* L., *A. rosea* L., to accumulate heavy metals (Cd, Pb, Cu, Ni, Zn) in their leaves and roots tissues under controlled conditions.
2. Assessment of the ability to translocate heavy metals between plant organs: determination of the ability of studied species to bioconcentrate and translocate HMs, along with the assessment of their physiological responses to stress, including evaluating oxidative stress parameters and antioxidative enzyme activity.
3. *In situ* plant and soil research: detection variations in heavy metal contamination in plants and soil at research sites representing the different land uses typical of urban areas.
4. Establishment of bioindicator recommendations: provide recommendations for the use of the studied plant species as bioindicators in environmental monitoring, considering their effectiveness in assessing heavy metal pollution in both urban and non-urban areas.

3. Materials and methods

3.1. Selection of plant species for the study

Aligned with the theme of 'Alternative Heavy Metal Garden,' this study meticulously selected five plant species renowned for their widespread distribution, ubiquity in various locales, and frequent emergence as weeds in urbanized environments. The species under investigation included *T. pratense* L., *R. acetosa* L., *A. rosea* L., *A. retroflexus* L., and *P. lanceolata* L. and *L. multiflorum* L. (serving as a control/reference). While each of these plants has been individually pre-tested in prior studies, they have not been collectively examined or comprehensively assessed, highlighting the novelty of the new approach. Furthermore, the physiological responses of these plants to heavy metal exposure have not been thoroughly investigated, adding another dimension of novelty to the research. This selection aimed to leverage the innate characteristics of these plants as potential bioindicators for air pollution. By evaluating them collectively, the investigation sought to identify the most effective bioindicator for heavy metals and study their physiological responses. Through this research, the aim was to pave the way for innovative applications of these common yet resilient plants in monitoring and mitigating heavy metal pollution in urban environments. The seeds were collected from different locations in Poland, including Wielkopolska rural areas and the Poznań agglomeration, with the seed material for *Lolium multiflorum* variety Ponto sourced from Norddeutsche Pfanzenzucht Hans-Georg Lembke KG (Germany). Each species possesses unique characteristics, such as native range, spread, and uses in traditional medicine and agriculture:

A. *Trifolium pratense* L., widely recognized as red clover, is a globally occurring clover species that serves various purposes such as forage provision, green manure, and honeybee nectar source. Originating from Europe, South, West, and Middle Asia, and North-West Africa, it is now commonplace in all continents except for the icy terrain of Antarctica. It significantly enhances soil fertility through its nitrogen-fixing capability. This versatile species frequently appears in meadows, pastoral lands, and country roadways (POWO, 2019).
B. *Rumex acetosa* L., also known as common sorrel, is a herbaceous plant widely distributed throughout Europe, Asia, and North Africa, and has spread to all continents except Australia and Antarctica. It is commonly used as a culinary herb and has a sour taste due to the presence of oxalic acid. Sorrel is often used in salads and soups, and

has also been used in traditional medicine to treat a variety of ailments (POWO, 2019).

C. *Amaranthus retroflexus* L., or red-root amaranth, is a species of flowering plant that is commonly considered a weed due to its ability to grow in a wide range of habitats and its production of a large number of seeds. It has been introduced into all continents except Antarctica. Certain regions cultivate it as a leafy vegetable and grain crop, and it's recognized for its medicinal properties in treating various conditions (POWO, 2019).

D. *Plantago lanceolata* L., also known as ribwort plantain, is a species of plantain that is widely distributed throughout Europe, North Africa, and Asia. It is commonly found in disturbed habitats such as roadsides, pastures, and fields. Ribwort plantain has a long history of use in traditional medicine, and is used to treat a variety of conditions, including respiratory problems, skin conditions, and digestive issues (POWO, 2019).

E. *Alcea rosea* L., commonly known as the hollyhock, was introduced to Europe from southwestern Asia as an ornamental plant prior to the fifteenth century. Over time, it has become a popular choice for ornamental purposes in urban areas and has spread as a wild species in the Americas, North Africa, and South Asia. This particular plant species has established itself not only as a cherished ornamental plant but also as a resilient and widespread wilderness species in various regions (POWO, 2019).

F. *Lolium multiflorum* L., commonly known as italian ryegrass, was used as a control species. This dynamic and fast-growing grass can exist as either an annual or a short-lived perennial. It finds frequent utilization as a forage crop and is often incorporated into turf production and erosion management strategies. Its natural habitat extends across Europe, except for Finland, and spans Western, Southern, and Central Asia, excluding Uzbekistan, as well as Northern Africa (Brunharo *et* al., 2019). With the exception of Antarctica, this species has been introduced to every continent. Despite its practical uses, it is often labeled as a weed due to its aggressive growth habit that outcompetes indigenous plants. Remarkably, the Ponto variety of Italian ryegrass exhibits phytoremediation potential, showing a remarkable capacity for extracting heavy metals from polluted soils (POWO, 2019; Cui *et* al. 2021).

3.2.Method of conducting experiments

1. First experiment:

The experiment took place during the 2021 growing season, starting in April with seeds planted in a greenhouse (16–18 °C, no artificial light) in 5 L pots containing a peat-sand mix (pH 6.8, N: 230 mg/L, P: 180 mg/L, K: 350 mg/L, Mg: 150 mg/L). Soil element concentrations were Cu: 4.151±0.032 mg/kg, Zn: 15.03±0.34 mg/kg, Cd: 0.091±0.004 mg/kg, and Pb: 4.302±0.052 mg/kg. Ten seedlings per pot were kept and irrigated with deionized water.

After 60 days, plants were moved to three sites in Poznań:

- Site A: Residential area (N: 52°23′53″; E: 16°57′36″).
- Site B: Botanical Garden (N: 52°25′14″; E: 16°52′39″).
- Site C: Single-family housing area (N: 52°25′50″; E: 16°54′58″).

Three pots per species were placed at each site for six weeks (June 1 to July 16, 2021), watered with distilled water, and shaded by natural vegetation. Air pollution data were obtained from the General Directorate for Environmental Protection. Heavy metal detection was done with inductively coupled plasma mass spectrometer (ICP-MS 7100x Agilent, Santa Clara, USA). After the obtained results bioconcentration (BCF) and translocation (TF) were calculated to assess heavy metal uptake. Physiological measurements included cell membrane stability, dry mass, relative water content, chlorophyll content, and photosynthetic activities. Oxidative stress and antioxidative enzyme activities, including hydrogen peroxide content, lipid peroxidation, protein quantification, and *in situ* detection of hydrogen peroxide, were also measured.

2. Second experiment:

In March 2022, seeds of each plant species were planted in individual 5 L pots containing a peat and sand mixture (pH 6.8, K: 350 mg/L, Mg: 150 mg/L, N: 230 mg/L, P: 180 mg/L) under greenhouse conditions (16–18°C), using deionized water for irrigation. Controlled heavy metal contamination was applied through irrigation with metal nitrate solutions at two levels: low and high. The plants received two treatment rounds, initially at 77 days and again after 10 days, to achieve doubled contamination levels. Nitrogen compensation was achieved using ammonium nitrate in N equivalents to the metal spiking, resulting in corresponding control treatments. The experiment had four variants (low contamination, high contamination, and their respective nitrogen controls) with three replicates each, totaling 60 pots (5 species × 3 replicates × 4 variants). Heavy metal detection was performed using an inductively coupled plasma mass spectrometer (ICP-MS 7100x Agilent, Santa Clara, USA). After the obtained results bioconcentration (BCF) and translocation (TF) were calculated to assess heavy metal uptake. Physiological measurements for control and treated species included such parameters as: cell membrane stability, dry mass, relative water content, and chlorophyll content. Oxidative stress and antioxidative enzyme activities, including hydrogen peroxide content, lipid peroxidation, protein quantification, and in situ detection of hydrogen peroxide, were also measured.

3. Third *in situ* bioindication investigation

The third phase of our research initiative entailed an extensive on-site bioindication investigation, focusing on *Trifolium pratense* L., conducted between May 5 and May 11, 2022. Soils and *Trifolium pratense* L. (leaves, roots) samples were collected from 8 different research sites: near the lake, individual houses area, an industrial area, a park, an old town, agricultural land, near a river and a high-density residential area. The research aimed to evaluate the concentration and translocation of the metals Cd, Cu, Cr, Ni, Pb, and Zn in the organs of *T. pratense* plants, based on soil content. The selection of these heavy metals for investigation was due to their common prevalence as pollutants in urban areas. Heavy metal detection was done with an inductively coupled plasma mass spectrometer (ICP-MS 7100x Agilent, Santa Clara, USA). To quantify heavy metal accumulation and translocation in the *T. pratense* the bioconcentration factor (BCF) and translocation factor (TF) were calculated.

3.3.Statistical analysis

Statistical analyses for the three experiments were performed using R Core (2014) and Statistica 13.1. In the first experiment, two-way ANOVA evaluated differences between species and locations, the post-hoc Scheffé test identified uniform groups ($\alpha \leq 0.05$), PCA assessed associations without prior assumptions, and heatmaps and cluster analyses visualized and identified similarities in heavy metal concentrations. In the second experiment, one-way ANOVA assessed sample differences, PCA visualized variable interdependence, and heatmaps and cluster analyses compared BCF and TF among plant species. In the third on-site bioindication investigation descriptive statistics assessed heavy metal concentrations, one-way ANOVA and post-hoc Scheffé tests evaluated differences, heatmaps visualized element concentrations, and cluster analyses identified distinctions among sites and sample types.

4. Characteristics of the obtained results

4.1. Common weeds as heavy metal bioindicators: a new approach in biomonitoring

The research results obtained in Experiment 1, as described in the scientific publication by Cakaj, A., Lisiak-Zielińska, M., Hanć, A., Małecka, A., Borowiak, K., & Drapikowska, M. (2023). Common weeds as heavy metal bioindicators: a new approach in biomonitoring. *Scientific Reports*, 13(1), 6926. DOI: https://doi.org/10.1038/s41598-023-34019-9, exhibited several important characteristics:

1. Species-specific variation: the study revealed that different exposed plant species exhibit varying degrees of potential for accumulating trace elements, demonstrating species-specific responses to heavy metal contamination.

2. Spatial variation: variations in heavy metal concentrations were observed among plants exposed in different study sites, indicating spatial variability in levels of metal contamination in both soil and plant tissue.

3. Metal content trends: the results showed a consistent trend in heavy metal content across all species and study sites, with the order of accumulation being Zn > Cu > Pb > Cd. This trend was observed in both soil and plant organs (roots and leaves).

4. Variability among elements: Cu, Zn, Cd, and Pb concentrations differed between root and leaf tissues across all species. Specific species showed variations in heavy metal accumulation.

5. Bioconcentration factors (BCF): BCF values exceeded 1 for Zn and Cd in all plant species showing effective bioaccumulation of these elements. *L. multiforum*, *A. rosea*, and *P. lanceolata* revealed the highest BCF values for Zn and Cd.

6. Translocation factors (TF): Translocation factors for Cu, Zn, Cd, and Pb varied among species. For example, *T. pratense* exhibited the highest Cu TF, while *T. pratense*, *A. retrofexus*, and *L. multiforum* showed high Zn TF. Cd TF was the highest in *T. pratense*, *P. lanceolata*, and *R. acetosa*. Pb TF values were notably high in *A. retrofexus* and *P. lanceolata*.

7. Physiological responses: the physiological response of the studied species to stress were adequate to the high content of the tested metals in the tissues. *P. lanceolata* revealed strong cell membrane stability and high chlorophyll content, while *A.*

retrofexus displayed notable dry mass content. Despite moderate water content, *R. acetosa* showed high photosynthesis activity. Furthermore, variations in photosynthesis parameters such as stomatal conductance and intercellular CO_2 concentration were observed in *P. lanceolata*, *A. retroflexus*, and *R. acetosa*, reflecting the diverse physiological strategies of these species.

8. The level of stress markers in detected species elevated after exposure (*in situ* hydrogen peroxide activity and MDA), antioxidative enzymes CAT and APOX activity was high, pointing to the greater detoxification efficiency, which provides better examined weeds resistance against trace metal-induced oxidative stress.

4.2.Plants as effective bioindicators for heavy metal pollution monitoring

The research results obtained in Experiment 2, as described in the scientific publication by Cakaj, A., Drzewiecka, K., Hanć, A., Lisiak-Zielińska, M., Ciszewska, L., & Drapikowska, M. (2024). Plants as effective bioindicators for heavy metal pollution monitoring. *Environmental Research*, 119222.bDOI: https://doi.org/10.1016/j.envres.2024.119222, exhibit several important characteristics:

1. HMs in soil: at low contamination levels, the trend for heavy metal content in the plants was Zn > Pb > Ni > Cd. *T. pratense* exhibited the lowest concentrations of Ni, Zn, Pb, and Cd, while *L. multiflorum* showed the highest concentrations of these metals. At high contamination levels, the trend was Pb > Zn > Ni > Cd. *R. acetosa* had the lowest concentrations of Ni, Zn, and Cd, whereas *P. lanceolata* had the lowest concentration of Pb. *T. pratense* and *L. multiflorum*, showed the highest concentrations for all the metals.

2. HMs in roots: at low contamination levels, the trend for heavy metal content was Zn > Ni > Pb > Cd. *A. retroflexus* had the lowest levels of Ni, Zn, Pb, and Cd, whereas *T. pratense* had the highest level of Ni, and *P. lanceolata* had the highest levels of Zn, Pb, and Cd. At high contamination levels, the trend was Zn > Pb > Ni > Cd. *A. retroflexus* had the lowest levels of Ni, Pb, and Cd, while *P. lanceolata* had the lowest level of Zn and the highest levels of Ni, Zn, Pb, and Cd.

3. HMs in leaves: at both contamination levels, the trend for heavy metal content was Zn > Ni > Pb > Cd. At the low contamination level, *T. pratense* had the lowest concentrations of Ni, Zn, Pb, and Cd, while *R. acetosa* had the highest concentrations of these metals. At the high contamination level, *T. pratense* again had lower

concentrations of Ni, Zn, Pb, and Cd, whereas *P. lanceolata* had the highest concentrations of Ni, Zn, and Cd, and *R. acetosa* had the highest concentration of Pb.

4. Bioconcentration factor (BCF): the highest BCF was noted for Zn, exceeding 2 in *T. pratense* (low level) and *P. lanceolata* (both levels). Ni and Cd had BCF values greater than 1, particularly in *T. pratense* and *L. multiflorum* (low level) and in *P. lanceolata* (both levels). Pb had BCF values below 1 across all species.

5. Translocation factor (TF): the highest translocation rates for Ni were observed in *R. acetosa*, *A. retroflexus*, and *P. lanceolata*, all exceeding the reference species *L. multiflorum*. Additionally, *R. acetosa* and *A. retroflexus* had the highest translocation factors (TF) for Pb at the low contamination level, both with TF values greater than 4. Conversely, *T. pratense* showed the lowest translocation ratios, with TF values exceeding 1 for Ni at both contamination levels and for Zn at the low contamination level.

6. Physiological Responses: at the low contamination level, *P. lanceolata* had the lowest dry mass, while *T. pratense* had the highest. At the high contamination level, *R. acetosa* had the lowest dry mass, while *T. pratense* had the highest. For cell membrane stability (MSI), *T. pratense* had the lowest MSI at the low contamination level, while *R. acetosa* had the highest. At the high contamination level, *A. retroflexus* had the lowest MSI, and *L. multiflorum* had the highest. In terms of relative water content (RWC), *P. lanceolata* had the lowest value at the low contamination level, and *A. retroflexus* had the highest. At the high contamination level, *R. acetosa* had the lowest RWC, while *T. pratense* had the highest.

7. Analysis of activity of oxidative stress parameters and level of enzymes of the antioxidative system: distinct physiological responses to heavy metal stress were observed among the investigated species, highlighting unique variations in their reactions. Hydrogen peroxide, malondialdehyde content, and enzymatic activities emerged as reliable indicators of plant stress induced by heavy metal solutions. Among studied species, the highest CAT activity was noticed in the leaves of *R. acetosa*, APOX activity was highest in the roots of *R. acetosa*. MDA content was higher in the leaves of *T. pratense*, *A. retroflexus*, and *P. lanceolata*. H_2O_2 elevated levels were also found in *T. pratense* and *A. retroflexus*.

4.3. *Trifolium pratense* and the heavy metal content in various urban areas

The research results obtained in *in situ* bioindication investigation, as described in the scientific publication by Cakaj, A., Hanć, A., Lisiak-Zielińska, M., Borowiak, K., & Drapikowska, M. (2023). *Trifolium pratense* and the heavy metal content in various urban areas. *Sustainability*, *15*(9), 7325. DOI: https://doi.org/10.3390/su15097325, showed several important characteristics:

1. HMs in soil: metal concentrations (Cd, Cu, Cr, Ni, Pb) varied across sites. Cr exhibited its highest concentration near the river, Cd in parks, and Ni, Cu, and Pb were the highest in industrial areas and the lowest in residential area.
2. HMs in roots: agricultural land displayed the highest concentrations of Cr and Ni in roots, while parks showed elevated Cu and Cd levels. Pb concentrations were highest in agricultural areas and lowest near the lake.
3. HMs in leaves: industrial area had the highest concentrations of Cr, Ni, and Pb in leaves. Cu concentrations were higher in parks, while Cd concentrations were elevated in agricultural land and lower near individual houses.
4. Bioconcentration and translocation factors: Cr and Ni had higher bioconcentration factors (BCF) in most research sites, especially in industrial areas and the old town. Ni's BCF was also notable near the lake, residential, and agricultural area, while Cu and Cd's BCF was elevated only in high-density housing area. Pb's BCF did not exceed 1. Additionally, Cr and Ni showed the highest translocation factors (TF), notably in industrial area and the old town.
5. Contamination factor: Cr and Ni showed low contamination levels across all sites. Cu exhibited medium contamination in industrial zones and parks, while Cd had medium contamination near river, industrial area, and park. Pb displayed considerable contamination in industrial area and medium levels near river, park, and the old town.
6. Bioindicator potential: *T. pratense* revealed a promising bioindicator of heavy metal contamination in urban environment.

5. Discussion

5.1. Heavy metal bioindicators

By using bioindicators, it is possible to assess the natural condition of a particular area or the extent of contamination it may have (Khatri and Tyagi, 2015; Parmar *et* al., 2016). They offer a cost-effective means of assessing environmental quality and permit straightforward sampling (Rucandio *et* al., 2011). The ability of plants to accumulate and tolerate heavy metals makes it a promising tool for monitoring selected heavy metals in urban areas (Malizia *et* al., 2012). The effectiveness of accumulation can be assessed using the bioconcentration factor (BCF) and the translocation factor (TF). These metrics are important for assessing the effectiveness of metal displacement, making them essential tools in identifying robust bioindicators (e.g., Galal *et* al., 2015; Baltrėnaitė *et* al., 2015; Lisiak-Zielińska *et* al., 2021).

In the first experiment a consistent trend was observed in heavy metal accumulation across all plant species and research sites: Zn>Cu>Pb>Cd, both in soil and plant organs (roots and leaves). All plant species exhibited a bioconcentration factor (BCF) greater than 1 for zinc (Zn) and cadmium (Cd), indicating their effective ability to accumulate these metals from the soil. Zinc, a commonly occurring heavy metal, is vital for the growth and development of plants and animals. However, an abundance of zinc can pose ecological risks to the surrounding environment (Tarish *et* al., 2024). While, cadmium (Cd) is widely acknowledged as a toxic substance (Yasin *et* al., 2024). *A. rosea* and *P. lanceolata* exhibited the highest BCF values for Cd, consistent with previous studies by Liu *et* al. (2008) and Ubeynarayana *et* al., (2021) highlighting their potential as hyperaccumulators of Cd. Similarly, these species showed effective Zn accumulation, supported by literature demonstrating Zn accumulation in *A. rosea* (Kaya and Gülser, 2018; Duan *et* al., 2022). For Cd translocation, *T. pratense*, *P. lanceolata*, and *R. acetosa* revealed high TF values, indicating efficient Cd transportation from roots to leaves (e.g., Nadgórska-Socha *et* al., 2015; Memić *et* al., 2023). Lead, a toxic heavy metal, presents substantial risks to both the environment and human health (Ali *et* al., 2019; Rahman and Singh, 2019). In the obtained results the accumulation of Pb was less effective compared to Zn and Cd, it was notable that none of the species showed a BCF value over 1. Additionally, an essential metal for the health of humans, animals, and plants is copper. However, excessive levels of copper can be harmful because it is non-biodegradable and highly toxic (Parmar and Thakur, 2013). In terms of Cu, several species including *L.*

multiflorum, *T. pratense*, *R. acetosa*, *A. retroflexus*, and *P. lanceolata* demonstrated Cu accumulation capabilities and rapid transport from roots to shoots. Previous studies have confirmed *T. pratense's* potential for Cu bioconcentration. *T. pratense* has been observed to accumulate Cu in their leaves in a linearly dependent manner with respect to soil content. Additionally, the fraction of Cu accumulated was quite high (25-40%) compared to the amount present in the soil (Malizia *et* al., 2012).

In the second experiment obtained results revealed variations in the concentrations of heavy metals (Ni, Zn, Cd, and Pb) among different plant species. In this study, *P. lanceolata* and *T. pratense* exhibited BCF values greater than 1 for Cd, surpassing those of the reference plant *L. multiflorum*, indicating their potential as efficient accumulators of Cd (Nadgórska–Socha *et* al., 2017). Furthermore, *P. lanceolata* exceeded a BCF value of 1 for Cd at the high concentration level, suggesting its promising characteristics as a bioindicator for heavy metals, consistent with previous findings (Dimitrova and Yurukova, 2005). Regarding Ni, *T. pratense, P. lanceolata*, and *L. multiflorum* showed BCF values exceeding 1, demonstrating their capacity to gather Ni from the surroundings. Additionally, all these species displayed TF values above 1 for Ni, indicating effective transportation from roots to above-ground tissues irrespective of pollution levels. These findings corroborate earlier studies that recognized *P. lanceolata* as a reliable indicator for Nickel (Dimitrova and Yurukova, 2005; Abate *et* al., 2022; Jordanovic *et* al., 2024). For Zn, *T. pratense*, *P. lanceolata*, *L. multiflorum*, and *R. acetosa* had BCF values higher than 1 with low Zinc levels, indicating their efficient accumulation of Zn and potential as bioindicators for Zn pollution even at lower contamination levels. Even with higher doses, their BCF values remained above 1, confirming their suitability as indicators, as supported by previous studies showing their Zinc accumulation abilities (Kurteva, 2009; Malizia *et* al., 2012; Bandiera *et* al., 2016). However, for Pb, none of the species showed a BCF value exceeding 1, suggesting that their ability to accumulate Pb in their tissues is limited. *R. acetosa*, however, demonstrated a TF value greater than 1 at both low and high concentration levels, indicating its effective movement of lead from roots to above-ground tissues. Likewise, *P. lanceolata* only exhibited a TF > 1 at the lower concentration level, implying its greater capacity for lead translocation when the soil has lower lead content, which aligns with previous research demonstrating the significant translocation of lead in *P. lanceolata* (Drava *et* al., 2019).

For a more practical approach, the third on-site bioindication investigation focused on *T. pratense*. Samples of *T. pratense* and soils were collected from different research sites and assessed to determine their potential as bioindicators across different types of land, including individual houses, near the lake and river, high-density residential area, industrial area, park, old town, and agricultural area. The concentrations of heavy metals including Pb, Cu, Cr, Ni, and Cd were analyzed in soils and plant organs (roots, leaves). The results revealed a correlation between land use and heavy metal concentrations in soils and *T. pratense* organs. These findings emphasize a promising discovery: *T. pratense* emerges as a reliable bioindicator, offering valuable insights for environmental monitoring.

5.2.Physiological responses

The presence of heavy metals in the environment can negatively affect plant ecosystems by altering their physiology, metabolism, growth, productivity, and ageing processes (e.g., Ghori et al., 2019; Hafeez et al., 2023). Nevertheless, many plants can gradually develop mechanisms to avoid and tolerate heavy metal stress (Islam and Sandhi, 2022). In the first experiment, plants show minor typical physiological symptoms of toxicity. However, it should be mentioned that, parameters such as dry mass, relative water content (RWC), and photosynthetic activities such as net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i), were generally higher in exposed plants compared to control species, except for *R. acetosa*, where a decrease was observed. Such physiological responses could be attributed to relatively low levels of heavy metals in the environment and short exposure durations. Heavy metal bioindicator plants possess the ability to accumulate high levels of heavy metals in their tissues and have developed detoxification mechanisms to tolerate metal stress (Stankovic *et* al., 2014; Gill, 2014; Yaashikaa et al., 2022). For instance, species that effectively accumulated Cd might have activated detoxification mechanisms to protect the photosynthetic apparatus (Wahid *et* al., 2010; El Rasafi *et* al., 2022), as observed in previous studies on other species such as *Amaranthus spinosus* (Dada, 2019). Contamination by heavy metals leads to the generation of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) (Gupta *et* al., 2015; Takallou *et* al., 2024). Higher levels of ROS were detected in all exposed plants compared to controls, particularly in *T. pratense*, *R. acetosa*, *A. rosea*, and *P. lanceolata*. Similar findings have been reported in studies, indicating that heavy metal stress induces ROS production, which in turn leads to increased antioxidant enzyme activities in various plant species (Bhaduri *et* al., 2012; Ghori *et* al., 2019). For better understanding how heavy metals affect physiological responses, the second experiment applied controlled contamination using metal nitrate solutions at two levels (low and high) through irrigation of the plant species. The accumulation in plant tissues of heavy metal ions caused various observable effects on the physiological activity of the plant species investigated, resulting in typical symptoms of metal toxicity. The dry mass of the investigated species varied across control and contamination levels. At low contamination, *P. lanceolata* had the lowest dry mass, suggesting susceptibility to heavy metal-induced growth inhibition. Conversely, *T. pratense* had the highest dry mass, indicating better stress tolerance. *T. pratense* also exhibited the lowest cell membrane stability (MSI) at the low contamination level, indicating increased vulnerability to membrane damage. *R. acetosa* had the highest MSI, indicating better membrane integrity in the presence of heavy metals. At high contamination, *A. retroflexus* had the lowest MSI, while *L. multiflorum* had the highest value, consistent with its known tolerance to heavy metals (Cui *et* al. 2021). Relative water content (RWC) analysis at both contamination levels showed a decrease compared to the control, indicating heavy metals' adverse impact on water retention capacity (Rucińska-Sobkowiak, 2016). This adverse effect on water relations and physiological processes due to heavy metals has been widely described (e.g., Borowiak and Fidler, 2014; Rucińska-Sobkowiak, 2016; Singh *et* al., 2023). Increased ROS levels were observed in all plants post-exposure, with *T. pratense*, *R. acetosa*, *A. rosea*, and *P. lanceolata* showing particularly high H_2O_2 levels. This increase was related to heightened antioxidant enzyme activity. The response of antioxidant enzymes to heavy metal stress varies by species (Gozdur *et* al., 2023; Moustakas, 2023). Among the species studied, *A. retroflexus*, *T. pratense*, and *R. acetosa* exhibited significant antioxidant responses. Similar findings have been reported by Lukatkin *et* al. (2021). Higher catalase (CAT) activity in plants treated with both contamination levels indicates an upregulation of this enzyme in response to increased H_2O_2 levels caused by heavy metal stress (Gechev *et* al., 2005). CAT plays a critical role in breaking down H_2O_2, protecting plants from oxidative damage (Aydin *et* al., 2022). These findings are consistent with studies showing CAT induction as a defense against heavy metal-induced oxidative stress (Štolfa *et* al., 2015; Aydin *et* al., 2022). The species-specific variation in CAT activity, with *L. multiflorum* showing higher activity in roots and *R. acetosa* in leaves, suggests different adaptation strategies. Higher ascorbate peroxidase (APOX) activities in the roots of *L. multiflorum* and *R. acetosa* after both contamination levels indicate their antioxidant defense mechanisms. APOX helps scavenge H_2O_2, suggesting a protective response to heavy metal pollution. Elevated malondialdehyde (MDA) levels in the leaves of *L. multiflorum*, *T. pratense*, *A. retroflexus*, and *P. lanceolata* after treatments signify oxidative damage from heavy metal exposure. Lipid peroxidation, indicated by MDA accumulation, is a common result of oxidative stress caused by heavy metals (Aydin *et* al., 2022).

6. Conclusions

In conclusion, this doctoral dissertation aimed to explore the heavy metal bioindicator potential of various plant species and to delve into their physiological responses under the influence of heavy metals. Through a comprehensive analysis, the effectiveness of selected plant species, including *T. pratense* L., *R. acetosa* L., *A. retroflexus* L., *P. lanceolata* L., and *A. rosea* L., as bioindicators for detecting and monitoring heavy metal was investigated. The study's findings can be summarized as follows:

1. Evaluation of heavy metal accumulation

The study confirmed that the selected plant species, including *Trifolium pratense* L., *Rumex acetosa* L., *Amaranthus retroflexus* L., *Plantago lanceolata* L., and *Alcea rosea* L., have varying capacities for heavy metal accumulation. In controlled conditions, *T. pratense* and *P. lanceolata* exhibited significant uptake and translocation of metals, especially Zn and Cd. *A. retroflexus* showed promise as a bioindicators for low-level Pb contamination. Despite exposure to heavy metals, plants displayed resilience in their physiological activities, with parameters such as dry mass, relative water content, and photosynthetic activity generally higher in exposed plants compared to controls. Increased levels of reactive oxygen species indicated oxidative stress, which was mitigated by increased antioxidant enzyme activities in certain species.

2. Determination of bioconcentration and translocation efficiency

The evaluation of the effectiveness of species in bioconcentration and translocation of heavy metals, together with the evaluation of physiological responses, showed their different abilities to accumulate and tolerate pollutants. *T. pratense* emerged as a potential bioindicator for Zn, Ni, and Cd, while *P. lanceolata* was effective for Zn and Cd. Physiological responses, such as increased antioxidant enzyme activities, indicated the plants' mechanisms to mitigate oxidative stress caused by heavy metal exposure.

3. *In situ* plant and soil research

The third research investigation underscored the efficacy of *T. pratense* as a reliable bioindicator across different urban environments. Moreover, red clover has the potential to accumulate HMs in plant organs. For instance, Cr's BCF exceeded 1 at most sites, except near the river, industrial area, and park. Ni's BCF exceeded 1 near the lake, high-density residential area, and agricultural land. The highest translocation factors (TF) for Cr and Ni were observed in industrial areas and old towns.

4. Establishment of bioindicator recommendations

Based on the physiological characteristics and bioaccumulation capacities, *T. pratense* and *P. lanceolata* are recommended as the most suitable bioindicators for heavy metal pollution. *T. pratense* showed remarkable adaptability and efficient uptake of Zn, Ni, and Cd, making it particularly valuable for environmental monitoring. *P. lanceolata* excelled in accumulating high concentrations of Zn and Cd.

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Common weeds as heavy metal bioindicators: a new approach in biomonitoring

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Environmental pollution by heavy metals affects both urban and non-urban areas of Europe and the world. The use of bioindicator plants for the detection of these pollutants is a common practice. An important property of potential bioindicators is their easy availability and wide distribution range, which means that they can be practically used over a wide area. Therefore, common and widely distributed weeds: *Trifolium pratense* L., *Rumex acetosa* L., *Amaranthus retroflexus* L., *Plantago lanceolata* L., ornamental species *Alcea rosea* L., and *Lolium multiflorum* L. var. Ponto were selected as a potential bioindicators of heavy metals (Cd, Pb, Cu, Zn). Plants were exposed in the same soil conditions in three sample sites in the Poznań city. It was found that all species had heavy metal accumulation potential, especially *A. rosea*, *P. lanceolata* and *L. multiflorum* for Zn (BCF = 6.62; 5.17; 4.70) and *A. rosea*, *P. lanceolata* for Cd (BCF = 8.51; 6.94). Translocation of Cu and Zn was the most effective in *T. pratense* (TF_{Cu} = 2.55; TF_{Zn} = 2.67) and in *A. retroflexus* (TF_{Cu} = 1.50; TF_{Zn} = 2.23). Cd translocation was the most efficient in *T. pratense* (TF_{Cd} = 1.97), but PB was the most effective translocated in *A. retroflexus* (TF_{Pb} = 3.09).. Based on physiological response to stress, it was detected an increasing level of hydrogen peroxide (H_2O_2) in roots and leaves of all samples, with the highest in all organs of *A. rosea*. Enzymatic activity levels of CAT, APOX, and also the marker of polyunsaturated fatty acid peroxidation MDA, were higher after 6 weeks of exposure in comparison to control samples and varied in time of exposure and between species and exposure. After the experiment, in almost all samples we detected a reduction of chlorophyll content and relative water content, but in efficiency of photosynthesis parameters: net photosynthesis rate, intercellular CO_2 concentration and stomatal conductance, we noted increased values, which proved the relatively good condition of the plants. The examined weeds are good bioindicators of heavy metal contamination, and their combined use makes it possible to comprehensively detection of environmental threats.

With the intensive development of human activities, urban areas have rapidly undergone significant and rapid changes. In urban areas, one of the most important urban pollutants are metals and metalloids[1,2]. Metals and metalloids are the subject of numerous studies because they are persistent and among the most widely disseminated industrial pollutants[3]. The main sources of these elements are natural sources, such as natural weathering of crust, erosion, and anthropogenic activities, like urban runoff, agricultural and industrial activities, and many others[4]. Exposure to heavy metals usually has subtle and chronic symptoms, moreover, exposure to airborne metals induces physiological responses in organisms and broad health effects in humans[5]. Also, the contamination of dietary substances by heavy metals is known to have a range of adverse effects on humans, animals and plants[6,7]. In plants, their toxicity varies depending on the specific metal, plant spieces, pH, soil composition, and chemical form. Certain heavy metals are considered to be essential for development and plant growth[8]. However, excess amounts of these elements can become toxic to plants[9], thus affecting plants only negatively[10].

The exposure of plants to unfavorable environmental conditions, including at higher concentrations of heavy metals, can cause an increase in the production of reactive oxygen species (ROS) such as singlet oxygen [(1) O_2], superoxide [$(O_2)^{-\cdot}$)], hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). ROS modifies proteins, damages DNA and causes free radical oxidation of unsaturated fatty acids or other lipids the product of which is MDA. The ROS detoxification process in plants is essential for the protection of plant cells, and therefore it seems that

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metal hyperaccumulating plants should have extremely efficient antioxidative and detoxicative defense mechanisms, enabling growth and development in a polluted environment[11]. Plant responses and tolerance to heavy metal stress are dependent on enzymatic antioxidants comprising ascorbate peroxidase (APOX), catalase (CAT), and the final product of polyunsaturated fatty acid peroxidation—malondialdehyde (MDA). These proteins take part in ROS detoxification in plants[12], and are present in practically all subcellular compartments. Usually, an organelle has more than one enzyme able to scavenge a single ROS[13]. As a result of oxidative stress, photosynthetic processes are disturbed, from electron transport to carbon bonding. Limitation of any of these processes within the photosynthetic apparatus reduces the ability of the chloroplast membrane to absorb light energy, increasing the ability to form oxidative radicals in the chloroplast, and as a consequence limits the productivity of photosynthesis[14].

Identifying areas with higher concentrations of heavy metals, guidelines, and effective legislation are necessary. In addition, these metals should be subject to mandatory monitoring due to their toxicity and possible bioaccumulation[4]. To control pollutants is a complex issue: the origin of pollutants and emission must be identified, critical emissions must be controlled, techniques must be developed that are sufficiently sensitive and low-cost to allow simultaneous measurement of multiple contaminants, risks and economic factors must be considered[15]. One inexpensive and simple method to determine the heavy metal concentration in the air and obtain information associated with the population's exposure to air pollutants in a particular ecosystem is biomonitoring[16]. Moreover, to obtain information about the changes in ecosystems, bioindicators can be used. Some plants are well known for their ability to accumulate trace elements from the environment. Therefore, they have been used in a number of monitoring investigations, providing low-cost information regarding environmental quality with the advantage of easy sampling. Various studies have used as bioindicators herbaceous plants (e.g., *Taraxacum officinale*, L., *Carduus nutans* L., *Plantago major* L., *Urtica dioica* L.), which are more common in urban environments (e.g.[17–20]).

Previous studies have indicate the ability of selected species to bioaccumulate heavy metals. However, so far, they have not been studied simultaneously, under the same contamination conditions, as well as taking into account the physiological response. The control of toxic elements contamination with the simultaneous use of commonly available weeds like *Plantago lanceolata* L.[21], *Amaranthus retroflexus* L.[22], *Trifolium pratense* L.[1], *Rumex acetosa*[23] and also known for the ability of trace metals accumulation, old ornamental plant (*Alcea rosea*)[24], seems to be a necessary procedure that enables comprehensive estimation of trace metals environment contamination. Selected weeds have the basic characteristics of bioindicators, such as: long life cycle, wide geographic ranges, large numbers of occurrence and ease of determination. Our research focused on checking whether selected weed species react in a characteristic way to changes in the environment (reaction to physical and chemical stress) depending on the place of occurrence. We made these arrangements by using the active bioindication based on the exposure and observation of specific plant species. This is where the purpose of our research lies—to evaluate widespread and very common bioindicators.

Considering the above, the main objectives of this study were as follows: (i) to determine the accumulation level of trace metals (Cu, Zn, Cd and Pb) in selected plant species exposed in even soil conditions in three research sites in the city; (ii) to assess the bioaccumulation potential of examined species; (iii) to establish translocation of metals from soil to above-ground parts; (iv) to study the physiological conditions of plants; (v) to determine the activity of oxidative stress parameters; and (vi) to assess the concentrations of enzymes of the antioxidative system.

Results

The content of copper, zinc, cadmium and lead in the soil used for pot culture and in the tissues (roots and leaves) of all samples of the studied species was examined, and then bioconcentration and translocation factors were calculated based on these results to assess bioaccumulation potential of examined species. Moreover, the plant physiological responses for stress were detected for all samples of examined species.

Heavy metal contents. The heavy metal contents for all species at all research sites showed the following tendency: Zn > Cu > Pb > Cd. This tendency was found for soil and plant organs (roots and leaves). In addition, for zinc and cadmium, the lowest values were mostly observed in the soil, while for copper and lead their content was generally the highest in the soil, with only a few exceptions (Suppl. Table S1). Analyzing the data in more detail, it was found that Cu, Zn, Cd and Pb concentrations in roots and leaves differ in all species. The highest Cu concentration in roots was found in *T. pratense* (2C: 20.38 mg kg^{-1}); also a high value was recorded in *R. acetosa* (3C: 10.51 mg kg^{-1}) and in *L. multiflorum* (1B: 8.30 mg kg^{-1}). The highest Cu accumulation in leaves was detected in *R. acetosa* (3C: 9.66 mg kg^{-1}), in *T. pratense* (2B: 9.20 mg kg^{-1}) and in *A. rosea* (4B: 8.13 mg kg^{-1}). The highest Zn concentration in roots was detected in *L. multiflorum* (1C: 81.13 mg kg^{-1}), *P. lanceolata* (6C: 80.45 mg kg^{-1}), *T. pratense* (2C: 68.49 mg kg^{-1}), *A. rosea* (4B: 55.73 mg kg^{-1}) and *A. retroflexus* (5A: 52.62 mg kg^{-1}). In *L. multiflorum* leaves the highest Zn concentration (1B: 172.45 mg kg^{-1}) was noted; high Zn concentration in leaves of *A. rosea* (4A: 135.85 mg kg^{-1}) and *P. lanceolata* (6C: 114.77 mg kg^{-1}) was also found. In the soil samples Zn concentration was lower than in plant tissues. The Cd amount varied in roots and leaves of studied species. In roots of *P. lanceolata* (Control: 0.69 mg kg^{-1}) we found the highest Cd concentration; also high Cd concentration was found in *A. rosea* (4A and 4B: 0.58 mg kg^{-1}) roots. In leaves of *A. rosea* (4B: 1.24 mg kg^{-1}) we observed the highest Cd amount; also in *L. multiflorum* leaves (1C: 0.79 mg kg^{-1}) and in *P. lanceolata* (6A: 1.11 mg kg^{-1}) high Cd concentration was detected in leaves. The highest Pb amount was found in *L. multiflorum* roots (1C: 1.32 mg kg^{-1}) as well as high Pb concentration in roots of *R. acetosa* (3C: 0.75 mg kg^{-1}). In leaf tissue of *L. multiflorum* the highest Pb concentration was detected (1A: 1.21 mg kg^{-1}), in *R. acetosa* Pb concentration in leaves reached 0.99 mg kg^{-1}, and in *P. lanceolata* it reached 0.77 mg kg^{-1}. Pb concentration in soil samples was higher than in plants. However, two-way ANOVA of species and site effect revealed significant influence ($\alpha \le 0.05$) of both factors on all analyzed trace elements levels in roots and leaves. The both factors were found to have no significant effect on the analyzed levels of these elements in soil, except of cadmium (one outlier observation in control) (Suppl. Table S2).

Based on the cluster analysis with the procedure for grouping objects and features (Fig. 1), taking into account all detected heavy metals, it can be found that there were differences between samples from sites A, B and C. The soil most contaminated by Pb, Cu and Zn and Cd was from site B—the Botanical Garden. Two groups of samples were formed. The first consisted of 1B, 1C, 4A and 4B, and 6C. The second consisted of four subgroups: the first subgroup included samples 6B, 2B, 6A and 1A; the second 5A, 2C; the third 3C, 4C, 5C; and the fourth 3A, 2A, 3B, 5B. Regarding the values of heavy metals in roots of detected species, the highest concentration of Zn in roots was detected in *L. multiflorum* (1B, 1C, 1A), *A. rosea* (4A, 4B) and in *P. lanceolata* (6A, 6C); the highest values of Cd in roots was detected in *A. rosea* (4A, 4B), *L. multiflorum* (1C), and *P. lanceolata* (6A) samples; the highest concentration of Pb in roots was noted in *L. multiflorum* (1B, 1C, 1A), *P. lanceolata* (6B), *R. acetosa* (3C), *A. rosea* (4C), *A. retroflexus* (5C) and in *T. pratense* (2A). In sequence, taking into consideration Cu in roots, we noted the highest values in *R. acetosa* (3C, 3A and 3B), *T. pratense* (2B, 2C), *A. rosea* (4B) and *P. lanceolata* (6A). Regarding the values of heavy metals in leaves of detected species, the highest concentrations of Zn were noted in *L. multiflorum* (1C), *P. lanceolata* (6C and 3C), *A. rosea* (4B), *A. retroflexus* (5A), and *T. pratense* (2C), while the highest amounts of Cd were detected in leaves of the following samples: *P. lanceolata* (6B, 6C), *A. rosea* (4A, 4B, 4C), *T. pratense* (2C), and *A. retroflexus* (5C). Relatively high values of Pb in leaves were detected in *L. multiflorum* (1C, 1A), *T. pratense* (2B), *R. acetosa* (3C, 3A) and *A. retroflexus* (5B), and finally, the highest concentrations of Cu in leaves were detected in the following samples: *T. pratense* (2C) and *R. acetosa* in (3C).

Bioconcentration and translocation factor. The bioconcentration factors (BCF) exceeded a value of 1 for Zn and Cd in all plant species. The highest values of Zn BCF were recorded in *L. multiflorum* (1C: $BCF_{Zn} = 6.62$), in *A. rosea* (4A: $BCF_{Zn} = 5.17$) and in *P. lanceolata* (6C: $BCF_{Zn} = 4.70$); however, the highest Cd BCF values were found in *A. rosea* (4B: $BCF_{Cd} = 8.51$), in *P. lanceolata* (6A: $BCF_{Cd} = 6.94$) and in 1C *L. multiflo-*

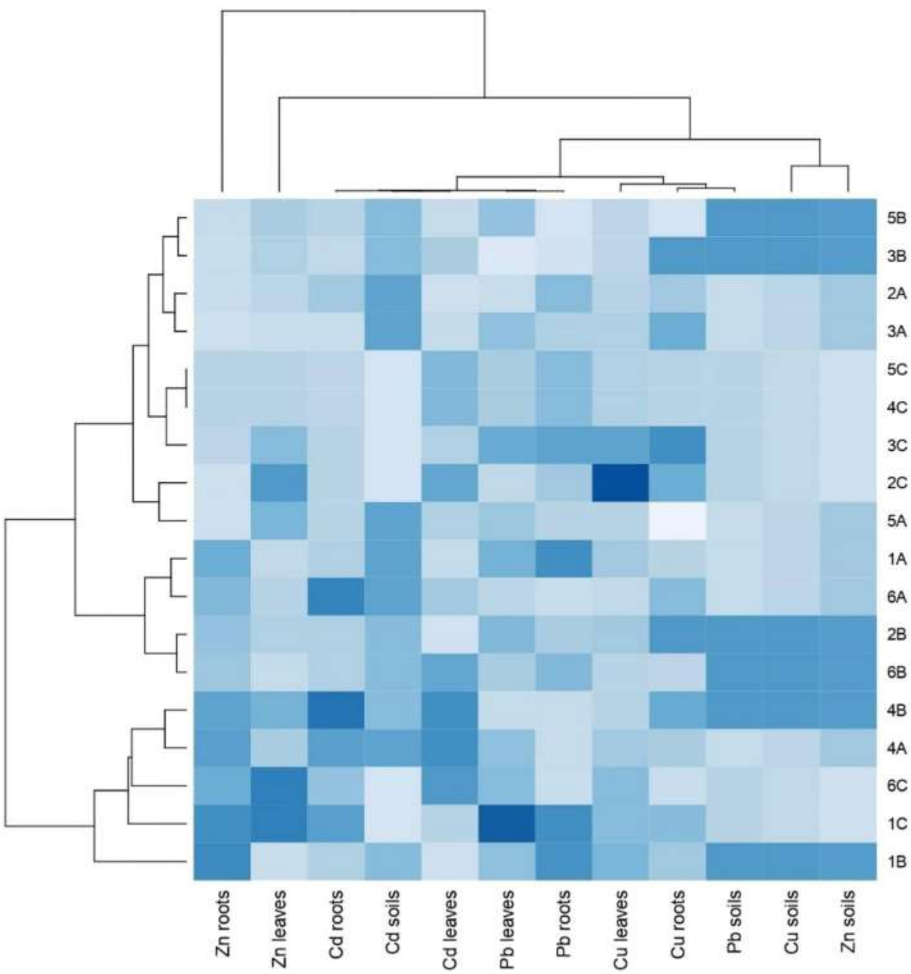

Figure 1. Heatmap and cluster analysis of heavy metal contents in soils, roots and leaves in examined samples at all research sites (abbreviations see "Materials and methods").

rum (BCF_{Cd} = 6.29). Cu and Pb bioconcentration was not as effective as the first two elements, but it is worth mentioning that Cu bioconcentration factors of all detected samples exceed bioconcentration of Pb. Taking into account translocation of detected HMs, the highest Cu TF value was detected in sample 2C of *T. pratense* (TF_{Cu} = 2.55), in sample 6C of *P. lanceolata* (TF_{Cu} = 1.55), in 5A sample *A. retroflexus* (TF_{Cu} = 1.50), and in all samples of *Lolium multiflorum* (TF_{Cu} = 1.31–1.08). The Zn translocation factor was the highest in *T. pratense* 2C (TF_{Zn} = 2.67), in all samples of *A. retroflexus* (TF_{Zn} = 2.23–1.05), and in *R. acetosa* (TF_{Zn} = 1.28–1.11). The highest Cd translocation factor was detected in 2C *T. pratense* (TF_{Cd} = 1.97), followed by *P. lanceolata* (TF_{Cd} = 1.51–1.24), and in *R. acetosa* (TF_{Cd} = 1.44–1.42). Regarding the Pb translocation factor, the highest values were detected in *A. retroflexus* (TF_{Pb} = 3.09), in *P. lanceolata* in 6B (TF_{Pb} = 2.25), and in sample 4A of *A. rosea* (TF_{Pb} = 1.90) (Table 1).

Physiological condition of species. After 6 weeks of the experiment, cell membrane stability (MSI) took values from 93.41% in *Alcea rosea* sample 4C to the highest values, more than 98%, in all *Plantago lanceolata* samples, and in samples of *Trifolium pratense*, *Rumex acetosa* and *Amaranthus retroflexus*. Dry mass content was highest (23.11%) in *Amaranthus retroflexus* sample 5A, and the lowest in *R. acetosa* (8.01%) in sample 3C. In all species higher dry mass contents was detected in comparison to control samples. RWC was the highest in *R. acetosa* sample 3A (95.25%) and the lowest in *A. rosea* sample 4C (62.53%). It should be noted that almost all samples were characterized by RWC above 90%. Chlorophyll *a* content ranged from 3.33 in *Amaranthus retroflexus* to 11.02 in *Rumex acetosa*. Chlorophyll *b* content ranged from 4.96 in *Trifolium pratense* to 0.9 in *Amaranthus retroflexus*. Chlorophyll *a* + *b* content ranged from 4.11 in *A. retroflexus* to 15.36 in *R. acetosa*. Chlorophyll *a/b* ratio ranged from 0.27 in *A. retroflexus* to 0.58 in *T. pratense*. Photosynthesis activity (P_N) ranged in detected species from 7.24 in *Rumex acetosa* to 28.28 in the *Trifolium pratense* sample. Stomatal conductance (g_s) ranged from 32.64 in *Amaranthus retroflexus* to 188.86 in *Trifolium pratense*. *C*i intercellular CO_2 concentration varies from 261.47 in *Amaranthus retroflexus* to 528.20 in *Plantago lanceolata* (Table 2).

The graphical representation of the results by analysis of the first two principal components for heavy metal accumulation in leaves, roots and photosynthesis activity parameters in all samples explained more than 47.81% of total variability (Fig. 2). A positive relationship was found between Zn-BCF, Cd-BCF in *L. multiflorum* samples (1A, 1B, 1C), *T. pratense* (2B) samples and in *A. rosea* (4B, 4C) samples. We also found a positive relationship between Pb TF and dry mass content in *P. lanceolata* (6B). Another large group consists of correlated Zn TF,

Plants		Bioconcentration factor (BCF)				Translocation factor (TF)			
		Cu	Zn	Cd	Pb	Cu	Zn	Cd	Pb
Lolium multiflorum	1A	0.48	**4.24**	**1.71**	0.18	**1.08**	0.30	0.45	0.58
	1B	0.35	**5.92**	**1.76**	0.16	**1.31**	0.17	0.25	0.50
	1C	0.64	**6.62**	**6.29**	0.18	**1.08**	0.50	0.23	**1.09**
	Control	0.43	**3.64**	**2.01**	0.11	**1.16**	0.36	0.22	0.73
Trifolium pratense	2A	0.53	**1.18**	**1.99**	0.11	0.72	**1.11**	0.24	0.43
	2B	0.51	**2.77**	**1.71**	0.07	0.64	0.47	0.22	**1.19**
	2C	0.71	**1.05**	**1.74**	0.08	**2.55**	**2.67**	**1.97**	0.62
	Control	0.73	**1.41**	**1.66**	0.08	0.99	**1.15**	0.33	0.62
Rumex accetosa	3A	0.67	0.87	0.49	0.07	0.62	**1.28**	**1.42**	**1.28**
	3B	0.51	**1.13**	**1.02**	0.03	0.48	**1.15**	**1.44**	0.86
	3C	0.86	**1.84**	**1.71**	0.15	**1.09**	**1.11**	0.92	0.76
	Control	0.98	**1.09**	**1.11**	0.04	**0.75**	**1.05**	1.20	0.96d
Alcea rosea	4A	0.52	**5.17**	**4.93**	0.05	0.99	0.29	0.73	**1.90**
	4B	0.45	**4.53**	**8.51**	0.04	0.58	0.42	0.46	**1.06**
	4C	0.51	**2.20**	**1.50**	0.11	0.91	0.69	**1.81**	0.67
	Control	0.80	**3.62**	**2.81**	0.04	0.78	0.54	0.65	**1.09**
Amaranthus retroflexus	5A	0.26	0.90	**1.30**	0.07	**1.50**	**2.23**	0.91	**1.16**
	5B	0.24	**1.30**	**1.47**	0.02	0.94	**1.05**	0.57	**3.09**
	5C	0.32	**1.11**	**1.37**	0.04	**1.17**	**1.50**	0.73	**1.71**
	Control	0.52	**1.09**	**1.62**	0.04	**1.01**	**1.73**	0.57	**1.13**
Plantago lanceolata	6A	0.61	**3.77**	**6.94**	0.04	0.55	0.37	0.22	**1.35**
	6B	0.31	**2.66**	**1.94**	0.10	0.80	0.41	**1.51**	0.62
	6C	0.44	**4.70**	**3.21**	0.04	**1.55**	0.70	**1.24**	**2.25**
	Control	0.76	**2.36**	**2.46**	0.06	**1.20**	0.53	**1.07**	**1.40**

Table 1. The bioconcentration (BCF) and translocation factor (TF) of Cu Zn, Cd and Pb in plant species from research sites (abbreviations see "Materials and methods"). Where: in BCF the highlighted values mean concentration in roots biomass and in TF the highlighted values means effective metals translocation within the plant.

Plants		MSI	Dray mass	RWC	Chl *a*	Chl *b*	Ch *a* + *b*	Ratio *b/a*	P_N	g_s	Ci
Lolium multiflorum	1A	97.99	20.00	94.30	5.84	1.91	7.74	0.33	10.67	85.84	471.82
	1B	94.30	14.85	91.89	7.33	2.39	9.58	0.32	16.43	123.64	436.27
	1C	96.82	16.19	64.57	6.00	1.64	7.60	0.26	15.18	115.29	507.37
	Control	98.10	10.48	96.51	9.41	2.98	12.35	0.32	10.77	70.05	233.93
Trifolium pratense	2A	96.64	19.97	93.56	7.95	4.00	12.80	0.50	20.71	108.62	331.63
	2B	98.27	20.50	93.36	5.78	2.01	7.74	0.35	16.30	188.86	518.13
	2C	96.76	16.93	90.74	8.79	4.96	14.84	0.58	28.28	172.52	459.57
	Control	95.86	11.51	93.77	17.85	5.30	22.89	0.30	9.10	38.43	254.63
Rumex accetosa	3A	97.66	12.73	95.25	5.15	2.01	7.14	0.39	7.24	38.28	357.50
	3B	98.83	14.30	90.90	6.55	2.51	9.04	0.38	7.49	75.96	506.33
	3C	97.03	8.01	92.08	11.02	4.33	15.36	0.39	13.47	99.45	517.57
	Control	94.95	11.86	89.68	7.06	2.19	9.16	0.31	18.27	116.93	284.43
Alcea rosea	4A	95.02	21.78	78.23	4.51	1.52	6.02	0.34	8.66	44.48	308.00
	4B	94.37	19.09	78.78	6.80	2.28	9.03	0.34	12.29	84.37	430.90
	4C	93.41	22.34	62.53	6.02	2.20	8.34	0.37	11.65	58.14	392.00
	Control	95.03	13.19	80.94	14.54	16.01	30.11	0.75	9.75	57.67	232.53
Amaranthus retroflexus	5A	97.44	23.11	91.75	3.76	1.13	4.81	0.30	18.21	81.08	261.47
	5B	98.21	22.07	95.38	3.33	0.90	4.11	0.27	15.42	77.16	341.03
	5C	97.38	14.19	94.11	10.27	4.06	14.84	0.39	8.45	32.64	309.50
	Control	94.13	9.26	91.04	12.83	3.21	15.73	0.25	6.70	57.67	232.53
Plantago lanceolata	6A	98.23	11.62	92.23	8.34	3.15	11.28	0.38	12.37	86.46	391.97
	6B	98.76	16.77	93.62	3.93	1.28	5.11	0.33	16.87	128.84	446.53
	6C	98.29	10.20	93.92	8.58	3.01	11.48	0.35	15.60	131.24	528.20
	Control	98.78	6.88	92.29	13.88	4.41	18.15	0.32	10.49	88.90	555.80

Table 2. Cell membrane stability—MSI (%), dry mass (%), relative water content—RWC (%), chlorophyll [Chl *a*, Chl *b*, Chl *a* + *b*, ratio *b/a*] (mg g^{-1}), and photosynthetic activities—net photosynthetic rate—P_N (μmol CO_2 m^{-2} s^{-1}), stomatal conductance—g_s (μmol CO_2 m^{-2} s^{-1}) and intercellular CO_2 concentration—Ci (*μ*mol CO_2 m^{-2} s^{-1}) detected for all samples (abbreviations see "Materials and methods").

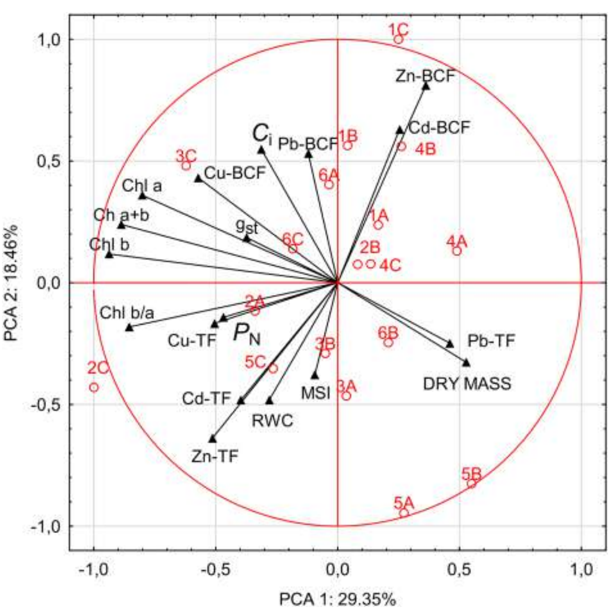

Figure 2. Principal component analysis of heavy metal concentrations, bioconcentration factor (BCF) and translocation factor (TF) in examined samples (abbreviations see "Materials and methods") in relation to photosynthesis activity parameters: net photosynthetic rate P_N, stomatal conductance g_s and intercellular CO_2 concentration and (*C*i), chlorophyll parameters and dry mass, relative water content (RWC) and cell membrane stability (MSI).

Cu-TF, Cd TF with relative water content (RWC), cell membrane stability (MSI), chlorophyll b/a coefficient and net photosynthetic rate – $P_{N.}$ of *T. pratense* (2A, 2C), *R. acetosa* (3A, 3B) and *A. retroflexus* (5C) samples. The last group was composed of Pb-BCF, Cu-BF correlated with chlorophyll content stomatal conductance (g_s) and intercellular CO_2 concentration (*Ci*) of *R. acetosa* (3C) and *P. lanceolata* (6A, 6C) samples.

The profiles of changes and the level of hydrogen peroxide values in all species were similar in roots and in leaves, with the highest amount of $H_2O_2 \approx 6$ (nmol $H_2O_2 \times min^{-1} \times$ mg protein^{-1}) in roots of *R. acetosa* and *A. rosea* (4A). The highest amount of $H_2O_2 \approx 5$ (nmol $H_2O_2 \times min^{-1} \times$ mg protein^{-1}) in leaves was detected in all samples of *T. pratense*, *A. retroflexus* and in *A. rosea* sample 4A (Fig. S1).

The profiles of changes and the level of CAT activity were similar in roots in leaves of *L. multiflorum*, *R. acetosa* and *A. retroflexus*. The highest activity of CAT ≈ 1.5 (nmol $H_2O_2 \times min^{-1} \times$ mg protein^{-1}) in roots and CAT ≈ 0.9 (nmol $H_2O_2 \times min^{-1} \times$ mg protein^{-1}) in leaves was noted in *L. multiflorum*. It is worth noting that in *A. retroflexus* and in *P. lanceolata* high activity of CAT ≈ 0.9 (nmol $H_2O_2 \times min^{-1} \times$ mg protein^{-1}) was noted in roots. In leaves the highest activity was noted in *L. multiflorum* CAT ≈ 0.9 (nmol $H_2O_2 \times min^{-1} \times$ mg protein^{-1}) and in A. retroflexus CAT ≈ 0.55 (nmol $H_2O_2 \times min^{-1} \times$ mg protein^{-1}) (Fig. S2).

APOX activities were generally higher in roots than in leaves. In roots, the highest activity (APOX ≈ 0.065) was noted in *L. multiflorum* and in *P. lanceolata*. In leaves APOX activity in all samples was high, with the highest activity of APOX ≈ 0.03 in *L. multiflorum* and in the *A. rosea* sample (Fig. S3).

The profiles of changes and the level of MDA activity were higher in roots than in leaves of *T. pratense*, *R. acetosa*, *A. rosea* and *A. retroflexus*. The highest level of MDA ≈ 12.0 in roots and in leaves (MDA ≈ 7.0) was detected in *A. rosea* sample 4A (Fig. S4).

The graphical representation of the results by the analysis of the first two principal components for heavy metal accumulation in leaves, roots and photosynthesis activity parameters in all samples explained more than 45% of the total variability (Fig. 3). A positive relationship was found between CAT activity in leaves and Cd and Cu in roots of *Plantago lanceolata* samples 6A and 6B and *Alcea rosea* sample 4B. APOX activities in roots were related to Zn in roots in *Alcea rosea* sample 4A. There was a positive relationship between hydrogen peroxide amount and Cu and Zn in leaves of *Plantago lanceolata* sample 6C. The next group consisted of APOX in leaves, MDA content in roots and the level of Pb in roots and leaves, the level of Cu in leaves in *A. rosea* 4C, *R. acetosa* 3C, *A.* retroflexus 5C and *T. pratense* 2C samples. Finally, there was a relationship between hydrogen peroxide in leaves, CAT activity in roots in *A. retroflexus* 5A and B, *R. acetosa* 3A and *T. pratense* 2A and B samples.

The most intensive fluorescence DHE, indicating the presence of H_2O_2 in leaves, was observed in the *T. pratense* (2A), *Rumex acetosa* (3A), A. rosea (4A) and *P. lanceolata* (6A) samples (Fig. 4).

Discussion

This comparative study of different weed species has shown their potential for accumulation of HMs. *Lolium multiflorum* var. Ponto was used as a known variety cumulating HMs[19,25,26], but in our study it was found that this variety showed similar possibilities of HM accumulation to the weed species selected for the experiment. Almost all the studied species showed significant potential for Zn and Cd accumulation compared to Cu and Pb. The efficiency of accumulation was expressed as the BCF factor, and the metal displacement efficiency as the translocation factor (TF). For a hyperaccumulator plant, both of these factors should be greater than unity[27]. Taking into account these two factors, the most effective bioindicator can be selected. Analyzing cadmium,

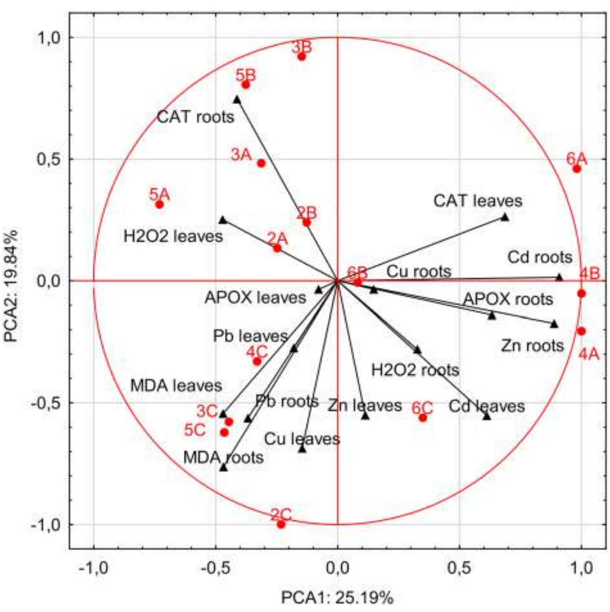

Figure 3. Principal component analysis of heavy metal concentration in examined samples (abbreviations see "Materials and methods") in relation to hydrogen peroxide—H_2O_2, APOX, CAT, MDA activities.

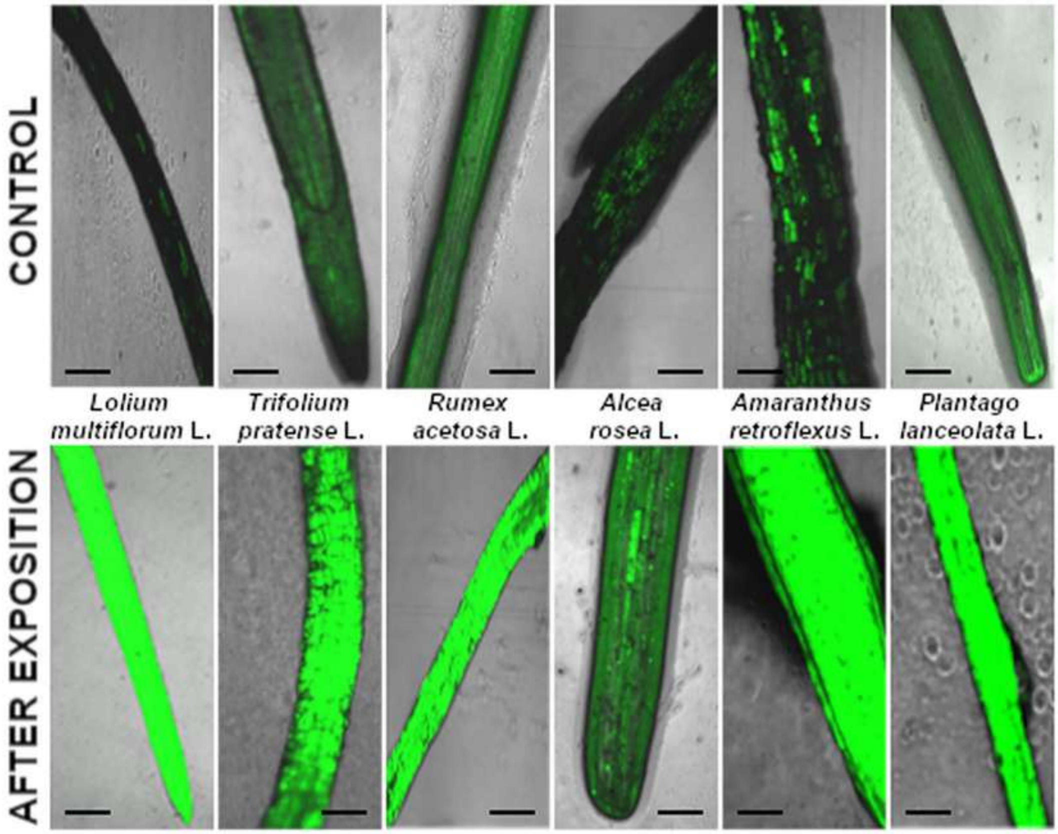

Figure 4. Fluorescent images show H_2O_2 production in leaves of examined species after exposure, (abbreviations see "Materials and methods"). The bar indicates 1 μm.

which is the metal that is the most toxic to plants and animals[28], the highest content of Cd was found in the roots of *A. rosea* and *P. lanceolata*. Very high bioconcentration coefficients calculated for these two species confirmed the potential for this element to be concentrated in these species' tissues. The usefulness of *A. rosea* as a hyperaccumulator of Cd was noted previously by Liu et al.[29] and Ubeynarayana et al.[30]. Zn was also effectively accumulated mainly in leaves by *A. rosea* and *P. lanceolata*. However, it is worth noting that Zn was well accumulated by all analyzed species. This ability was also confirmed by bioconcentration factors determined for this species. Zinc as an essential micronutrient in plant nutrition is naturally taken up by plants, and the efficiency of uptake depends on soil pH and phosphorus levels. In the countries of the European Union, soil is polluted with zinc, due to the use of sewage sludge for fertilization purposes or composts made from them[31]. The amounts of Zn in unpolluted soils typically are lower than 125 ppm (125 mg kg^{-1}) and in plants growing in these soils this metal concentration varies between 0.02 and 0.04 mg g^{-1} (20–40 mg kg^{-1}) dry weight[32]. In our study, the highest concentration of Zn (≈ 29 mg/kg) was detected in Botanical Garden soil (B), which could be the consequence of use of fertilizers in this area. However, according to Polish regulations on the permissible content of substances causing risk to human health and the environment, the obtained results do not exceed the standards for soil at urban areas (Cu = 200 mg kg^{-1}, Zn = 500 mg kg^{-1}, Cd = 2 mg kg^{-1}, Pb = 200 mg kg^{-1})[33]. Duan et al.[34] noted that Zn was accumulated more in roots than in leaves of *A. rosea*, which was confirmed in our study; the most effective accumulation of Zn was also detected in *A. rosea* as well as in *P. lanceolata* and *L. multiflorum* var. Ponto. We confirmed earlier information about mobility of Zn in plant tissues. The best Zn-TF transport efficiency was detected in *R. acetosa*.

Longnecker et al.[35] reported that in plants tolerant of toxic levels of Zn, accumulation was observed in the root cortex and in their leaves. We detected that cadmium was effectively accumulated in the roots, but also efficiently transported to the leaves of *A. rosea*, *P. lanceolata*, *T. pratense* and *R. acetosa* samples, as evidenced by high translocation factor (TF) values. If we take into account Zn and Cd together, we should remember about the interaction between Zn and Cd, which consists in the mutual inhibition of the accumulation and sometimes translocation of elements in the plant[36].Recent research showed that the use of excess Zn, along with exposure to Cd under hydroponic conditions, mitigated Cd toxicity in plants by increasing all phenols and chlorophylls in the leaves, thereby mitigating the adverse effects of Cd on photosynthetic function and oxygen secretion activity[37]. The authors suggested that these mechanisms are involved in Zn detoxification and protection against Cd-induced structural and functional damage of the photosynthetic membranes.

Pb accumulation was not so effective as mentioned earlier for Zn and Cd, but it is worth pointing out that a relatively high concentration was found in leaves of all examined species, the highest in *L. multiflorum* and *R.*

acetosa. Barrutia et al.[38] established that *R. acetosa* had the potential for bioconcentration of Pb (and also Zn and Cd), from highly contaminated mine soil. However, our research did not confirm the ability to hyperaccumulate this element, which could be due to there being a relatively small amount in the soil of the studied sites. Lead was efficiently transported to the above part of the plant in selected samples of all examined species, especially in *A. retroflexus* and also in *A. rosea* and *P. lanceolata*. Mobility of this ion in *Amaranthus spinosus* was also confirmed by Yingping et al.[39]. In *Limbarda crithmoides* and *Helianthus annuus* Pb was not efficiently transported from roots to the leaves[40] and it was only accumulated in roots.

Taking into consideration Cu, we noted that *L. multiflorum*, *T. pratense*, *R. acetosa*, *A. retroflexus* and *P. lanceolata* showed the ability to accumulate copper and its quick transport from the roots to the above-ground parts of the plant. Malizia et al.[1] confirmed that *T. pratense* has the potential for copper bioconcentration.

Our results also demonstrate a synergistic interaction between Cu, Cd and Zn during translocation of these elements in shoots of *Trifolium pratense* and *Amaranthus retroflexus* (Fig. 2). Other researchers have also noted that *Amaranthus retroflexus* accumulates metals such as Cd, Ni, Pb and Cu in the aboveground parts[41]. In *Rumex acetosa* an interaction between translocation of Cd and Zn (Fig. 2) was found, whereas other authors[42] reported that Zn induced a decrease in Cd uptake and a simultaneous increase in Zn accumulation in tomato plants. This suggested strong competition between Zn and Cd for the same membrane transporters.

In the presence of heavy metal ions, the plants in our study did not exhibit characteristic symptoms of their toxic effect on physiological activity. Dry mass, RWC, net photosynthetic rate P_N, stomatal conductance g_s and intercellular CO_2 concentration *Ci* in plants after exposure were higher than in control species; only in one species—*R. acetosa*—did we detect a decrease in these parameters compared to the control (Table 2). This may be due to relatively small amounts of heavy metals in the environment and relatively short exposure. The metal hyperaccumulating plants have an ability to accumulate a relatively high level of HMs in their plant tissues, and they have developed a number of detoxification mechanisms for acclimation and tolerance of metals. The mechanism of Cd tolerance has been extensively studied in many species[43]. Our research showed that species that accumulated Cd effectively triggered detoxification mechanisms to protect the function of the photosynthetic apparatus against Cd stress[44]. Other authors observed a decrease in all photosynthesis parameters in *Amaranthus spinosus*[39] and delayed chlorophyll fluorescence in *Lemna minor*[45].

Contamination of plant tissues by heavy metals leads to the formation of ROS such as hydrogen peroxide (H_2O_2)[12]. We observed an increase in the level of ROS compared to control plants in all plants after exposures. We observed higher levels of H_2O_2 in all plant organs of *T. pratense*, *R. acetosa*, *A. rosea* and *P. lanceolata*. The increase in ROS production in plants was associated with an increase in the activity of antioxidant enzymes.

All examined species were characterized by the potential for accumulation of heavy metals. However, an effective bioaccumulation process will depend on active detoxifying enzymes and the regulation of primary defense enzymes. We always observed the induction of antioxidant enzyme activity in roots and leaves of plants, although there were no significant differences between the researched plants. Heavy metals modify membrane properties by interacting with functional groups of membrane proteins and lipids. As a lipid peroxidation marker[46], the measurement of malondialdehyde (MDA) content is used. In our research, MDA level activity after 2 weeks was higher than after 6 weeks of exposure. The increase in MDA was induced by both essential metals such as Zn and non-essential metals such as Pb. It was detected that a higher MDA level in roots and leaves in *Lolium multiflorum*, *Trifolium pratense* and *Amaranthus retroflexus* was correlated with high amounts of Pb in roots (Fig.). Lukatkin et al.[22] reported that MDA levels in roots and leaves of *Amaranthus retroflexus* were correlated with high levels of Zn. Ascorbate peroxidase (APOX) isoforms play important and direct roles as protective elements against adverse environmental conditions[42]. The decrease in membrane lipid peroxidation observed after 6 weeks may be due to activation of the ROS-inactivating antioxidant system. In our study APOX activities were generally higher in leaves than in roots in all species. Activity of APOX was definitely lower than catalase, especially in the above-ground parts, which means that this enzyme complements CAT catalytic activity. APOX may be responsible for controlling the levels of H_2O_2 as signal molecules, and the CAT function is to remove large amounts of oxygen during oxidative stress[12]. The profiles of changes and the activity level of CAT were different between leaves and roots, but it is worth noting that in all sites with exposure of *A. retroflexus* higher activity values of CAT were noted. Mohamed et al.[47] showed in *Brassica juncea* that the higher activity of antioxidant enzymes offers greater detoxification efficiency, which provides better plant resistance against trace metal-induced oxidative stress.

The results show the high accumulation potential of these species and their adequate physiological response to stress. In order to detect environmental pollution, data from all tested species should be obtained, and such a procedure will more effectively determine the levels of risk for heavy metals environment contamination.

Conclusions

Based on the obtained results, it can be concluded that all species showed varied but generally great potential for high accumulation of detected trace elements. The concentrations of all elements in plant tissues were dependent on species, organ (root vs. shoot), and species-organ interactions. The physiological response of the studied species to stress was correlated with the high content of the tested metals in the tissues. Plants exposed in different study sites showed different concentrations of trace elements in their tissues. Due to the varying degree of the tested species ability to accumulate trace elements, in order to estimate the degree of environmental pollution by these compounds, we recommend the simultaneous use of all species of weed which were tested in this work.

In the future, finding a bioindicator among weeds that would have a whole set of excellent bioindicating features, would provide a simple and cheap early warning system against the negative effects of changes in the ecosystem.

Materials and methods

In order to evaluate the bioaccumulation ability of Cd, Pb, Cu and Zn by 6 selected plant species, an experiment was organized. The contents of these elements, their bioconcentration and translocation in plants and soil were also determined, as well as the condition of the plants was evaluated. In addition, the results were analyzed using statistical analysis.

Materials. Species for the study were selected due to their common, wide range of occurrence, generally throughout Europe. For this investigation we selected species often found as weeds and as ornamental species in urbanized areas:

Lolium multiflorum L. (no. 1) is native to all Europe (except Finland), Western, Southern and Central Asia (except Uzbekistan), as well as Northern Africa. It was introduced to the Americas, South and East Africa, Australia and East Asia (Hultén and Fries, 1986; POWO, 2019). For our purposes we used the *Lolium multiflorum* variety Ponto, obtained from Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Germany). It displayed phytoremediation potential for heavy metals[48].

Trifolium pratense L. (no. 2) commonly known as red clover. It is a species native to: Europe, South, West and Middle Asia as well as North-West Africa. Introduced and widespread in all continents except Antarctica[49,50]. The seeds were collected by the authors from Wielkopolska rural areas;

Rumex acetosa L. (no. 3), also known as common sorrel, is a herbaceous plant native to Europe, Asia, and North Africa (Morocco), and now it has spread to all continents except Australia and Antarctica[49,50]. The seeds were collected by the authors from Wielkopolska rural areas;

Alcea rosea L. (no. 4), the common hollyhock; it was imported into Europe from southwestern Asia as an ornamental plant species before the fifteenth century. Since then, it is common ornamental plants in cities and widespread in the Americas, North Africa, and South Asia as a wilderness species[49,50]. In the present experiment we used *Alcea rosea* L. The seeds were collected by the authors from Wielkopolska rural areas;

Amaranthus retroflexus L. (no. 5), the red-root amaranth, is now found nearly worldwide. It is a species native to Mexico. It was introduced into all continents except Antarctica[49,50]. The seeds were collected in the Poznań agglomeration;

Plantago lanceolata L. (no. 6), ribwort plantain; this remarkably widespread species is native to all Europe, North Africa and West, South and Middle Asia but has been introduced extremely widely elsewhere and now occurs e.g. in both Americas, Australia, New Zealand, Japan and in South and East Africa, where it thrives at high altitude[49,50]. The seeds were collected by the authors from Wielkopolska rural areas.

We confirm that all methods including collection of plant material, were carried out in accordance with relevant guidelines and regulations.

Organization of the experiment. The experiment was carried out during the growing season in 2021. The experiment started in April with planting the seeds in the control greenhouse conditions (temperature 16–18 °C, no artificial light). 5 L pots with a standard mixture of peat and sand were used (pH 6.8, N: 230 mg L^{-1}, P: 180 mg L^{-1}, K: 350 mg L^{-1}, Mg: 150 mg L^{-1}). The content of the tested elements in potting soil at the beginning of the experiment was 4.151 ± 0.032 mg kg^{-1} for Cu, 15.03 ± 0.34 mg kg^{-1} for Zn 0.091 ± 0.004 mg kg^{-1} for Cd, and 4.302 ± 0.052 mg kg^{-1} for Pb. Seeds of each species were sown in individual pots in equal amounts. After germination, ten of the most vital and largest seedlings were left in the pot. During germination and cultivation in the greenhouse, deionized water was used for plant irrigation. After 60 days, the plants were taken to exposure sites with various environmental conditions. The three exposure sites were selected for these investigations, located in Poznań city. The first site (site A) was located in a residential area located on the right bank of the Warta river (N: 52°23′53″; E: 16°57′36″), in the eastern part of the Poznań city (Fig. 5). High-density built-up areas (multi-family housing) dominated in the surrounding area of this research site. The second exposure site (site B) was located in the Botanical Garden of the Adam Mickiewicz University in Poznań on the left bank of the Warta river (N: 52°25′14″; E: 16°52′39″), in the western part of the city. The immediate surroundings were green areas, whereas in the further surroundings of exposure site C there was a low-density built-up area (single-family housing) and main road (N: 52°25′50″; E: 16°54′58″). At each site, three pots with plants of a given species were placed (in total, there were 18 pots per site). The exposure of samples lasted 6 weeks (from June 1 to July 16, 2021). During the exposure, the plants were watered with distilled water and protected from the direct sun (shadowed) by naturally occurring higher vegetation, without negative effect for air flow. Air pollution at research sites during exposition, were provided by the General Directorate for Environmental Protection (Table S3).

Heavy metal analysis. *Preparation samples.* In the laboratory, the plant samples were first purified with deionized water using Milli-Q Advantage A10 Water Purification Systems, Merck Millipore (Merck, Darmstadt, Germany), and separated into leaves and roots. The soil samples from all pots were sieved (2 mm). To achieve constant dry weight, the plant and soil samples were dried at 40 ± 3 °C in an electric oven (FD115, Binder, Germany). Digestion of the powdered samples of plant (homogenous samples from each pot) and soil were carried out in the CEM Mars 5 Xpress microwave mineralization system (CEM, USA). From each plant, 0.3000 ± 0.0001 g of leaves or roots were placed in a Teflon vessel with 8 mL of concentrated (65%) HNO_3 (analytical purity, Merck, Darmstadt, Germany) and 1 mL of H_2O_2 (Merck, Darmstadt, Germany). The program of digestion included the following stages—first stage: temperature to 80 °C, 10 min, power 600 W; second stage: temperature 140 °C, 12 min, power 1200 W; third stage: temperature 185 °C, 15 min, power 1200 W. After the digestion steps using Qualitative Filter Papers (Grade 595: 4–7 μm Whatman, GB), the solutions were filtered, placed in flasks and made up to a final volume of 15 mL with deionized water. The analysis of the element's

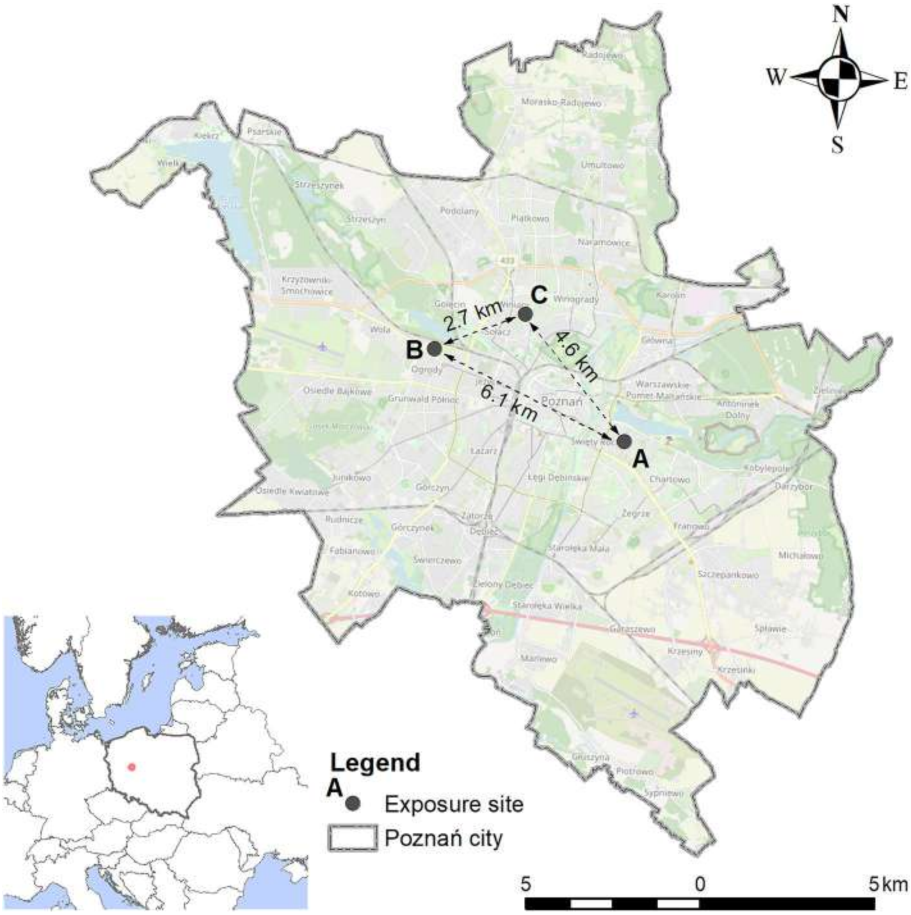

Figure 5. Localization of exposure sites in Poznań city with information about distance between them (source: own study based on data from National Geodetic and Cartographic Resource and © OpenStreetMap and contributors CC-BY-SA).

concentration in the soil was performed in accordance with the PN-EN 16174 standards. Procedural blanks and reference materials were carried out in the same way as the samples in each digestion run.

Analytical procedure. Elemental analysis of Cu, Zn, Cd and Pb was carried out using an inductively coupled plasma mass spectrometer (ICP-MS 7100 × Agilent, Santa Clara, CA, USA) equipped with an octopole reaction system (ORS), MicroMist concentric nebulizer, quartz Scott double pass spray chamber, Ni cones, and a quadrupole mass spectrometer. The instrumental parameters were optimized using the Tuning Solution (Agilent). The typical instrument operating conditions for ICP-MS spectrometers were as follows: 1550 W for RF power, 15 L min^{-1} for plasma gas flow rate, 0.98 L min^{-1} for nebulizer gas flow rate, 0.9 L min^{-1} for auxiliary gas flow rate. For the reduction of spectral interferences, helium mode was used. The non-spectral and matrix interferences were reduced by diluting the samples and using an internal standard solution containing 10 μg L^{-1} Rh introduced in parallel with all analyzed solutions. High purity argon (99.999%) was used as a nebulizer, auxiliary, and plasma gas for the ICP-MS (Messer, Chorzów, Poland). Calibration solutions were prepared by appropriate dilution of 10 mg L^{-1} of multielemental stock solution in 5% HNO_3 (Multi-Element Calibration Standard 3, PerkinElmer, MA, USA). The calibration curves were constructed in the concentration ranges: 0.05–50 μg L^{-1} for Cd and Pb and 0.1–200.0 μg L^{-1} for Cu and Zn.

Quality assurance. To evaluate trueness and establish the traceability of the measurement result, certified reference materials (CRM) were used: NIST SRM 1570a Trace Elements in Spinach Leaves (USA), NIST SRM 2711a Montana Soil. The validation parameters linearity, precision, limits of detection (LOD) and trueness were evaluated. The linearity of the calibration curve was calculated as the correlation coefficient (R), the value of which is greater than 0.9996 for all analytes. The LOD for determined elements were calculated according to LOD = 3.3 S/b, where S means standard deviation of the results obtained for the blank samples and b is the sensitivity (n = 5). The LOD values were as follows: Cd 0.007 μg g^{-1}, Cu 0.036 μg g^{-1}, Pb 0.008 μg g^{-1} and Zn 0.092 μg g^{-1}. Precision values were calculated as the coefficient of variation (CV) (%) ranging from 0.8 to 2.3% for all elements. Trueness was evaluated by applying the certified reference materials and expressed as recovery (%). Recovery values ranged from 97 to 102% for plants and from 93 to 98% for soil, respectively. The results of Student's t-test also confirmed that there were no significant differences between the measured concentration ± SD and the certified concentration ± standard uncertainty.

Accumulation and translocation factor. To estimate the efficiency of heavy metals' phytoextraction by the studied plant species from three research sites, two factors were calculated: bioconcentration and translocation. The ratio of heavy metal accumulation in root samples to heavy metal accumulation in soil samples was used to calculate bioconcentration factor (BCF) of heavy metals[51]:

$$BCF = HM\ concentration\ in\ roots\ \left(\mathrm{mg\ kg^{-1}DW}\right) /\ HM\ concentration\ in\ soil\ \left(\mathrm{mg\ kg^{-1}DW}\right).$$

Translocation factor (TF) is efficiency of the heavy metals' transference to above-ground biomass[52], with leaves and roots used in the analysis:

$$TF = HM\ accumulation\ in\ leaves\ \left(\mathrm{mg\ kg^{-1}DW}\right) /\ HM\ accumulation\ in\ roots\ \left(\mathrm{mg\ kg^{-1}DW}\right).$$

Analysis of physiological conditions of plants. *Determination of chlorophyll content.* To determine chlorophyll content, the experiment was performed in a laboratory, where plants were brought from the three locations of the experiment. In order to avoid chlorophyll degradation, the experiment was carried out in subdued light during analyses and the storage period. Three replicates for each plant sample were made. To determine the content of chlorophyll in plants, first undamaged leaves of the plant were cut and weighed approximately 0.100 g. The weighed samples were cut into smaller pieces and placed in a test tube, then 5 mL of 99.5% DMSO was added, the test tubes were closed with a stopper and they were placed in the refrigerator for 24 h. After 24 h, samples were placed in a water bath at approximately 65 °C for 30–45 min to extract chlorophyll from the leaf blade. Then, the chlorophyll extract was transferred to a 1 cm cuvette and the absorbance was measured on the Hach Lange DR-2800 spectrometer, at three wavelengths: 645 nm, 652 nm, and 663 nm. In parallel time, dry matter determination was carried out for each plant sample. The chlorophyll content in samples was calculated using Arnon's formula[53].

Cell membrane stability (MSI). To determine cell membrane stability from each plant, 2 cm^2 of green leaves (without injury) were cut. Leaves which were cut were rinsed three times with double distilled water and were placed in 25 mL glass beakers, immersed in 10 mL of distilled water then covered with aluminum foil and were put in a refrigerator for 24 h. The same process was repeated after 24 h: the distilled water was removed, leaves were rinsed and were put in the same glass beakers, immersed in 10 mL of double distilled water, covered with aluminum foil and were put in a refrigerator for the next 24 h. After 24 h samples were taken out of the refrigerator and at room temperature their initial conductance was measured. After each measure samples were covered with aluminum foil. Then samples were autoclaved at 0.5 atm, 105 °C for 30 min. After those processes samples were cooled at 25 °C and final conductance was measured. Their respective electric conductivities C1 and C2 were measured by conductivity meters. The membrane stability index was calculated using the equation according to Almeselmani et al.[54] formula.

Relative water content (RWC). Leaf relative water content (RWC) estimation was done by cutting 3–4 pieces of leaf blade (without injuries). The pieces were weighed and placed in glass beakers. Leaves were pureed in 100 mL of distilled water (completely submerged) and covered with aluminum foil, and they were placed in the refrigerator for 12 h. After 12 h, water was removed from the glass beakers and the samples were dried with tissue paper and then were weighed again. Then after weighing samples, they were placed again in the same beakers and dried at 60 °C for 70 h. After 70 h, the samples were cooled in a desiccator and were weighed again. The RWC value was calculated according to the formula by[55].

Dry matter content in leaves. The drying-weight method was used to determine dry matter content in leaves. For each plant three replications were done. About 1 g of the plant's leaves were cut and were placed into a beaker, which were closed with a watch glass and placed in a dryer for 24 h. Plants were dried at 105 °C until plants had a constant weight. The dry matter content was calculated based on the weight of the plants before and after drying, using formula by Ostrowska et al.[56].

Analysis of activity of oxidative stress parameters and level of enzymes of the antioxidative system. *Photosynthesis.* At the beginning, in the middle, and at the end of each exposure series we measured three intensity parameters: net photosynthesis (P_N), intercellular CO_2 concentration (C_i,) stomatal conductance (g_s). For measurement, matured leaves were selected without mechanical injury. Gas exchange analysis was performed between 09:00 and 15:00 with the aid of the portable photosynthesis system C_i 340aa (CID Bioscience Inc., Camas, WA, USA). To ensure similar conditions of measurements in the leaf chamber, stable conditions were provided: CO_2 inflow concentration (410 µmol (CO_2) mol^{-1}), photosynthetic photon flux density (PPFD) 1000 µmol (photon) m^{-2} s^{-1}, a chamber temperature of 25 °C, and relative humidity of 50 ± 3%.

Hydrogen peroxide content. The hydrogen peroxide content was determined using the method described by Patterson et al.[57]. The decrease in absorbance was measured at 508 nm using a UV–VIS spectrophotometer (Shimadzu Scientific Instruments, Japan). The reaction mixture contained 50 mM phosphate buffer (pH 8.4) and reagents, 0.6 mM 4-(-2 pyridylazo) resorcinol, and 0.6 mM potassium-titanium oxalate (1:1). The corresponding concentration of H_2O_2 was determined against the standard curve of H_2O_2.

Determination of antioxidative enzyme activities. The activity of catalase (CAT, EC 1.11.1.6) was determined by directly measuring the decomposition of H_2O_2 at 240 nm for 3 min as described by Aebi[58] in a 50 mM phosphate buffer (pH 7.0) containing 5 mM H_2O_2 and enzyme extract. CAT activity was determined using the extinction coefficient of 36 mM^{-1} cm^{-1} for H_2O_2. The activity of ascorbate peroxidase (APOX, EC 1.11.1.11) was assayed using the method described by Nakano and Asada[59] by monitoring the rate of ascorbate oxidation at 290 nm (extinction coefficient of 2.9 mM^{-1} cm^{-1}) for 3 min. The reaction mixture consisted of 25–50 µL of supernatant, 50 mM phosphate buffer (pH 7.0), 20 µM H_2O_2, 0.2 mM ascorbate, and 0.2 mM EDTA.

Measurement of lipid peroxidation and protein quantification. Malondialdehyde (MDA) content was determined by reaction with thiobarbituric acid (TBA) as described by Heath and Packer[60]. Total soluble protein contents were determined according to the method of Bradford[61] using the Bio-Rad assay kit with bovine serum albumin as a calibration standard.

In situ detection of hydrogen peroxide. For the in vivo determination of hydrogen peroxide we used a modified version of the method described by Afzal et al.[62]. All plant specimens were submerged for 12 h in 4 µM dichlorodihydrofluorescein diacetate (DCFH-DA) in 5 mM dimethyl sulfoxide (DMSO). After rinsing with 50 mM phosphate buffer (pH 7.4), the roots were observed with a confocal microscope (Zeiss LSM 510, Axiovert 200 M, Jena, Germany) equipped with no. 10 filter sets (excitation 450–490 nm, emission 520 nm or more).

Statistical analysis. Descriptive statistical analysis was performed to assess the concentrations of heavy metals in examined plant species from different samples and also concentration of defense system and physiological parameters. Statistical analysis was performed for 72 pots (separately for leaves, roots and soil). All samples followed assumptions of distribution normality and homogeneity. Analysis of variance (two-way ANOVA) was used to assess the significance of differences between species and location for all parameters, and finally, the Scheffé test was applied to show the existence of uniform groups of objects (soils, roots and leaves, separately) ($\alpha \leq 0.05$). Principal component analysis (PCA) was performed to evaluate associations between elemental contents and different cities and determine interactions between independent variables (relations between elemental contents in species, location and physiological parameters), without any a priori assumptions. Cluster analyses with procedure grouping objects and features, were performed using R platform (R Core 2014), to find similarities between sites, species, and heavy metal accumulations. Data were visualized using heat maps to compare the concentration of a particular group of elements in plants and soils at specific research sites, with two-dimensional variables (research sites, element) represented by colors.

Statistical analyses were carried out using statistical software (Statistica 13.1) and R computer platform (R Core, 2014).

Data availability

All data included in this study are available upon request by contact with the corresponding author.

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Author contributions
A.C.-Conceptualization, field research, biochemical analysis, partly draft preparation. M.L.Z.-Conceptualization, biochemical analysis, partly draft preparation, edition. A.H.-Chemical analysis, partly draft preparation. A.M.-Biochemical analysis, confocal microscopy analysis. K.B.-Biochemical analysis, partly draft preparation. M.D.-Conceptualization, statistics evaluation, partly draft preparation and edition.

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Plants as effective bioindicators for heavy metal pollution monitoring

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ABSTRACT

This study investigated the bioindicator potential of *Amaranthus retroflexus* L., *Plantago lanceolata* L., *Rumex acetosa* L., and *Trifolium pratense* L. including the use of *Lolium multiflorum* L. as a reference species, for heavy metal pollution monitoring, in particular Zinc (Zn), Cadmium (Cd), Nickel (Ni), and Lead (Pb). Controlled heavy metal contamination was applied through irrigation with metal nitrate solutions two levels of contamination (low and high). The study also focused on analyzing heavy metals concentration in plant tissues and related physiological responses. Distinct physiological responses to heavy metal stress were observed among the investigated species, highlighting unique variations in their reactions. Hydrogen peroxide, malondialdehyde content, and enzymatic activities emerged as reliable indicators of plant stress induced by heavy metal solutions. *P. lanceolata* displayed elevated Zn concentrations in both roots and leaves (3271 ± 337 and 4956 ± 82 mg kg^{-1}). For Pb, *L. multiflorum* and *P. lanceolata* showed highest root concentrations (2964 ± 937 and 1605 ± 289 mg kg^{-1}), while *R. acetosa* had higher leaf concentration (1957 ± 147 mg kg^{-1}). For Ni, *L. multiflorum* had the highest root concentration (1148 ± 93 mg kg^{-1}), and *P. lanceolata* exhibited the highest leaf concentration (2492 ± 28 mg kg^{-1}). *P. lanceolata* consistently demonstrated the highest Cd concentrations in both roots (126 ± 21 mg kg^{-1}) and leaves (163 ± 12 mg kg^{-1}). These results provide valuable insights for selecting effective bio-indicator species to establish control strategies for heavy metal pollution.

1. Introduction

Heavy metals are a major environmental concern due to their toxicity and detrimental effects on living organisms and ecosystems (Elnabi et al., 2023). These metals are frequently referred to as "silent killers" when their density is greater than 5 g cm^{-3} (Orji et al., 2018). The sources of heavy metals in the environment can be natural, including volcanic activity, weathering of metal containing rocks, erosion, and other geologic processes (Morais et al., 2012), or anthropogenic such as urban runoff, agriculture, and industrial processes (Popoola et al., 2018). In fact, nearly all human activities can generate heavy metals as a byproduct (Gaur and Adholeya, 2004). The main pathways of exposure to heavy metals include ingestion, inhalation, and dermal contact, leading to harmful health effects such as acute and chronic kidney injury, and disorders of the nervous system (Mahurpawar, 2015; Junaid et al., 2017; Rehman et al., 2018; Al Osman et al., 2019). In addition to their toxic effects on human health, heavy metals can also impact the soil and water ecosystems, leading to the decline of plant and animal populations and affecting food chain dynamics (Pujari and Kapoor, 2021). The heavy metals accumulation in the environment can result in long-term impacts on the human health and environment (Martin and Griswold, 2009).

Due to the co-occurrence of pollutants and the frequent exposure to combinations of pollutants rather than single chemicals, determining the effects of pollution on living organisms is difficult. While field measurements and dispersive models can offer insight into the sources of pollution, these methods have limitations in evaluating the biological impact of pollutants on ecosystems and are mostly limited to small geographic areas (Wolterbeek, 2002). To overcome these issues, the use of plants as bioindicators have emerged as efficient complementary tools to traditional methods of investigation. The plants can provide information on the presence of harmful amounts in the soil or air and any

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alterations in the behavior or bioavailability of contaminants in the plant tissues (Pellegrini et al., 2014; Świsłowski et al., 2022). The determination of heavy metal concentration in the environment and the assessment of the population's exposure to pollutants in a specific ecosystem can be achieved through biomonitoring, which is a cost-effective approach (e.g., Hoodaji et al., 2012; Çobanoğlu Özyiğitoğlu, 2020). The terms "bioindicator" and "biomonitor" are used to describe an organism, or a part of it, that serves as an indicator of the presence of pollutants through concentrations, specific symptoms, or morphological changes (Hoodaji et al., 2012; Asif et al., 2018). Effective bioindicators possess several crucial characteristics, as outlined by Hilty and Merenlender (2000), Füreder and Reynolds (2003) and Holt and Miller (2011). These criteria include high sensitivity to environmental stressors, quantifiable responses to contamination levels, and ease of observation. They should be resilient, well-distributed spatially, and well-studied, with established ecology and life history. Affordability of surveying, abundance, and public interest are some characteristics that are relevant from an economic and social perspective. Additionally, biological relevance encompasses early warning capabilities, measurable alterations in response to stress, and serving as sentinel indicators for human populations.

Some plant species can accumulate toxic trace elements from the environment in their above-ground portion, making them useful in monitoring as they provide cost-effective information about environmental quality (Rucandio et al., 2011). Trees, ornamental plants, crops, and non-vascular plants are used as bioindicators (Sawidis et al., 2011). For monitoring heavy metal pollution, lichens and mosses are commonly used as bioindicators (Budzyńska-Lipka et al., 2022; Mao et al., 2022; Vergel et al., 2022; Saib et al., 2023). These organisms have long lifespans, making them valuable long-term integrators of atmospheric deposition (Bargagli, 2016; Świsłowski et al., 2021). However, despite their usefulness, planting them in urban and industrial areas is challenging due to the absence of roots and a well-developed cuticle (Shahid et al., 2017; Ramesh and Gopalsamy, 2020). Therefore, vascular plants such as *Senecio vulgaris* L., *Poa annua* L., *Polygonum aviculare* L. (Salinitro et al., 2019), *Taraxacum officinale* (Lisiak-Zielińska et al., 2021), *Carduus nutans* L (Radulescu et al., 2013), *Plantago major* L. (Mazur et al., 2015) have been used as alternative bioindicators of heavy metal pollution. Furthermore, unlike lichens and mosses, vascular plants possess cuticles and robust root systems, making them well-suited for growth in urban and industrial environments (Baluška and Mancuso, 2021). The quicker growth rate of some herbaceous plants also facilitates a faster assessment of heavy metal exposure (Riaz et al., 2021). Previous studies have showed that heavy metal accumulation in different portion of plants, such as leaves, stems or roots can indicate the level of exposure and potential environmental risk (e.g., Sarma et al., 2012; Alengebawy et al., 2021). Furthermore, vascular plants, particularly evergreen species, have been studied as passive samplers and bioindicators of airborne trace elements in industrial and urban centers (Pellegrini et al., 2014), and remote sites (Dadea et al., 2017). For instance, *Amaranthus retroflexus* L. is a robust bioindicator for Lead (Pb) and Nickel (Ni) contamination (Lukatkin et al., 2021), and *Trifolium pratense* L. has a high bioaccumulation potential for Zinc (Zn), Cadmium (Cd), and Ni (Malizia et al., 2012; Cakaj et al., 2023a). Wang et al. (2003) have identified *Rumex acetosa* L. as efficient for at accumulating Zn, Cd and Pb in polluted environments, and Dimitrova and Yurukova (2005), underscored the bioaccumulation proficiency of *Plantago lanceolata* L. for various heavy metals, including Pb, Cd and Zn.

However, despite the existing literature emphasizing the increased accumulation potential of these selected species (e.g., Gucwa-Przepióra et al., 2016; Lukatkin et al., 2021; Pietrelli et al., 2022), there is a critical need to better understand their comparative bioaccumulation potential in different contamination levels. To overcome this knowledge gap, our study aimed to quantify the bioaccumulation potential of different weed species (*Amaranthus retroflexus* L., *Plantago lanceolata* L., *Rumex acetosa* L., and *Trifolium pratense* L.) as bioindicators of heavy metal pollution. To establish their efficacy, we systematically compared their performance against the well-established reference bioindicator, *Lolium multiflorum* L. var. Ponto (Cakaj et al., 2023a). Notably, a critical gap in the existing literature pertains to the limited exploration of the intricate physiological responses exhibited by the selected weed species when directly confronted with heavy metals. Therefore, our research also aimed to investigate the complex physiological response of selected weed species when exposed to two distinct levels of heavy metal contamination. Thus, by conducting this investigation, we aim to shed light on their suitability as reliable bioindicators across varying contamination scenarios.

2. Materials and methods

2.1. Selection of plant species

In this study, five plant species were selected, i.e. *Amaranthus retroflexus* L., *Lolium multiflorum* L., *Plantago lanceolata* L., *Rumex acetosa* L., and *Trifolium pratense* L. (e.g., Malizia et al., 2012; Lukatkin et al., 2021; Sipos et al., 2023). The plant species were chosen based on broad spatial distribution, low requirements, and their frequent emergence as weeds in urbanized environments (Cakaj et al., 2023a, 2023b). Each species possesses unique characteristics, including its native range, spread, and traditional uses in medicine and agriculture. In 2020, seeds of the given species were gathered from two medium-sized plant breeding companies in Poland and Germany.

2.2. Organization of the experiment and sampling

In March 2022, seeds of each plant species were planted in individual pots (5 L) with a mixture of peat and sand (pH 6.8, K: 350 mg L^{-1}, Mg: 150 mg L^{-1}, N: 230 mg L^{-1}, P: 180 mg L^{-1}) under greenhouse conditions (ambient air temperature 16–18 °C), and deionized water has been used for plant watering (Cakaj et al., 2023a). The plants were subjected to heavy metal contamination through surface irrigation with solutions containing heavy metals ions. This irrigation method aimed to replicate the conditions of heavy metal exposure. The heavy metals incorporated in the study included Cd, Pb, Ni, and Zn, and were introduced as metal nitrates ($Cd(NO_3)_2 \times 4H_2O$, $Pb(NO_3)_2$, $Ni(NO_3)_2 \times 6H_2O$, $Zn(NO_3)_2 \times 6H_2O$) dissolved in distilled water, resulting in a mixed metal ion solution. The threshold value (M1) is defined as the concentration at which the irrigation solution initiates soil concerns, as it introduces heavy metal ions to replicate conditions of heavy metal exposure. Based on the dry matter content and soil moisture weight for each pot, the solutions were formulated to achieve either threshold value (M1) or low guideline value (M2) for each metal (Table 1). The plants underwent two rounds of treatment during the experiment, i.e. initial (at 77 days of the cultivation), and repeated (after 10 days) to gradually achieve doubled M1 and M2 values. To detect soil contamination and areas at ecological and health risks, guideline values were set by the Ministry of the Environment, Finland (2007) for industrial areas, i.e., high guideline values, and for other land uses, i.e. low guideline values (Tóth et al., 2016).

To compensate the nitrogen (N) input, ammonium nitrate (NH_4NO_3) dissolved in distilled water was added to soil in N equivalents accordingly to metal spiking, and resulting N1 and N2 treatments were used as

Table 1
Guideline values for metal concentration in soil (mg kg^{-1} dry weight) in Europe (Tóth et al., 2016).

Metal	Threshold value (M1)	Low guideline value (M2)	High guideline value
Cadmium, Cd	1	10	20
Lead, Pb	60	200	750
Nickel, Ni	50	100	150
Zinc, Zn	200	250	400

a control for each M treatment (130.6 and 212.3 mg N kg^{-1} dry weight, respectively). The experiment was structured into four variants: M1, M2, N1, and N2. Each variant comprised 3 replicates, resulting in a total of 60 pots (each pot contained 3 plants). In the experiment, which involved 5 species with 3 replicates each and 4 variants (M1, M2, N1, and N2), a total of 60 pots were used (5 species × 3 replicates × 4 variants). The physical samples collected from these pots included roots, leaves, and soil. For root samples, there were 15 samples per variant (5 species × 3 replicates), resulting in a total of 60 root samples across all variants. Similarly, there were 60 leaf samples and 60 soil samples, each corresponding to the number of pots. Therefore, the experiment involved a total of 180 physical samples, distributed as follows: 60 root samples, 60 leaf samples, and 60 soil samples.

2.3. Heavy metals analysis

A full description of the analytical procedures, and quality control and assurance, can be found in Supplementary Materials, while a shortened version is presented in the main text.

2.3.1. Samples preparation

After 20 days following the initial treatment, plants were harvested, and the rhizosphere soil samples were collected by gently shaking the root system of each plant. The resulting subsamples were combined into one soil sample per pot. Plant samples were purified using an ultrapure water to remove impurities, surface dried with a paper tissue and divided into roots and shoots. For metal analysis, the plant and soil samples were dried (~40 °C) in an electric oven and digested with a microwave mineralization system. From each plant, small fraction of leaves or roots were placed in Teflon vessels with nitric acid and hydrogen peroxide (H_2O_2). The solutions were filtered using qualitative filter papers, placed in flasks, and diluted with deionized water.

2.3.2. Analytical procedure

Elemental analysis of Cd, Pb, Ni and Zn were carried out using an inductively coupled plasma mass spectrometer (ICP-MS 7100x Agilent, USA) equipped with an Octopole Reaction System (ORS), and the conditions, instrumental settings, and optimization parameters were previously described by Cakaj et al. (2023b) and summarized in Supplementary Materials.

2.3.3. Quality control and assurance

The certified reference materials (NIST SRM 1570a Trace Elements in Spinach Leaves and NIST SRM 2711a Montana Soil) and the analytical blanks were analyzed once per 12 samples to check the accuracy and ensure the traceability of the measurements. Validation parameters (linearity of the calibration curve, accuracy, and limits of detection: 0.007; 0.012; 0.008; 0.092 μg/g for Ni, Pb, Zn, and Cd) were examined using the correlation coefficient ($r > 0.9997$) and coefficient of variation (CV = 0.8–2.3%) for all elements. No significant differences between the measured and certified concentrations were found (Student's t-test).

2.4. Bioconcentration and translocation factors

Bioconcentration factor (BCF) was calculated using the ratio of metals content in roots (mg kg^{-1} dry weight) to its content in the rhizosphere soil (mg kg^{-1} dry weight) and provides information on the plant species' ability to uptake and concentrate heavy metals from the soil (Cohen et al., 1998). A value of BCF greater than 1 indicates accumulation in roots biomass, while a BCF lower than 1 indicates no accumulation. The translocation factor (TF) was calculated as the ratio of metals content in leaves (mg kg^{-1} dry weight) to that in roots (mg kg^{-1} dry weight). This factor indicates the plant capacity to transport heavy metals from roots to leaves, stems or flowers (Yu and Zhou, 2009). A value of TF greater than 1 indicates that metals are translocated within the plant effectively (Dinu et al., 2020).

2.5. Determination of chlorophyll content

The methods given by Hiscox and Israelstam (1979) and Shoaf and Lium (1976) were used to determine the chlorophyll content. The absorbance of the extract was measured at λ = 645, 663 and 652 nm using a Hach Lange DR-2800 spectrometer, and the following formulas were used to calculate chlorophyll (Chl) content:

$$\text{Chl a} = (12.7 * D_{\lambda=663} - 2.7 * D_{\lambda=645}) * V / (1000 * DW)$$

$$\text{Chl b} = (22.9 * D_{\lambda=645} - 4.7 * D_{\lambda=663}) * V / (1000 * DW)$$

$$\text{Chl a+b} = 27.8 * D_{\lambda=652} * V / (1000 * DW)$$

where $D_{\lambda=663}$, $D_{\lambda=645}$, and $D_{\lambda=652}$ represent the absorbance values at λ = 663 nm, 645 nm, and 652 nm, respectively, V the total extract volume (mL), and DW the dry mass of the sample (g).

2.6. Cell membrane stability of leaves

The cell membrane stability index (MSI) was calculated as following: MSI (%) = 1 - (C1/C2) × 100 where C1 and C2 are the conductivity of the sample before and after autoclaving (μS), respectively (Almeselmani et al. (2011).

2.7. Relative water content of leaves

The relative water content (RWC) was calculated as following: RWC (%) = (fresh weight - dry weight)/(turgid weight - dry weight) × 100 as suggested by González and González-Vilar (2001).

2.8. Dry matter content in leaves

The dry matter content (Wd) of leaves was determined using the drying-weight methodology described by Ostrowska-Gumkowska and Ostrowska-Czubenko (1991), and calculated as:

$$W_h = [(m_1 - m_2)/m_1] \times 100$$

$$W_d\ (\%) = 100 - W_h$$

where W_h is the weight related to the hygroscopic water, m_1 and m_2 are the sample weight before and after drying (g), respectively.

2.9. Oxidative stress parameters and level of enzymes of the antioxidative system

2.9.1. Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) was determined according to Patterson et al. (1984) using UV-VIS spectrophotometry. The reaction mixture consisted of 50 mM phosphate buffer (pH = 8.4), 0.6 mM 4-(2 pyridylazo)-resorcinol, and 0.6 mM potassium-titanium oxalate (1:1, v/v). The absorbance at 508 nm was measured using a Hach Lange DR-2800 spectrometer, and the corresponding concentration of H_2O_2 was determined using a standard curve prepared for an array of H_2O_2 solutions.

2.9.2. Determination of antioxidative enzyme activities

The enzymes were extracted with leaves and roots of each sample. Catalase activity (CAT) measured by monitoring the decomposition of H_2O_2 at 240 nm using a Hach Lange DR-2800 spectrometer for 3 min in a 50 mM phosphate buffer (pH 7.0) containing 5 mM H_2O_2 and the enzyme extract. Ascorbate peroxidase activity (APX) was assayed spectrophotometrically by monitoring the rate of ascorbate oxidation at 290 nm for 3 min using a reaction mixture of 25–50 μL extract, 50 mM phosphate buffer (pH 7.0), 20 μM H_2O_2, 0.2 mM ascorbate, and 0.2 mM EDTA according to the method described by Nakano and Asada (1981).

2.9.3. Lipid peroxidation and protein quantification

The malondialdehyde (MDA) content was measured using a colorimetric method based on its reaction with thio-barbituric acid (Heath and Packer, 1968). The total soluble protein content was assessed following the Bradford method (Bradford, 1976), utilizing the Bio-Rad assay kit and bovine serum albumin as a standard for calibration.

2.9.4. In situ detection of hydrogen peroxide

In situ H_2O_2 was detected using a modified method by Afzal et al. (2003). Plant samples were soaked in a 4 μM solution of 2′,7′-Dichlorodihydrofluorescein diacetate in 5 mM dimethyl sulfoxide for 12 h. After rinsing with 50 mM phosphate buffer (pH = 7.4), the roots were examined under a confocal microscope (Zeiss LSM 510, Axiovert 200 M, Jena, Germany) with filter set no. 10 (excitation at λ = 450–490 nm, emission with a 520 nm long-pass filter).

2.10. Statistical analysis

Data were checked for normal distributions with the Kolmogorov-Smirnov D test. For all parameters, a one-way analysis of variance was performed to determine the significance of differences between samples. The principal component analysis was used to analyze and visualize the dependence among variables, i.e., heavy metals concentrations, physiological and stress parameters, and plant species, within different sites. To identify similarities between plant species in heavy metal accumulation, cluster analyses were performed. The heavy metal content in roots and leaves of all species were presented through heatmaps, a two-dimensional data visualization using color to represent numerical values. All the calculations and statistical analyses were performed with Statistica 13.1 and R (R Core Team, 2014).

3. Results

3.1. Heavy metal content in rhizosphere soils and plants

The soil samples from different plant species exhibited variabilities of heavy metal content, with the following trend: Zn > Pb > Ni > Cd at the M1 level and Pb > Zn > Ni > Cd at the M2 level (Table S1). At the M1 level, *T. pratense* showed the lowest Ni, Zn, Pb, and Cd mean concentrations (respectively, ~277; 759; 314; and 6 mg kg^{-1}), and higher mean concentrations were reported for *L. multiflorum* (~501; 1417; 752; 10 mg kg^{-1}, respectively). At the M2 level, *R. acetosa* exhibited the lowest concentrations of Ni, Zn, and Cd (~877; 1464; and 84 mg kg^{-1}, respectively), while *P. lanceolata* had the lowest Pb concentration (~2240 mg kg^{-1}). Higher levels were found in *L. multiflorum* (~1241; 1947; 4106; and 122 mg kg^{-1}, respectively) and *T. pratense* (1096; 1868; 2588; and 103 mg kg^{-1}, respectively) for Ni, Zn, Pb, and Cd.

In terms of root samples, the trend for heavy metal content was: Zn > Ni > Pb > Cd at the M1 level and Zn > Pb > Ni > Cd at the M2 level (Table S1). At the M1 level, *A. retroflexus* displayed the lowest levels of Ni, Zn, Pb, and Cd (~229; 1069; 111; and 6.6 mg kg^{-1}, respectively) and the highest concentrations were noted in *T. pratense* for Ni (~432 mg kg^{-1}) and in *P. lanceolata* (~2208; 289; and 14 mg kg^{-1}, respectively) for Zn, Pb, and Cd. At the M2 level, *A. retroflexus* had the lowest concentrations of Ni, Pb, and Cd (~615; 927; and 59 mg kg^{-1}, respectively), while *P. lanceolata* had the lowest Zn concentration (~158 mg kg^{-1}). At the M2 level, higher levels of Ni, Zn, Pb, and Cd were found in *P. lanceolata* (~1086; 3271; 1605; and 126 mg kg^{-1}, respectively).

In the case of leaves, the trend for heavy metal content was at M1 and M2 levels: Zn > Ni > Pb > Cd at both levels (Table S1). At the M1 level, *T. pratense* exhibited the lowest Ni, Zn, Pb, and Cd mean concentrations (~507; 1727; 120; and 7 mg kg^{-1}, respectively), while *R. acetosa* had the highest levels (~1397; 3756; 704; and 22 mg kg^{-1}, respectively). At the M2 level, *T. pratense* displayed lower mean concentrations (~1277; 2479; 638; and 80 mg kg^{-1}, respectively), while *P. lanceolata* had the highest concentrations of Ni, Zn, and Cd (~2492; 4956; 163 mg kg^{-1}, respectively), and *R. acetosa* had the highest Pb concentration (~1957 mg kg^{-1}).

3.2. Classification of heavy metal content in roots

Based on the cluster analysis (Fig. 1), it can be found that there were differences in root samples between the two levels of soil spiking. For roots at the M1 level, cluster analysis unveiled two distinct groups, one comprising *R. acetosa* (CM1) and *A. retroflexus* (EM1), and the second group includes *L. multiflorum* (AM1), *T. pratense* (BM1) and *P. lanceolata* (FM1). Also, when examining the heavy metal content in the root systems two groups were revealed, with only Zn the first group, and Cd, Ni, and Pb in the second group. Furthermore, a detailed indicated variations in contents of Cd, Ni, Pb, and Zn among all investigated species. *P. lanceolata* (FM1), *L. multiflorum* (AM1) exhibited the highest content of Cd, while for Pb and Ni the highest values was noted for *L. multiflorum* (AM1) and *R. acetosa* (CM1), and for Zn for L. *multiflorum* (AM1), *P. lanceolata* (FM1) and *T. pratense* (BM1).

At the M2 level, cluster analysis unveiled two distinct groups, one group included *L. multiiflorum* (AM2) and *P. lanceolata* (FM2), and the second group includes two subgroups, *T. pratense* (BM2) alone and *R. acetosa* (CM2) and *A. retroflexus* (EM2). Also, when examining the heavy metal contents in roots two groups were revealed, one includings Cd and Ni and the second Pb and Zn. Furthermore, a detailed analysis using color intensity in the heatmap indicated variations in Cd, Ni, Pb, and Zn content among investigated species. Based on the heatmap analysis the highest content of Cd and Zn, was observed in *P. lanceolata* (FM2) and *L. multiflorum* (AM2), Ni in L. *multiflorum* (AM2), *P. lanceolata* (FM2) and *T. pratense* (BM2), and Pb in roots of *L. multiflorum* (AM2) and *P. lanceolata* (FM2).

3.3. Classification of heavy metal content in leaves

Based on the cluster analysis (Fig. 2), it can be found that there were differences between leaf samples at the two applied levels. At the lower level (M1), cluster analysis unveiled two main groups, one group with *T. pratense* (BM1) only and the second group included other species. Also, when examining the heavy metal content in leaves two groups were found, with Zn in the first group and remaining metals in the second group. Furthermore, a detailed analysis using color intensity in the heatmap indicated variations in the content of Cd, Ni, Pb, and Zn among the investigated weed species. Based on the heatmap analysis, the highest concentration of Cd, Pb, Ni, and Zn was observed in the *R. acetosa* (CM1) and (FM1). While at the M2 addition level, cluster analysis unveiled two distinct groups, one group included *T. pratense* (BM2) and *A. retroflexus* (EM2), and the second group with two subgroups: the first subgroup included *R. acetosa* (CM2) and the second subgroup *L. multiflorum* (AM2) and *P. lanceolata* (FM2). Furthermore, the highest Cd content leaves at the M2 treatment was observed for *P. lanceolata* (FM2) and *R. acetosa* (CM2), Pb for *R. acetosa* (CM2), and Ni and Zn for L. *multiflorum* (AM2) and *P. lanceolata* (FM2).

3.4. Bioconcentration and translocation factor

The highest values of the BCF among investigated metals (Table 2) were noted for Zn and exceeded 2 in the case of *T. pratense* (at M1) and *P. lanceolata* (at both treatments), and for the two species the uptake of Zn was increased compared to the reference species *L. multiflorum*. In a contrary, *R. acetosa* and *A. retroflexus* accumulated Zn to significantly lesser extend with the BCF values below 1 (excluding *R. acetosa* at M1). Furthermore, Ni and Cd demonstrated comparable uptake rates, characterized by BCF greater than 1, particularly observed in the case of *T. pratense* and *L. multiflorum* at M1, and in both treatments for *P. lanceolata* (Table 2). Uptake of Cd by roots of *T. pratense* and *P. lanceolata* was more intense compared to the reference *L. multiflorum* at both levels applied. In the case of Pb, the BCF values did not exceed 1.

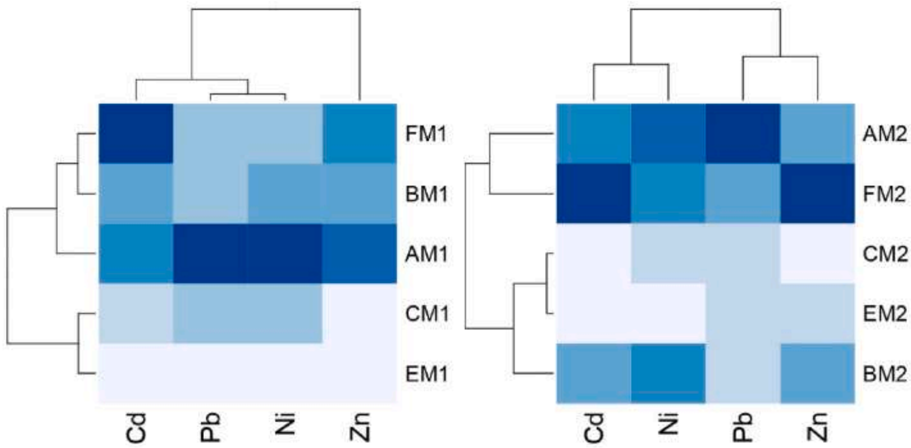

Fig. 1. Heatmap and cluster analysis related to heavy metal content in roots of all species in two levels M1(AM1- *L. multiflorum*, BM1-*T. pratense*, CM1-*R. acetosa*, EM1-*A. retroflexus*, and FM1-*P. lanceolata*) and M2 (AM2- *L. multiflorum*, BM2-*T. pratense*, CM2-*R. acetosa*, EM2-*A. retroflexus*, and FM2-*P. lanceolata*), where darker color indicated higher heavy metal concentration.

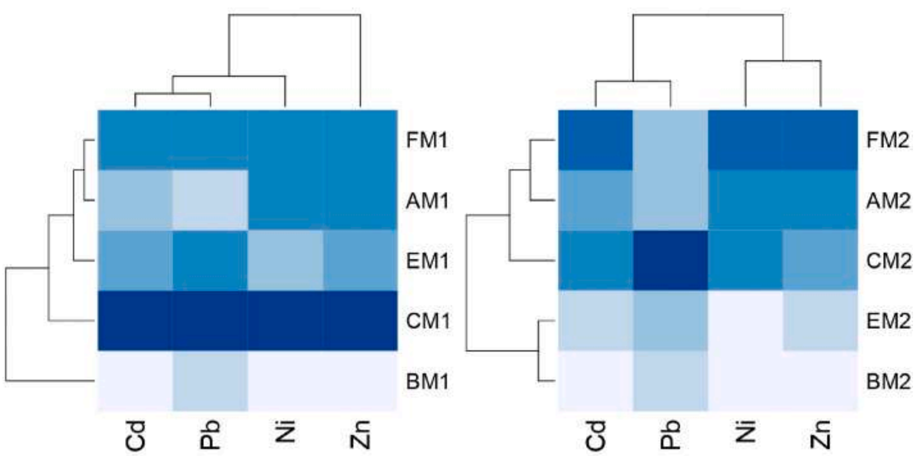

Fig. 2. Heatmap and cluster analysis related to heavy metal content in leaves of all species in two levels M1(AM1- *L. multiflorum*, BM1-*T. pratense*, CM1-*R. acetosa*, EM1-*A. retroflexus*, and FM1-*P. lanceolata*) and M2 (AM2- *L. multiflorum*, BM2-*T. pratense*, CM2-*R. acetosa*, EM2-*A. retroflexus*, and FM2-*P. lanceolata*), where darker color indicated higher heavy metal concentration.

Table 2
The bioconcentration factor (BCF) and translocation factor (TF) of Ni, Zn, Cd and Pb in plant species from two levels M1 (AM1-*L. multiflorum*, BM1-*T. pratense*, CM1-*R. acetosa*, EM1-*A. retroflexus*, FM1-*P. lanceolata*; M2) and M2 (AM2-*L. multiflorum*, BM2-*T. pratense*, CM2-*R. acetosa*, EM2-*A. retroflexus*, FM2-*P. lanceolata*). The BCF and TF ≥ 1 are given in boldface.

Species and HMs level		BIOCONCENTRATION FACTOR				TRANSLOCATION FACTOR			
		Ni	Zn	Cd	Pb	Ni	Zn	Cd	Pb
Lolium multiflorum	**AM1**	**1.25**	**1.74**	**1.10**	0.77	**1.62**	**1.27**	**1.16**	0.30
	AM2	0.93	**1.21**	0.83	0.72	**1.98**	**1.79**	**1.42**	0.27
Trifolium pratense	**BM1**	**1.56**	**2.53**	**1.72**	0.79	**1.17**	0.90	0.70	0.48
	BM2	0.96	**1.27**	0.92	0.39	**1.22**	**1.04**	0.84	0.63
Rumex acetosa	**CM1**	0.91	**1.14**	0.85	0.35	**3.75**	**2.92**	**2.75**	**2.98**
	CM2	0.84	0.96	0.71	0.37	**3.15**	**2.82**	**2.66**	**2.06**
Amaranthus retroflexus	**EM1**	0.47	0.82	0.65	0.15	**3.59**	**2.58**	**2.34**	**4.17**
	EM2	0.59	0.86	0.61	0.31	**2.21**	**1.94**	**1.83**	0.86
Plantago lanceolata	**FM1**	**1.05**	**2.16**	**1.73**	0.54	**2.75**	**1.47**	**1.33**	**1.41**
	FM2	**1.18**	**2.12**	**1.46**	0.72	**2.30**	**1.52**	**1.29**	0.60

Considering metal translocation from roots to shoots, the highest rate was noted in the case of Ni for *R. acetosa* > *A. retroflexus* > *P. lanceolata* with the TF values exceeding the reference (*L. multiflorum*). Similarly, other metals were intensively translocated in *R. acetosa* and *A. retroflexus* with the highest TF value for Pb in the case of *A. retroflexus* at M1 (>4). For *T. pratense*, the lowest translocation ratio was noted with the TF values exceeding 1 in the case of Ni (both treatments) and Zn (at M1).

3.5. *Physiological condition of species*

Variations in physiological responses were observed among the investigated plant species at both M1 and M2 levels (Table 3). At the M1 level, *P. lanceolata* exhibited the lowest dry mass (~9%), whereas *T. pratense* demonstrated the highest dry mass (19%). At the M2 level, *R. acetosa* displayed the lowest dry mass (~8%), while *T. pratense* exhibited the highest dry mass (20%). Regarding the MSI, *T. pratense* demonstrated the lowest MSI value (94%) at the M1 level, whereas *R. acetosa* displayed the highest CSM (97%). At the M2 level, the lowest MSI was observed in *A. retroflexus* (92%), while the highest was detected in *L. multiflorum* (97%).

Analyzing the RWC, *P. lanceolata* exhibited the lowest value (81%) at the M1 level, whereas *A. retroflexus* showed the highest value (95%). In contrast, at the M2 level, *R. acetosa* displayed the lowest RWC (84%), and *T. pratense* exhibited the highest RWC (98%) (Table 3). Examining chlorophyll-a content at the M1 level, the values ranged from 9.0 mg g^{-1} in *T. pratense* to 11.6 mg g^{-1} in *L. multiflorum*. At the M2 level, the range was from 8.7 mg g^{-1} in *T. pratense* to 11.3 mg g^{-1} in *A. retroflexus*. Furthermore, the chlorophyll-b content varied between 3.0 mg g^{-1} in *R. acetosa* (M1 level) and 4.4 mg g^{-1} in *T. pratense* (M1 level), and about 3.8 mg g^{-1} in *T. pratense*, *R. acetosa,* and *A. retroflexus* (M2 level). The combined chlorophyll content (chla + b) ranged from 11.9 mg g^{-1} in *R. acetosa* (M1 level) to 16.5 mg g^{-1} in *L. multiflorum* (M1 level), and from 12.4 mg g^{-1} in *L. multiflorum* (M2 level) to 15.1 mg g^{-1} in *A. retroflexus* (M2 level).

3.5.1. Production of ROS and enzyme antioxidant activity

The levels of catalase activity (CAT) activity and profiles of changes observed after M1 and M2 treatment were generally higher in comparison to control samples across all species examined (Figs. S1–S4; Table S2). Notably, *L. multiflorum* indicated higher CAT values in roots, while *R. acetosa* displayed the highest CAT activities in leaves (CAT ~ 0.280 μmol H_2O_2 min^{-1} mg $protein^{-1}$). Moreover, ascorbate peroxidase activities (APOX) were found to be higher in roots of both *L. multiflorum* and *R. acetosa* samples, with the highest APOX activities recorded as APOX ~0.022 μmol H_2O_2 min^{-1} mg $protein^{-1}$. When considering the profiles of changes and the level of malondialdehyde activity, leaves of *L. multiflorum*, *T. pratense* (MDA ~ 15 μmol H_2O_2 min^{-1} mg $protein^{-1}$), *A. retroflexus*, and *P. lanceolata* exhibited higher MDA activities. The levels of H_2O_2 in both roots and leaves showed similar patterns across all species, with the highest amount recorded in *L. multiflorum* leaves (~20 μmol H_2O_2) and in *T. pratense* and *A. retroflexus* (~10 μmol H_2O_2).

The first two principal components, PCA1 and PCA2, jointly account for 69% of the total variation. The scatter plot of the PCA reveals distinct groups among the samples from the two treatments, M1 and M2. The first group consists of *T. pratense, A. retroflexus,* and *P. lanceolata*, which are predominantly associated with MDA activity in leaves. The second group comprises *L. multiflorum* samples, which are related to H_2O_2 levels in both leaves and roots, as well as CAT activity predominantly in roots, with a lesser effect in leaves. The third group includes *R. acetosa*, which is related to APOX activity in both leaves and roots, as well as MDA activity in leaves (Fig. 3). Additionally, at the M1 level, the highest intensity of fluorescence from dihydroethidium, indicating the presence of H_2O_2 in roots, was observed in *L. multiflorum*, *T. pratense*, and *R. acetosa*. Conversely, at the M2 level, the most intense fluorescence from DHE was observed in *R. acetosa* (Fig. 4).

4. Discussion

4.1. Bioindicator for heavy metals

Efficient monitoring of heavy metals pollution in the environment is a major concern in order to establish suitable control strategies for pollution mitigation. To overcome this issue, bio-indicators such as plants can play a crucial role in providing fruitful information about the extent and severity of contamination by heavy metals (Cakaj et al., 2023a). By using bio-indicators, we can gain a better understanding of the overall health of the environment and the potential impacts of heavy metals on ecosystems (Singh and Singh, 2020). In this study, we investigated the bioaccumulation efficiency of five plant species recognized for their capability to accumulate heavy metals (Klumpp et al., 2009), i. e., *Amaranthus retroflexus* L., *Lolium multiflorum* var. Ponto (reference), *Plantago lanceolata* L., *Rumex acetosa* L., and *Trifolium pratense* L. The present study showed variations in heavy metal (Ni, Zn, Cd, and Pb) concentrations among different plant species. *T. pratense* exhibited the lowest levels of metals, while the highest levels were observed in *A. retroflexus*, an efficient plant species for the phytoremediation of Ni- and Pb-contaminated soils (Lukatkin et al., 2021). Conversely, *L. multiflorum* and *T. pratense* exhibited the highest concentrations of the heavy metals at the M2 level.

Table 3
Mean value of dry mass (%), cell membrane stability (MSI %), dry mass (%), relative water content (RWC%), chlorophyll [Chl *a*, Chl *b*, Chl *a*+*b* (mg g^{-1}); n = 3], where: M1 represents the lower treatment (AM1-*L. multiflorum*, BM1-*T. pratense*, CM1-*R. acetosa*, EM1-*A. retroflexus*, FM1-*P. lanceolata*), N1 representing the treatment control for M1 (AN1-*L. multiflorum*, BN1-*T. pratense*, CN1-*R. acetosa*, EN1-*A. retroflexus*, FN1–*P. lanceolata*), and M2 represents the higher treatment (AM2-*L. multiflorum*, BM2-*T. pratense*, CM2-*R. acetosa*, EM2-*A. retroflexus*, FM2-*P. lanceolata*), N2 representing the treatment control for M2 (AN2-*L. multiflorum*, BN2-*T. pratense*, CN2-*R. acetosa*, EN2-*A. retroflexus*, FN2–*P. lanceolata*).

Parameter Species and HMs level		Dry mass (%)	MSI (%)	RWC (%)	Chl a (mg g^{-1})	Chl b (mg g^{-1})	Chl a+b (mg g^{-1})
Lolium multiflorum	**AM1**	**14 ± 1**	**96 ± 1**	**93 ± 2**	**11.57 ± 0.57**	**4.07 ± 0.20**	**16.54 ± 0.77**
	AN1	16 ± 1	97 ± 0.3	96 ± 1	10.12 ± 0.50	3.74 ± 0.18	14.15 ± 0.68
	AM2	**15 ± 2**	**97 ± 1**	**96 ± 5**	**9.27 ± 0.46**	**3.14 ± 0.15**	**12.43 ± 0.61**
	AN2	15 ± 1	97 ± 1	98 ± 1	11.58 ± 0.57	4.48 ± 0.22	16.79 ± 0.79
Trifolium pratense	**BM1**	**19 ± 2**	**94 ± 1**	**89 ± 2**	**9.02 ± 0.45**	**4.36 ± 0.21**	**14.24 ± 0.67**
	BN1	20 ± 1	97 ± 1	91 ± 3	9.88 ± 0.49	5.51 ± 0.27	17.00 ± 0.76
	BM2	**20 ± 3**	**96 ± 1**	**98 ± 1**	**8.71 ± 0.43**	**3.77 ± 0.18**	**13.06 ± 0.61**
	BN2	19 ± 1	97 ± 2	91 ± 2	8.98 ± 0.44	6.11 ± 0.30	17.18 ± 0.74
Rumex acetosa	**CM1**	**9 ± 1**	**97 ± 1**	**82 ± 3**	**9.17 ± 0.45**	**3.02 ± 0.15**	**11.88 ± 0.60**
	CN1	8 ± 1	98 ± 1	91 ± 2	14.06 ± 0.70	5.17 ± 0.25	19.01 ± 0.95
	CM2	**8 ± 1**	**96 ± 1**	**84 ± 4**	**11.18 ± 0.55**	**3.78 ± 0.18**	**14.65 ± 0.73**
	CN2	9 ± 1	96 ± 1	90 ± 1	10.38 ± 0.51	3.60 ± 0.18	13.72 ± 0.69
Amaranthus retroflexus	**EM1**	**14 ± 1**	**95 ± 1**	**95 ± 1**	**10.28 ± 0.51**	**3.22 ± 0.16**	**13.32 ± 0.67**
	EN1	16 ± 1	98 ± 1	99 ± 1	13.26 ± 0.66	4.37 ± 0.21	17.51 ± 0.87
	EM2	**14 ± 1**	**92 ± 2**	**90 ± 3**	**11.27 ± 0.56**	**3.79 ± 0.19**	**15.14 ± 0.75**
	EN2	15 ± 1	98 ± 1	99 ± 1	11.27 ± 0.56	3.88 ± 0.19	15.22 ± 0.75
Plantago lanceolata	**FM1**	**9 ± 1**	**97 ± 1**	**81 ± 6**	**10.58 ± 0.52**	**3.43 ± 0.17**	**13.64 ± 0.69**
	FN1	10 ± 1	98 ± 1	93 ± 4	13.46 ± 0.67	4.78 ± 0.24	18.03 ± 0.91
	FM2	**12 ± 1**	**96 ± 1**	**86 ± 4**	**10.51 ± 0.52**	**3.34 ± 0.16**	**13.51 ± 0.68**
	FN2	10 ± 1	96 ± 2	92 ± 2	13.08 ± 0.65	4.87 ± 0.24	18.27 ± 0.89

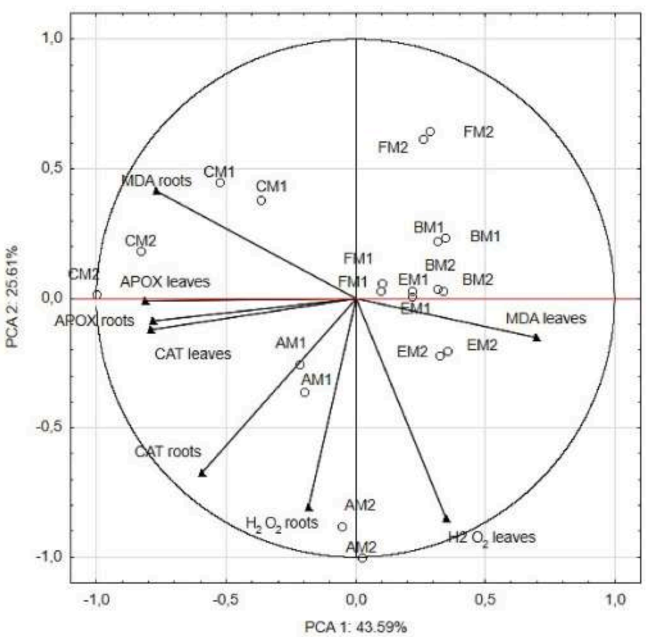

Fig. 3. Principal Component Analysis for plant species (M1: AM1-*L. multiflorum*, BM1-*T. pratense*, CM1-*R. acetosa*, EM1-*A. retroflexus*, FM1-*P. lanceolata*; M2: AM2-*L. multiflorum*, BM2-*T. pratense*, CM2-*R. acetosa*, EM2-*A. retroflexus*, FM2-*P. lanceolata*) in relation to Hydrogen peroxide level (H_2O_2), Ascorbate peroxidase (APOX), Catalase (CAT), and Malondialdehyde (MDA) activities.

In the roots of plant species, A. *retroflexus* exhibited the lowest concentrations of Ni, Cd, and Pb in both the M1 and M2 levels. This suggests that *A. retroflexus* has a relatively lower capacity for metals uptake and accumulation in its roots compared to the other species. On the other hand, at the M1 level, *T. pratense* showed higher concentrations of Ni, Zn, Cd, and Pb compared to *A. retroflexus*. This indicates that *T. pratense* has a higher capacity for metals uptake and accumulation in its roots (Malizia et al., 2012). Similarly, *P. lanceolata* exhibited higher concentrations of Zn, Cd, and Pb at the M1 level, indicating its ability to accumulate these metals in its roots. While, at the M2 level, *L. multiflorum* and *P. lanceolata* displayed the highest concentrations of Ni, Cd, and Pb in their roots. Furthermore, the study conducted by Gucwa-Przepióra et al. (2016) and Pietrelli et al. (2022) revealed that *P. lanceolata* exhibited a high capacity for the uptake of heavy metals.

Because the soil used for plant cultivation was deliberately irrigated with a heavy metal solution, it resulted in increased metal concentrations, thereby simulating a contaminated environment. Under such conditions, certain plant species may show enhanced efficiency in the uptake and accumulation of metals in their roots. Metallic pollutants into soil have detrimental consequences for plant life (Jia et al., 2018). Through the uptake of metal ions via their roots from soil solutions, plants can either transfer these ions to their shoots or store them within their roots, a process influenced by the specific traits of each plant species (Stankovic et al., 2014). The capacity of heavy metal uptake by plants depends on the cultivar, plant species, developmental stage, soil type, pH levels, temperature, chemical and bioavailability characteristics of element, its redox potential, cation exchange capacity, dissolved oxygen levels, and the quantity and quality of root exudates (Filipović-Trajkovic et al., 2012; Stankovic et al., 2014). The BCF and TF values provide important insights into the extent and efficiency of metals uptake by plants, and their potential for use in environmental monitoring (Eid and Shaltout, 2016). Their values were previously calculated to assess the uptake and translocation of heavy metals in different plant species and to identify potential bio-indicators of heavy metals pollution (Ladislas et al., 2012; Cakaj et al., 2023a, 2023b). In the case of hyper-accumulator plants, the BCF and TF should exceed a value of 1 (Yanqun et al., 2005).

The BCF values for Cd surpassed 1 in the case of *P. lanceolata, T. pratense*, and *L. multiflorum*. Specifically, *P. lanceolata* and *T. pratense* displayed higher BCF values for Cd compared to the reference plant *L. multiflorum*. These findings underscore their potential as efficient accumulator of Cd. Conversely, only *P. lanceolata* exceeded a BCF value of 1 for Cd at the M2 level. The species has demonstrated promising characteristics as a bio-indicator for heavy metals as observed by Dimitrova and Yurukova (2005). Their study involved an analysis of data collected from urban and non-ferrous industrial sites, revealing that leaves of *P. lanceolata* can effectively serve as bio-indicators for Zn, Pb, and Cd (Dimitrova and Yurukova, 2005). In our study, four species showed a TF value greater than 1, indicating their ability to transport Cd from the roots to the above-ground tissues. The exception was *T. pratense* at both the Cd and Pb levels applied, suggesting a limited translocation of Cd and Pb from the roots to the shoots. In relation to TF and *T. pratense*, Bidar et al. (2009) have found a limit in the translocation of Cd, Pb, and Zn from the roots to the shoots. This may indicate that the species has mechanisms aimed to restrict the uptake of Cd and Pb to the above-ground tissues, potentially reducing the risk of Cd and Pb accumulation and its negative impacts on plant physiology (Bidar et al., 2009).

For Ni, the BCF values at lower dose (M1) were greater than 1 for *T. pratense*, *P. lanceolata*, and *L. multiflorum*, indicating their ability to accumulate Ni from the surrounding environment. At higher dose (M2), only *P. lanceolata* showed a BCF over 1 proving its higher capacity for Ni accumulation in comparison to the other species under elevated heavy metals content in the soil. In terms of Ni translocation, all species displayed a TF value greater than 1 at both the M1 and M2 levels. This indicates the efficient translocation of Ni from the roots to the above-ground tissues regardless of pollution level. The use of *P. lanceolata* as a suitable bio-indicator has been previously recognized by Dimitrova and Yurukova (2005), making it a valuable bio-indicator for assessing the presence and accumulation of Ni.

Zinc is a key micronutrient for all living organisms with a crucial role in biological processes (growth, development, defense); however, its excessive amounts can be harmful to plants (Ghori et al., 2019; Obasi and Akudinobi, 2020). At low Zn dose, *T. pratense*, *P. lanceolata*, *L. multiflorum*, and *R. acetosa* exhibited a BCF >1. This indicates their capacity to efficiently accumulate Zn from the surrounding environment and can be used as effective bio-indicators for Zn pollution, even at lower pollution levels. When subjected to higher dose, the BCF >1 confirmed the suitability of *P. lanceolata*, *T. pratense* and *L. multiflorum* as promising bio-indicators, specifically in situations characterized by elevated contamination levels. Kurteva (2009) has demonstrated that *P. lanceolata* possesses the capacity to accumulate higher quantities of Zn. Similarly, Malizia et al. (2012) provide robust evidence supporting that *T. pratense* is a reliable bio-indicator for Zn contamination assessment. Additionally, *R. acetosa* showed the highest efficiency in transporting Zn at both M1 and M2 doses as previously reported by Bandiera et al. (2016) and Cakaj et al. (2023b).

Lead is a toxic heavy metal that poses significant risks to the environment and human health (Ali et al., 2019). None of the species showed a BCF value over 1, indicating limited Pb accumulation in their tissues. *R. acetosa* displayed a TF value greater than 1 at both the M1 and M2 levels, indicating its ability to efficiently translocate Pb from roots to the above-ground tissues. *R. acetosa*, exhibited a notable preference for several metallic elements (Bandiera et al., 2016). *P. lanceolata*, on the other hand, exhibited a TF $>$ 1 only at the lower level, proving its higher translocation ability at lower Pb content in the soil. Other studies also demonstrated intense Pb translocation in *P. lanceolata* (e.g., Cakaj et al., 2023b).

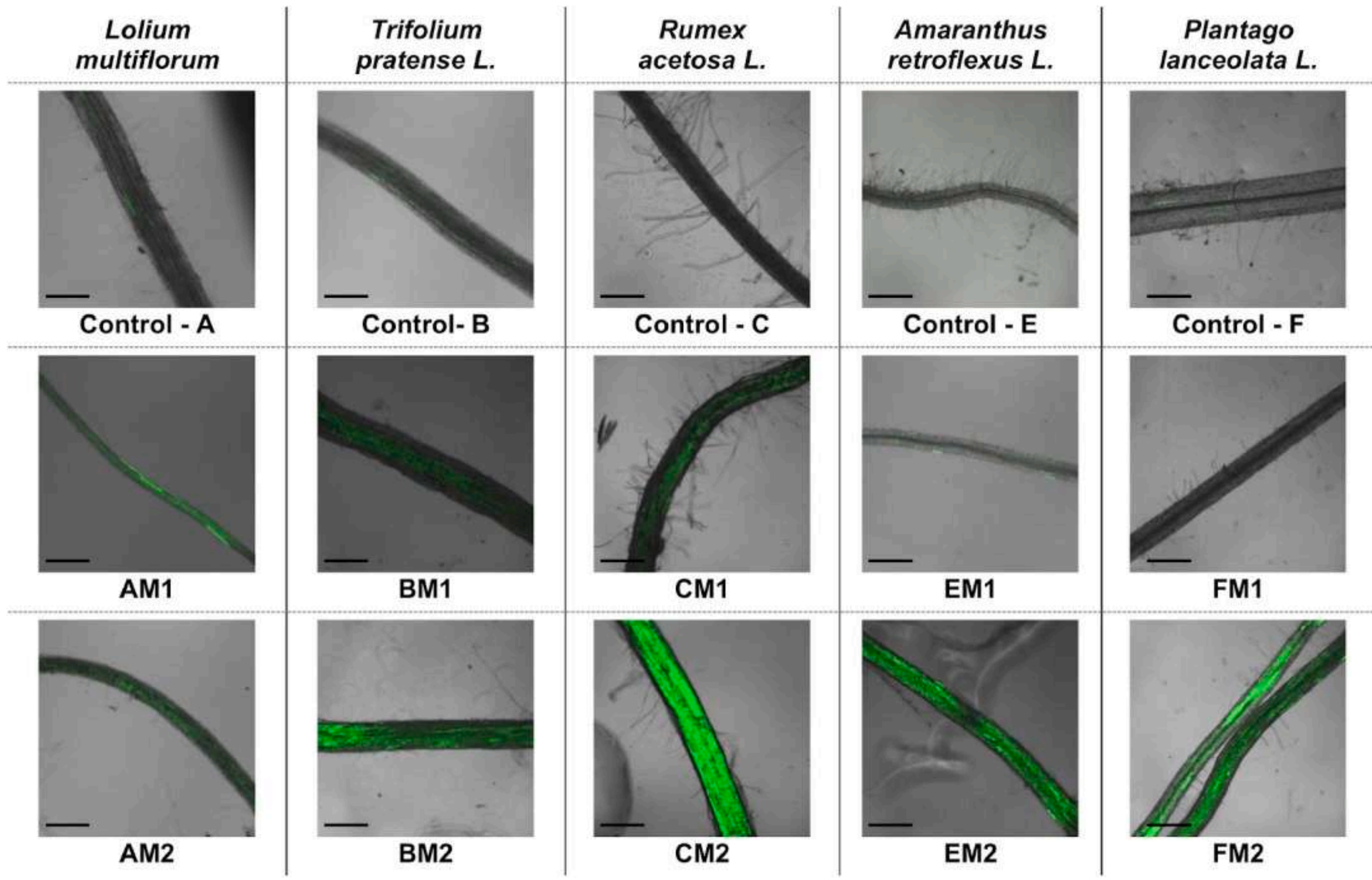

Fig. 4. Fluorescent images show H_2O_2 production in roots of examined species after treatment. At the top – the control and at the middle and bottom the treated species (M1: AM1-*L. multiflorum*, BM1-*T. pratense*, CM1-*R. acetosa*, EM1-*A. retroflexus*, FM1-*P. lanceolata*; M2: AM2-*L. multiflorum*, BM2-*T. pratense*, CM2-*R. acetosa*, EM2-*A. retroflexus*, FM2-*P. lanceolata*). The bar indicates 1 μm.

4.2. Physiological response

The occurrence of heavy metals in environment can have adverse effects on plants ecosystems, directly impacting their physiology, metabolism, growth, productivity, and senescence (e.g., Ghori et al., 2019; Hafeez et al., 2023). However, most plants can gradually develop an avoidance mechanism and tolerance mechanism in the heavy metal stress environment (Islam and Sandhi, 2022). The term "avoidance" refers to a plant's capacity to limit the uptake of metals, preventing significant internal metal concentrations. On the other hand, "tolerance" refers to the ability of plants to survive and thrive even in the presence of high concentrations of metals within their tissues (Hossain et al., 2012). In our study, the presence of heavy metal ions elicited diverse and discernible effects on the physiological activity of plant species under investigation, resulting in characteristic symptoms of metal toxicity.

The dry mass in investigated species differed at the control, and at the M1 and M2 level. At the M1 level, *P. lanceolata* had the lowest dry mass (9%) and may indicate susceptibility to heavy metal-induced growth inhibition. In contrast, *T. pratense* had the highest dry mass (19%) indicating a better tolerance to stress. *T. pratense* exhibited the lowest cell membrane stability (MSI) at the M1 level (94%), indicating increased vulnerability to membrane damage. *R. acetosa* had the highest MSI (97%), indicating better membrane integrity in the presence of heavy metals. At the M2 level, *A. retroflexus* had the lowest MSI (92%), while *L. multiflorum* had the highest value (97%), which is an expected result due to the known tolerance of this plant species to heavy metals (Cui et al., 2021). The results of the Relative Water Content (RWC) analysis for both M1 and M2 levels compared to control revealed a decrease in RWC among the selected plant species, indicating the adverse impact of heavy metals on their water retention capacity (Rucińska-Sobkowiak, 2016). The adverse impact of heavy metals on water relations in plants and on physiological processes has been widely described (e.g., Borowiak and Fidler, 2014; Singh et al., 2023).

Compared with the control species, the chlorophyll-a and chlorophyll-b levels decreased under heavy metal stress, implying impaired photosynthetic efficiency and reduced plant productivity (Zengin and Munzuroglu, 2005; Singh et al., 2022). The presence of heavy metals can impact the chlorophyll content in plants (Soran et al., 2023; Moustakas et al., 2022). Previous studies have reported that the presence of higher concentrations of heavy metals, textile dyes and salts is linked to the reduction of chlorophyll contents (Tripathi et al., 2017; Aly and Mohamed, 2012). *L. multiflorum* at M1 level and *R. acetosa* at M2 level had the highest combined chlorophyll content (chlorophyll a+b), indicating resilience to heavy metal-induced chlorophyll degradation. Many studies have observed reductions in the overall chlorophyll content of various plant species when exposed to heavy metals such as Cd, Pb, Ni, Cu and Hg (Panda et al., 2003). Hence, chlorophyll pigments seem to be an unequivocal sign of heavy-metal injury in plants (Shakya et al., 2008). These results provide valuable insights into the diverse responses of plant species to heavy metal pollution. The variations in dry mass, MSI, RWC, and chlorophyll content among the studied species underscore their differential tolerance and adaptation mechanisms. In addition, the presence of heavy metals in plant tissues leads to formation of reactive oxygen species, like H_2O_2 (Sytar et al., 2013; Małecka et al., 2021). The antioxidant response and the stress tolerance is species-specific (Gozdur et al., 2023; Moustakas, 2023). Among the tested species, the most appropriate responses to the levels of H_2O_2 and MDA activity in relation to heavy metal content in plant organs were observed in *A. retroflexus*, *T. pratense*, and *R. acetosa*. The reaction of the last species was also reported by Lukatkin et al. (2021). The observed higher CAT activity levels in plants treated with M1 and M2 indicate an upregulation of this enzyme in response to increased H_2O_2 levels caused by stress like heavy metal exposure (Gechev and Hille, 2005). CAT plays a crucial role in the breakdown of H_2O_2, thus protecting plants from oxidative damage induced by heavy metals (Aydin et al., 2022). The findings align with previous studies demonstrating the induction of CAT as a defense mechanism against heavy metal-induced oxidative stress (Štolfa et al., 2015; Aydin et al., 2022). The variation in CAT between species, with *L. multiflorum* showing higher activity in roots and *R. acetosa* displaying the highest activity in leaves, suggests species-specific adaptation strategies to heavy metal stress. On the other hand, CAT in *R. acetosa* leaves may indicate a distinct adaptation strategy to mitigate heavy metal-induced oxidative stress in its aerial tissues. The higher APOX activities observed in the roots of *L. multiflorum* and *R. acetosa* following M1 and M2 treatments provide further evidence of their antioxidant defense mechanisms against heavy metal-induced oxidative stress. APOX acts as an essential enzyme involved in scavenging H_2O_2, and its increased activity in roots suggests a protective response to heavy metal pollution. The elevated levels of malondialdehyde (MDA) in the leaves of *L. multiflorum*, *T. pratense*, *A. retroflexus*, and *P. lanceolata* after M1 and M2 treatments signify higher oxidative damage resulting from heavy metals exposure. Lipid peroxidation is a common consequence of oxidative stress caused by heavy metals, leading to MDA accumulation, and indicating the extent of cellular damage (Aydin et al., 2022). The similar patterns of H_2O_2 levels observed in both roots and leaves across all species suggest that heavy metals exposure induces reactive oxygen species accumulation throughout the plant (Aydin et al., 2022). Variations in H_2O_2 levels between species may be attributed to their individual tolerance mechanisms and the degree of oxidative stress imposed by heavy metals treatments.

5. Conclusion

This study provided insights into heavy metal concentrations and bioaccumulation efficiency across diverse plant species, elucidating their physiological responses to metal pollution. The results revealed considerable variability in metal concentrations, underscoring the distinct capacities of plants to accumulate and tolerate pollutants. Based on the obtained results *Trifolium pratense* emerged as a versatile indicator, demonstrating consistent efficacy for Zn, Ni, and Cd in both root and leaf samples. *Plantago lanceolata* exhibited effectiveness for Zn and Cd, and *Amaranthus retroflexus* showed promise as a bioindicator for low-level Pb contamination. Regarding Pb, none of the plant species examined exhibited a bioconcentration factor exceeding 1. However, *Trifolium pratense* had a similar value at the lower contamination level as *Lolium multiflorum* and *Plantago lanceolata* displayed a similar pattern at the higher contamination level, just as for *Lolium multiflorum*. In terms of physiological responses, *Trifolium pratense* demonstrated notable adaptability, exhibiting higher dry mass, membrane stability, and chlorophyll content. In contrast, *Plantago lanceolata* consistently displayed lower dry mass and relative water content, while *Rumex acetosa* exhibited lower membrane stability. Therefore, based on their physiological characteristics and bioconcentration values, *Trifolium pratense* and *Plantago lanceolata* stand out as the most suitable bioindicators for heavy metal pollution, displaying consistent efficacy across multiple contaminants. *Trifolium pratense* demonstrated remarkable adaptability and efficient uptake of Zn, Ni, and Cd, while *Plantago lanceolata* excelled in accumulating high concentrations of Zn and Cd. These recommended species can serve as effective tools for environmental monitoring and assessment, contributing to a broader understanding of heavy metal contamination in diverse ecosystems.

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CRediT authorship contribution statement

Arlinda Cakaj: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kinga Drzewiecka:** Writing – review & editing, Project administration, Methodology, Investigation, Data curation. **Anetta Hanć:** Writing – original draft, Methodology, Conceptualization. **Marta Lisiak-Zielińska:** Writing – review & editing, Visualization, Supervision, Methodology, Data curation. **Liliana Ciszewska:** Visualization, Software, Conceptualization. **Maria Drapikowska:** Writing – review & editing, Visualization, Supervision, Software, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.119222.

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Trifolium pratense and the Heavy Metal Content in Various Urban Areas

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Abstract: Effective biomonitoring strategies are essential for identifying and assessing the sources and levels of contamination of heavy metal pollutants in urban areas, given their negative impacts on human health and the environment. This study aimed to assess the potential of common weed, *Trifolium pratense* as a bioindicator of heavy metal contamination in various land uses in urban areas, with a focus on Cd, Cu, Cr, Ni, and Pb. The results have shown that Cr and Ni had high bioconcentration factor (BCF) values in most sites, in comparison with Cu, Cd and Pb. Contamination factor (CF) values varied across all sites. The industrial area and old town sites had the highest translocation factor (TF) values for Cr and Ni, indicating greater transport of these metals from roots to aerial parts of plants. Differences between heavy metals (HMs) according to land use were observed; especially, Pb and Cu were more concentrated in soils than other heavy metals in industrial areas. Overall, these findings suggest that *Trifolium pratense* is a promising bioindicator for heavy metal contamination in various land uses in urban areas, making it a potentially valuable tool for monitoring heavy metal pollution in cities of the northern hemisphere.

Keywords: biomonitoring; red clover; plant uptake; soil pollution; aerial pollution

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1. Introduction

Urban environments are recognized as significant sources of hazardous and noxious pollutants that can contaminate the air, water, and soil [1]. Atmospheric pollution is considered one of the most pressing environmental problems affecting urban regions [2]. Human activities, such as industrial, municipal, and commercial operations, generate a diverse range of toxic pollutants that can negatively impact the health and well-being of living organisms [3,4].

Among the pollutants emitted through various human activities, industries, heating, and transportation in urban environments, heavy metals are a significant source of concern and a major pollutant [1,5]. Conventionally defined as elements with an atomic density greater than 5 g cm^{-3} and atomic numbers >20 [6], heavy metals are known for their ability to bioaccumulate, inherent toxicity, and long-lasting presence in various environments, making them commonly referred to as environmental pollutants [7]. Once heavy metals persist in the environment, their removal is challenging [8]. Unfortunately, due to the increasing number of pollution sources, low air quality, and inappropriate urban planning, human exposure to heavy metals has risen dramatically, especially in urban and industrial areas [9]. In the environment, accumulated heavy metals can be transferred to humans by contaminated water, inhaling polluted air, and consuming plants grown in contaminated soil [10]. So, the problem of heavy metals is widespread [11]. For humans, heavy metal exposure is a significant contributor to various health problems, including developmental retardation, immune system dysfunction, several types of cancer, endocrine disruption, neurological effects, kidney damage, and other disorders [8]. Furthermore, the concentration of heavy metals in plants can have negative impacts on plant growth, yield, and the environment [12]. For example, chromium (Cr) is a known carcinogenic metal that can accumulate in plant tissues, resulting in decreased growth and yield [13,14]. Cadmium (Cd) is a toxic heavy metal that can cause leaf chlorosis, stunted growth, and reduced root length [15]. Copper (Cu) is an essential micronutrient for plant growth but can be toxic at high concentrations, causing oxidative stress and decreased photosynthesis [16]. Nickel (Ni) is a heavy metal that can accumulate in plant tissues, causing oxidative damage and reduced growth [17]. Zinc (Zn) is an essential micronutrient that is toxic at high concentrations, causing chlorosis and reduced growth [12]. Lead (Pb) is a toxic heavy metal that can cause decreased photosynthesis and chlorosis, as well as neurological and developmental effects in humans and animals [18,19]. Therefore, their concentrations must be monitored and analyzed to assess and mitigate environmental heavy metal pollution [7]. Consequently, over the years, a large number of studies have analyzed the accumulation of heavy metals in different components, such as soils [20,21], water [20,22], sediments [22], and the tissues of living organisms [23]. Additionally, a good deal of research over the years has shown that a suitable choice to indicate the accumulation of heavy metals in different environments was to use plants, known as bioindicators [24,25].

In order to monitor the state of the environment, bioindicators are an adequate tool [26]. Bioindicators include species, communities, and biological processes used to assess the environment's quality. Due to their moderate tolerance to environmental variability, bioindicators effectively indicate the environment's condition [24,27]. Furthermore, an effective bioindicator plant for heavy metal pollution should possess the ability to efficiently accumulate heavy metals in its tissues, thereby serving as a sensitive detector of environmental pollution. In addition, the plant should have a wide distribution range, allowing for its use as a bioindicator across multiple geographic areas, and exhibit a moderate tolerance to environmental variability to grow in a variety of soil types and conditions. These criteria are essential in selecting appropriate bioindicator plants for monitoring heavy metal pollution in urban areas, ensuring that reliable and comprehensive data can be obtained to assess the environmental quality and potential risks associated with pollution [24,27,28].

Therefore, worldwide, in different environments, analyses of the heavy metal content in potential bioindicators have been conducted, such as lichens, mosses, and vascular plants [25,29]. Among wild plants, *Trifolium pratense* L. has received attention as a possible bioindicator plant for heavy metals [5]. *Trifolium pratense* L. (Red Clover) is a wild plant belonging to the legume (*Leguminosae*) family [30]. Due to its ability to accumulate heavy metals and other contaminants from the soil, it has been proposed as a potential bioindicator plant [5,31–34]. Therefore, several studies have investigated the use of red clover as a bioindicator of soil and air pollution (e.g., [5,35–37]). Overall, the use of *Trifolium pratense* L. as a bioindicator plant has shown promising results, although more research is needed to fully understand its potential and limitations.

The main aim of the present study was to evaluate *T. pratense* as a heavy metal bioindicator in urban areas of the representative city concerning land use. *Trifolium pratense* L. was selected as a potential bioindicator plant for heavy metal contamination in urban areas due to its widespread distribution, high biomass production, and ability to accumulate heavy metals in its tissues.

Moreover, the research was carried out to assess the concentration and translocation of the metals Cd, Cu, Cr, Ni, Pb, and Zn in the organs of *T. pratense* plants depending on the soil content. The heavy metals investigated in this study were chosen due to their prevalence as common pollutants in urban areas [38,39].

2. Materials and Methods

2.1. Study Area

Our study was conducted in Poznań, located in western Poland (52°24′30″ N, 16°56′03″ E). With an area of approximately 261.91 km^2 and a population of around 530,000 inhabitants, Poznań ranks as the fifth-largest city in Poland in terms of population and the eighth largest in terms of area [40]. It is known for its temperate continental climate, which is characterized by warm summers and cold winters [41]. In Poznań, transport and built-up areas cover 43.5% of the total urban area. Industrial sites are predominantly located near the major roads in the western, eastern, and southeastern areas of Poznań, as well as in the surrounding areas. The surrounding areas of the city are home to the city's developing automotive industry and agro-food sector. The most common soil textures are sand and sandy loam, with an average pH of 6.5–8.0 [42].

2.2. Experimental Materials

Experimental materials were one-year-old specimens of *Trifolium pratense* L. collected from 8 different research sites: individual houses area, area near the lake (15 m from shore), near a river (16 m from the riverbank), a high-density residential area, an industrial area, a park, an old town, and agricultural land (Table 1, Figure 1). Only plants in identical vegetative stages that showed no evident damage (such as discoloration, insect or disease indications), were gathered. Due to the key role of soil as the main factor modulating the physiological response of plants, and thus directly affecting the process of heavy metal accumulation, soil samples were collected in the same places where *T. pratense* grew.

Table 1. Sample sites.

No.	Description of Sample Sites	Code
1	individual houses	POZ01
2	area near the lake	POZ02
3	area near the river	POZ03
4	high-density residential area	POZ04
5	industrial area	POZ05
6	Park	POZ06
7	old town	POZ07
8	agricultural land	POZ08

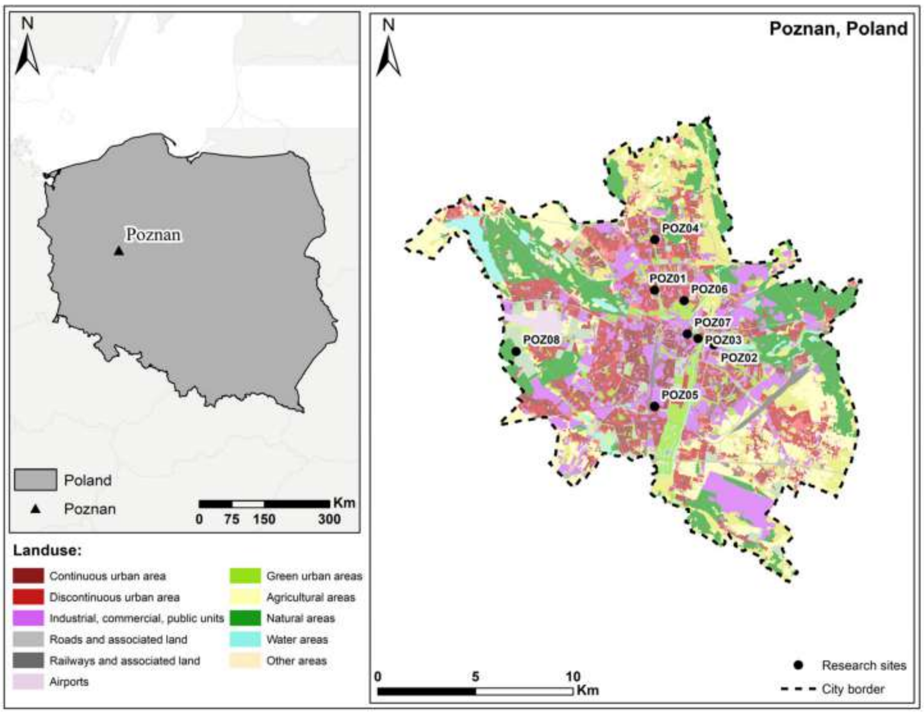

Figure 1. Location of research sites in Poznan (POZ01-POZ08) (source: own study based on Urban Atlas 2018).

2.3. Soil and Plants Sampling

Trifolium pratense L. and soil samples from particular localizations were collected between 5 and 11 May 2022. Soil materials and plant samples were taken from sample sites (Table 1), each one covered square-shaped areas with a 20 to 50 m^2 range. Nine specimens of *T. pratense* were collected for each square-shaped area, from each rooting zone, and 0.5 kg of soil samples were collected in a layer of 0–20 cm depth. Separate plastic boxes were used to store the gathered samples of soil and plants before being delivered to the lab. We confirm that all methods, including the collection of plant material, were carried out in accordance with relevant guidelines and regulations. Voucher specimens were deposited in the herbarium of the Department of Ecology and Environmental Protection, Poznań University of Life Sciences.

2.4. Sample Preparation and Digestion Procedure

In the laboratory, the plant samples were first purified with deionized water using Milli-Q Advantage A10 Water Purification Systems, Merck Millipore (Merck, Darmstadt, Germany), and separated into leaves and roots. The soil samples were sieved (2 mm). To achieve constant dry weight, the plant and soil samples were dried at 40 ± 3 °C in an electric oven (TC 100, SalvisLAB, Rotkreuz, Switzerland) for 120 h. Digestion of the crushed in an agate mortar sample was carried out in the CEM Mars 5 Xpress microwave mineralization system (CEM, Matthews, NC, USA). From each plant or soil sample, 0.3000 ± 0.0001 g was added to a 55 mL vessel containing 8 mL of concentrated (65%) HNO_3 Suprapur® (Merck, Darmstadt, Germany) and 1 mL of H_2O_2 for ultratrace analysis (Merck, Darmstadt, Germany). The program of digestion included three steps: ramp to temperature to 180°C—20 min; hold temperature at 180 °C—20 min; cool to room temperature—20 min. After the digestion steps using Qualitative Filter Papers (Grade 595: 4–7 µm Whatman, Kent, UK), the solutions were filtered, placed in flasks and made up to a final volume of 15.0 mL with deionized water. Reagents blank solutions were prepared in the same way as samples. The pH and electrical conductivity of the soil samples were measured using a pH meter and a conductometer, specifically the WTW Multi 3630 IDS EETF model. For pH measurement, a 1 N KCl solution with a proportion of 1:2.5 (10 g soil/25 mL KCl) was used. For electrical conductivity measurement, a proportion of 1:5 (10 g soil/50 mL demineralized H_2O) was used.

2.5. Heavy Metal Determination

The determination of cadmium (Cd), chromium (Cr), nickel (Ni), copper (Cu), and lead (Pb) in plant samples was carried out using an inductively coupled plasma mass spectrometer (ICP-MS). The instrument used was the Agilent 7700x (Agilent, Santa Clara, CA, USA), which is equipped with a collision/ reaction cell (Octopole Reaction System, ORS), and was operated in no-gas and helium modes. The sample was introduced into argon (Linde Gas, Cracow, Poland) plasma via a MicroMist concentric nebulizer, quartz Scott double pass spray chamber and a quartz torch with a quartz injector. The operating conditions for the inductively coupled plasma mass spectrometry (ICP-MS) were optimized daily using the Tuning Solution (Agilent). The instrumental parameters were as follows: 1550 W for radiofrequency (RF) power, 15 L min^{-1} for plasma gas flow rate, 0.98 L min^{-1} for nebulizer gas flow rate, and 0.9 L min^{-1} for auxiliary gas flow rate. The ORS mode with helium gas (Linde Gas, Poland) was used in order to eliminate spectral interferences. To reduce non-spectral interferences, a 10 µg L^{-1} solution of 103 Rh was used as an internal standard. High-purity argon (99.999%) was used as a nebulizer, auxiliary, and plasma gas for the ICP-MS (Messer, Chorzów, Poland). An external calibration curve was prepared by preparing a set of 5 standard solutions in the concentration ranges of 0.05–50 µg L^{-1} for Cd, Cr, Ni, and Pb, and 0.05–100.0 µg L^{-1} for Cu.0–5 µg L^{-1}. Calibration solutions were prepared by diluting a 10 mg L^{-1} multielement stock solution in 5% HNO_3 (Multi-Element Calibration Standard 3, PerkinElmer, Waltham, MA, USA) [43].

2.6. Quality Assurance

To assess the precision and trueness of the analytical procedure-certified reference material (CRM), Trace Elements in Spinach Leaves (1515, NIST, Gaithersburg, MD, USA) and the method of standard additions were used [44]. The linearity of the calibration curves was calculated as a coefficient of correlation R, which was greater than 0.9996 for all analytes. The LOD was calculated according to the equation LOD = 3.3 SBL, where SBL is the standard deviation of repeated blank measurements. The LOD values were as follows: Cd 0.003 μg g^{-1}, Cr 0.006 μg g^{-1}, Cu 0.008 μg g^{-1}, Ni 0.006 μg g^{-1}, and Pb 0.006 μg g^{-1}. The LOQ was calculated as three times the LOD value. Precision values were calculated as the coefficient of variation (CV) (%), which ranged from 0.8% to 2.5% for all elements. Trueness was evaluated as recovery (%), which ranged from 97% to 103%, respectively. The results of the Student's t-test confirmed that there were no significant differences between the measured concentration ± standard deviation and the certified concentration ± standard uncertainty.

2.7. Ratios of Accumulation, Translocation and Contamination

Pollution of plants and soils with heavy metals was assessed by the bioconcentration factor (BCF), the translocation factor (TF), and the contamination factor (CF). The BCF reflects a plant's ability to accumulate and translocate heavy metals, and is calculated as the ratio between the concentrations of heavy metals in plants and in soils [45]. The heavy metal bioconcentration factor (BCF) in plants was calculated with the formula (1), which expresses the ratio between the concentration of trace elements in root samples and the concentration of trace elements in soil samples [46]:

$$\text{BCF} = \text{heavy metal concentration in roots (mg kg}^{-1}\text{ DW)/heavy metal concentration in the soil (mg kg}^{-1}\text{ DW)} \quad (1)$$

To assess the ability of heavy metals to move from plant roots to other organs, we calculated the translocation factor (TF), which is a measure of a compound's capacity for translocation. The TF can be used to evaluate the extent to which heavy metals are transferred from one plant organ to another [47]. With the following formula (2), according to Yu and Zhou [48], the translocation factor (TF) was calculated as the ratio between elements' concentration in leaves and their concentration in the roots:

$$\text{TF} = \text{heavy metal content in leaves (mg kg}^{-1}\text{ DW)/heavy metal content in roots (mg kg}^{-1}\text{ DW)} \quad (2)$$

The contamination factor (CF) is a useful single index for monitoring heavy metal contamination [49]. It provides an effective means of quantifying the extent to which heavy metals have contaminated a given area. The CF for heavy metals can be calculated using the following formula (3):

$$CF = C^i/Cn^i \quad (3)$$

where C^i is the mean accumulation of the element in the soil, and Cn^i is the reference level for the element. The value of contamination factor allows classification of the degree of pollution in following way:

- CF < 1—LCF—low contamination factor,
- 1 ≤ CF < 3—MCF—moderate contamination factory,
- 3 ≤ CF < 6—CCF—considerable contamination factor,
- CF ≥ —VHCF—very high contamination factor [49].

According to [50] the reference levels for heavy metals are: Cd—0.41 mg kg^{-1}; Cr—59.5 mg kg^{-1}; Ni—29 mg kg^{-1}; Cu—38.9 mg kg^{-1}; Pb—27 mg kg^{-1}.

2.8. Statistical Analyses

Statistical analyses were carried out using statistical software (R Core, 2014) and Statistica 13.1. A descriptive statistical analysis was performed to assess the concentrations of heavy metals in the examined plant species from different samples and also the concentration of defense system and physiological parameters. To assess the significance of differences between heavy metal levels in plant species, one-way analysis of variance (ANOVA) and post hoc Scheffé test were used. Data were visualized using heatmaps to compare the concentration of a particular group of elements in plants and soils at specific research sites, with two-dimensional variables (research sites, element) represented by colors. Specifically, darker colors correspond to higher concentrations, while lighter colors represent lower value. In addition, to discover distinctions and similarities among sites, sample types, and element accumulations, cluster analysis was also carried out.

3. Results

3.1. Soils Characteristics and Concentration of Heavy Metals in Soils

In research sites of Poznań, the soil pH ranged from 6.372 (high-density residential area) to 7.506 (industrial area), while the electrical conductivity (EC) ranged from 0.05 mS cm^{-1} (high-density residential area) to 0.221 mS cm^{-1} (park) (Table 2).

Table 2. Soil parameter at research sites (3 replications for each site).

Research Site	pH	EC [mS cm^{-1}]
Individual houses (POZ01)	6.886 ± 0.012	0.102 ± 0.002
Area near the lake (POZ02)	7.209 ± 0.013	0.101 ± 0.001
Area near the river (POZ03)	6.414 ± 0.031	0.088 ± 0.004
High-density residential area (POZ04)	6.372 ± 0.034	0.050 ± 0.003
Industrial area (POZ05)	7.506 ± 0.025	0.147 ± 0.004
Park (POZ06)	7.314 ± 0.032	0.221 ± 0.003
Old town (POZ07)	7.033 ± 0.023	0.096 ± 0.002
Agricultural land (POZ08)	7.353 ± 0.014	0.106 ± 0.004

The concentrations of various metals (Cd, Cu, Cr, Ni, and Pb) in soil samples were found to vary across different areas. Specifically, the highest concentration of Cr was observed near the river (POZ03), while the lowest concentration occurred in the high-density residential area (POZ04). For Cd, the highest concentration was found in the park soils (POZ06), while the lowest concentration was observed in the high-density residential area (POZ04). The lowest concentrations of Ni, Cu, and Pb were also observed in the high-density residential area (POZ04). On the other hand, their highest concentrations were observed in the industrial area (POZ05) (Table 3).

In the graph (Figure 2), the standardized results of heavy metal concentration in soils concerning land use were presented. Heavy metals concentration formed two main groups. The first group includes the industrial area (POZ05) and park (POZ06), and the second main group includes other research sites. From the intensity of the heatmap (the darker the color, the higher uptake of the heavy metal), it can be noted that soils from the industrial area (POZ05) and from the park (POZ06) compared with other research sites revealed an elevated level of all heavy metals (Cr, Cu, Ni, Cd, and Pb). However, from the intensity of the heatmap color in the soils of the industrial area (POZ05), these soils are noted to have a higher concentration of Pb, Ni, and Cu. Furthermore, in the park (POZ06), the soil is noted to have a higher concentration of Cd and Cu. Moreover, it can be noted that soils near the river (POZ03) revealed an elevated level of Cd and Cr (Figure 2).

3.2. Content of Heavy Metals in Plant Roots

Concerning roots, the highest concentration of Cr and Ni was noted in agricultural land (POZ08), and their lowest concentration was observed in the industrial area (POZ05). Cu and Cd's highest concentration was observed in the park (POZ06). On the other hand, for Pb, the highest concentration was observed in roots of agricultural land, and the lowest concentration was observed near the lake (POZ02) (Table 3).

Table 3. The HMs content (mg kg^{-1} DW) in soil and plants (mean ± SD), where different letters (a–f) indicate means are significantly different ($p < 0.05$) in the same row according to the post hoc Scheffé test.

HMs		POZ01	POZ02	POZ03	POZ04	POZ05	POZ06	POZ07	POZ08
soil	Cr	14.2 ± 1.22 ^{d}	8.69 ± 0.574 ^{de}	47.3 ± 4.62 ^{a}	5.714 ± 0.403 ^{e}	38.3 ± 4.75 ^{b}	21.4 ± 1.26 ^{c}	8.797 ± 0.158 ^{de}	8.487 ± 0.669 ^{de}
	Ni	10.9 ± 0.823 ^{c}	5.43 ± 0.391 ^{d}	10.7 ± 1.10 ^{c}	4.04 ± 0.285 ^{d}	22.3 ± 2.81 ^{a}	13.9 ± 0.778 ^{b}	5.70 ± 0.097 ^{d}	6.30 ± 0.567 ^{d}
	Cu	15.9 ± 1.25 ^{bc}	10.1 ± 0.652 ^{c}	21.3 ± 1.78 ^{b}	6.38 ± 0.274 ^{c}	78.5 ± 9.74 ^{a}	72.6 ± 4.22 ^{a}	20.2 ± 0.273 ^{b}	12.7 ± 1.13 ^{bc}
	Cd	0.321 ± 0.030 ^{de}	0.386 ± 0.010 ^{d}	0.971 ± 0.021 ^{b}	0.143 ± 0.010 ^{f}	0.853 ± 0.025 ^{c}	1.12 ± 0.123 ^{a}	0.322 ± 0.009 ^{de}	0.268 ± 0.003 ^{d}
	Pb	13.6 ± 1.08 ^{e}	12.0 ± 0.805 ^{e}	39.5 ± 3.33 ^{bc}	7.99 ± 0.445 ^{e}	90.4 ± 14.6 ^{a}	49.1 ± 3.3 ^{b}	27.7 ± 0.458 ^{cd}	15.4 ± 1.22 ^{de}
roots	Cr	32.5 ± 0.771 ^{b}	38.3 ± 2.25 ^{b}	35.9 ± 0.037 ^{b}	54.8 ± 2.24 ^{a}	7.10 ± 0.314 ^{d}	20.8 ± 0.717 ^{c}	18.3 ± 0.648 ^{c}	60.6 ± 4.71 ^{a}
	Ni	9.97 ± 0.322 ^{b}	11.2 ± 0.617 ^{b}	10.4 ± 0.383 ^{b}	15.6 ± 0.779 ^{a}	3.78 ± 0.081 ^{d}	6.5 ± 0.226 ^{c}	5.6 ± 0.179 ^{c}	16.8 ± 1.22 ^{a}
	Cu	6.47 ± 0.081 ^{f}	7.71 ± 0.482 ^{ef}	10.5 ± 0.485 ^{d}	6.98 ± 0.230 ^{ef}	19.5 ± 0.746 ^{b}	24.3 ± 1.04 ^{a}	15.1 ± 0.663 ^{c}	8.56 ± 0.688 ^{e}
	Cd	0.084 ± 0.003 ^{f}	0.336 ± 0.006 ^{c}	0.463 ± 0.035 ^{a}	0.178 ± 0.008 ^{e}	0.404 ± 0.008 ^{b}	0.498 ± 0.023 ^{a}	0.257 ± 0.006 ^{d}	0.156 ± 0.006 ^{e}
	Pb	1.06 ± 0.020 ^{f}	1.00 ± 0.055 ^{f}	3.25 ± 0.121 ^{c}	1.45 ± 0.081 ^{e}	8.65 ± 0.210 ^{a}	4.74 ± 0.169 ^{b}	4.66 ± 0.186 ^{b}	2.04 ± 0.166 ^{d}
leaves	Cr	3.48 ± 0.039 ^{e}	7.49 ± 0.382 ^{d}	15.46 ± 0.327 ^{c}	15.8 ± 1.047 ^{c}	52.4 ± 3.20 ^{a}	9.53 ± 0.273 ^{d}	35.8 ± 1.25 ^{b}	36.9 ± 1.38 ^{b}
	Ni	1.33 ± 0.037 ^{e}	2.71 ± 0.156 ^{d}	5.03 ± 0.170 ^{c}	4.92 ± 0.253 ^{c}	15.3 ± 0.777 ^{a}	3.06 ± 0.144 ^{d}	11.2 ± 0.613 ^{b}	10.9 ± 0.326 ^{b}
	Cu	6.28 ± 0.111 ^{e}	6.40 ± 0.358 ^{e}	7.49 ± 0.173 ^{d}	6.54 ± 0.466 ^{e}	11.2 ± 0.659 ^{b}	13.0 ± 0.377 ^{a}	8.58 ± 0.301 ^{c}	6.60 ± 0.277 ^{de}
	Cd	0.008 ± 0.003 ^{e}	0.012 ± 0.001 ^{e}	0.061 ± 0.007 ^{b}	0.032 ± 0.003 ^{d}	0.109 ± 0.003 ^{a}	0.044 ± 0.006 ^{c}	0.040 ± 0.002 ^{cd}	0.111 ± 0.004 ^{a}
	Pb	0.176 ± 0.004 ^{e}	0.340 ± 0.017 ^{de}	0.955 ± 0.025 ^{c}	0.416 ± 0.036 ^{d}	3.60 ± 0.232 ^{a}	1.04 ± 0.028 ^{c}	2.48 ± 0.109 ^{b}	1.06 ± 0.040 ^{c}

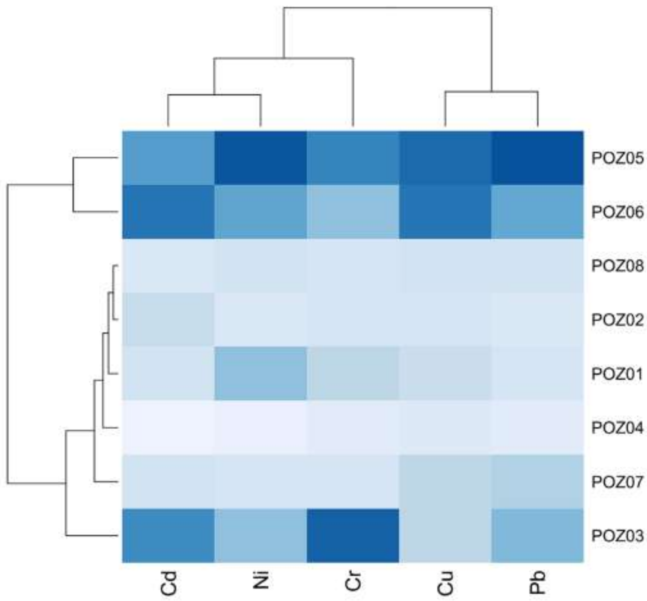

Figure 2. Heatmap and cluster analysis of heavy metal (Cd, Cu, Cr, Ni and Pb) concentration in soils from all research sites.

The standardized results of the analysis of heavy metals concentration in roots in relation to land use revealed the grouping of heavy metals into two main groups (Figure 3). The first main group included two subgroups; the first one includes a high-density housing area (POZ04) and agricultural land (POZ08), while the second subgroup includes individual houses (POZ01), near the lake (POZ02), and near the river (POZ03). The second main group included two subgroups; the first included the industrial area (POZ05), the second one—the park (POZ06) and the old town (POZ07). From the intensity of heatmap color (the more the intensity of the color is dark, the more the uptake of the heavy metal is high), it can be noted that the highest Cr and Ni concentration is noted in the agricultural land (POZ08) and the high-density housing area (POZ04). Pb and Cu's highest concentration was noted in the industrial area (POZ05), park (POZ06), and the old town (POZ07). At the same time, Cd's highest concentration was observed in the park (POZ06), near the river (POZ03), and in the industrial area (POZ05) (Figure 3).

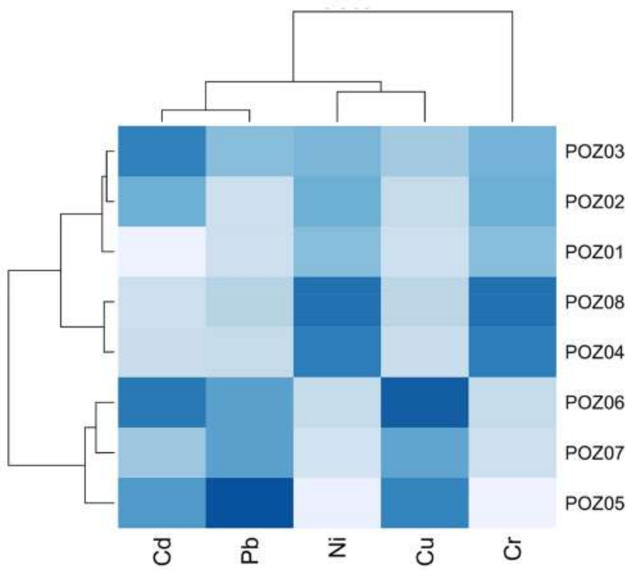

Figure 3. Heatmap and cluster analysis of heavy metal (Cd, Cu, Cr, Ni and Pb) concentration in roots from all research sites.

3.3. Content of Heavy Metals in Plant Leaves

In leaves, the highest concentration of Cr, Ni, and Pb was observed in the industrial area (POZ05), and their lowest concentration was observed in the individual houses (POZ01). For Cu, the highest concentration was observed in the park (POZ06), and the lowest concentration was observed in the agricultural land (POZ08). On the other hand, concerning Cd, the highest concentration was observed in agricultural land (POZ08), and the lowest concentration was observed in individual houses (POZ01) (Table 3).

The standardized results of analyzed heavy metal concentrations in *T. pretense* leaves in relation to land use revealed the grouping of heavy metals into two main groups. The first group includes the industrial area (POZ05), the old town (POZ07), and the agricultural land (POZ08), and the second group includes other research sites. From the intensity of the heatmap color (the darker the color, the higher the uptake of the heavy metal), it can be noted that Pb, Ni, and Cr were revealed to be present in higher concentrations in the leaves of the industrial area (POZ05). In addition, it can be noted that Cu revealed a higher concentration in the industrial area (POZ05), but the concentration is lower than in the park leaves (POZ06). In addition, Cr and Ni revealed higher concentrations in the old town (POZ07) and the agricultural land (POZ08) (Figure 4).

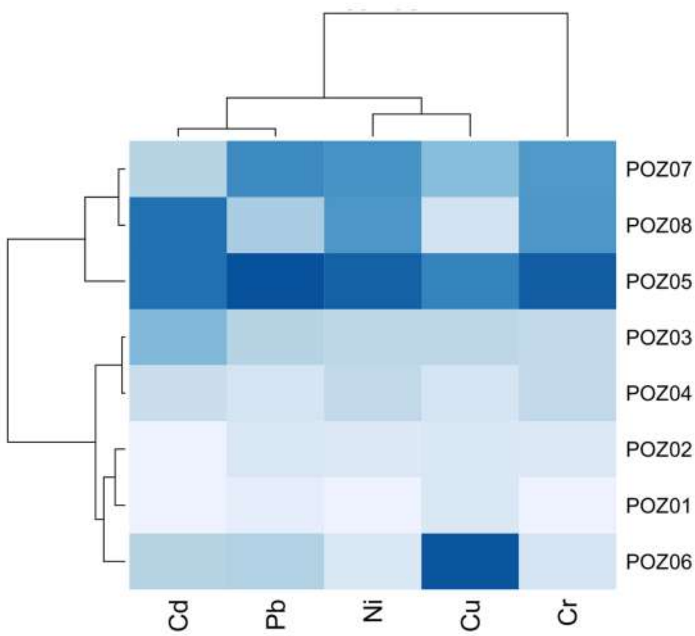

Figure 4. Heatmap and cluster analysis of heavy metal (Cd, Cu, Cr, Ni and Pb) concentration in leaves from all research sites.

3.4. Bioconcentration (BCF), Translocation (TF) and Contamination Factor (CF)

The bioconcentration factor (BCF) of chromium (Cr) in comparison with other heavy metals (Ni, Cu, Cd, and Pb) exceeded a value of 1, in most of the research sites except those near the river (POZ03), industrial area (POZ05), and park (POZ06). The nickel (Ni) bioconcentration factor (BCF) exceeded a value of 1 near the lake (POZ02), in the high-density residential area (POZ04), and on the agricultural land (POZ08). In contrast, the bioconcentration factor of Cu and Cd only exceeded a value of 1 in the high-density residential area (POZ04). On the other hand, the bioconcentration factor of Pb did not exceed the value of 1 in any research site. While concerning the translocation factor (TF), Cr and Ni recorded the highest translocation factor. Both elements exceeded the value of 1 in the industrial area (POZ05) and the old town (POZ07). Whereas other heavy metals did not exceed the value of 1 in any research site. Moreover, for the contamination factor (CF), Cr, Ni, Cu, Cd, and Pb showed different contamination values at all research sites. Cr and Ni, all research sites, were recorded to have a low contamination factor (LCF). In the case of

Cu, the medium contamination factor (MCF) was recorded in the industrial area (POZ05) and the park (POZ06), whereas other research sites recorded a low contamination factor (LCF). In addition, as for the Cd, a medium contamination factor (MCF) was recorded in the river (POZ03), industrial area (POZ05), and the park (POZ06), while a low contamination factor (LCF) was recorded in other research sites. Additionally, in the case of Pb in the industrial area (POZ05) a considerable contamination factor (CCF) was recorded, while in the river (POZ03), park (POZ06), and the old town (POZ07) a medium contamination factor (MCF) was noted, whereas other research sites recorded a low contamination factor (LCF) (Table 4).

Table 4. The bioconcentration, translocation and contamination factor of Cd, Cu, Cr, Ni and Pb in research sites.

Research Sites	BCF Where: The Highlighted Values Mean Concentration in Roots Biomass					TF Where: The Highlighted Values Mean Effective Metals Translocation within the Plant					CF Where: The Highlighted Values Mean Moderate or Considerable Contamination Factors				
	Cr	Ni	Cu	Cd	Pb	Cr	Ni	Cu	Cd	Pb	Cr	Ni	Cu	Cd	Pb
POZ01	**2.291**	0.919	0.412	0.259	0.078	0.107	0.135	0.972	0.088	0.167	0.238	0.371	0.404	0.793	0.500
POZ02	**4.448**	**2.085**	0.765	0.87	0.084	0.196	0.241	0.829	0.038	0.339	0.144	0.185	0.257	0.942	0.439
POZ03	0.779	0.976	0.492	0.48	0.082	0.422	0.488	0.718	0.133	0.297	0.731	0.365	0.543	**2.374**	**1.451**
POZ04	**9.679**	**3.907**	**1.104**	**1.26**	0.184	0.288	0.312	0.931	0.176	0.284	0.095	0.138	0.162	0.347	0.293
POZ05	0.186	0.171	0.249	0.477	0.095	**7.428**	**4.038**	0.572	0.269	0.418	0.638	0.762	**2.003**	**2.069**	**3.369**
POZ06	0.973	0.473	0.337	0.442	0.097	0.46	0.465	0.535	0.09	0.222	0.357	0.474	**1.853**	**2.734**	**1.807**
POZ07	**2.077**	0.985	0.748	0.797	0.169	**1.956**	**1.997**	0.569	0.156	0.532	0.148	0.197	0.519	0.787	**1.027**
POZ08	**7.004**	2.609	0.668	0.59	0.13	0.618	0.666	0.777	0.712	0.53	0.143	0.218	0.327	0.652	0.573

4. Discussion

In the presented study, as a potential bioindicator for heavy metals in an urban area, *Trifolium pratense* was used. Based on the obtained data, the spatial distribution of heavy metals (Cd, Cu, Cr, Ni and Pb) in the analyzed research sites was irregular. These differences in heavy metal concentrations in urban areas can result from many factors. Besides parameters, such as the type and number of vehicles and traffic that affect air pollution in urban areas [1], heavy metal concentrations can also be different due to other human activities and the natural background (land use propose, green areas, construction zones, industrial sites, roadside), demographic factors, soil type, and its parameters [25]. In addition, topography and meteorological conditions will determine their dispersion and transformation. Therefore, there is considerable diversity in the content of metals in urban soils in various locations [51]. In the presented study, the research sites were divided into two groups based on the content of heavy metals in the soil (first: industrial area and park; second: individual houses, near the lake, near the river, high-density housing area, the old town, and agricultural land).

The soils of the industrial area (POZ05) were characterized by the highest concentration of Pb (90.4 mg kg^{-1}) and Cu (78.5 mg kg^{-1}). In addition, Ni (22.2 mg kg^{-1}) in this research site was higher than in other research sites. Lead (Pb), which is one of the most common elements [52], has many applications in various fields, such as industrial, agricultural, and household uses [53]. The high concentration of Pb in the soils of the industrial area (POZ05) can be attributed to its widespread use in various industries, including mining, smelting, and battery manufacturing. Similarly to Pb, metals such as Cu, Cd, and Zn can originate from sewage sludge, landfills, vehicle transport, geochemical processes, and industries [52]. Furthermore, the high concentrations of Cu and Ni in the soils of the industrial area can be attributed to the discharge of industrial effluents, as well as anthropogenic activities such as vehicular emissions [53]. The high concentrations of Pb, Cu, and Ni found in the soil of the industrial area suggest that anthropogenic activities in the area have significantly contributed to heavy metal pollution and the relation between land use and heavy metals.

The plant's ability to accumulate and tolerate heavy metals makes it a promising tool for monitoring selected heavy metals in the soil [5] of urban areas. That land use may have a relationship with the amounts of heavy metals is also concluded by Degórska [54], Lisiak-Zielińska et al. [25], and Adamu and Nganje [55]. Degórska [54] also found a significant relationship between the content of heavy metals in soil and land use in her investigation. Urban environments, which have experienced industrialization and urbanization, have shown a considerable increase in heavy metals such as Pb, Cd, Cu, and Zn on streets. Lisiak-Zielińska et al. [25] utilized *Taraxacum officinale* as a bioindicator of heavy metals in their study. The results showed that the heavy metal content in soil was primarily related to land use type, as determined by the geochemical background. Similarly, Adamu and Nganje [55] concluded that the elevation of heavy metal content in the surface soils of Benue State was related to urbanization and poor land planning and use. These findings underscore the importance of effective land management and planning in mitigating heavy metal pollution and preserving soil quality.

Furthermore, concerning Cd and Cr, the soils of the park (POZ06) were noted to have the highest Cd (1.13 mg kg^{-1}) concentration, and those near the river (POZ03) were noted to have the highest Cr (35.9 mg kg^{-1}) concentration. The cement-soil road bases in this area were probably the cause of the increased amounts of this element because chromium is mainly leached from the cement–ground foundation. The concentration of Cd can be related to the fact that these two areas are quite popular for recreation. Tourism activities, including the intensification of traffic and other related human activities, have been shown to contribute to the pollution of cadmium (Cd). As a result, it is clear that the occurrence and cycling of Cd in tourism environments are common phenomena [56]. The study conducted by Lisiak-Zielińska et al. [25] in Poznan also found similar results, where *Taraxacum officinale* accumulated higher amounts of Cd in areas near the lake, which is a popular recreational site. The increased traffic and other human activities associated with tourism were identified as contributing factors.

The roots of plants in the agricultural land (POZ08) were found to have higher concentrations of Cr and Ni. Heavy metals from human activities can significantly influence agricultural soils, leading to the bioconcentration of Cr and Ni in the roots of plants. The bioconcentration factor (BCF) for Cr and Ni in agricultural soil was found to exceed the value of 1, indicating that these elements were transported from the soil to the plant roots. The metals taken from the soil are initially stored in the plant roots. These metals can then be translocated to the aboveground plant parts through the xylem sap, which is driven by the plant's respiration power [57]. These findings are supported by other studies [58,59], which demonstrate that chromium can be permanently bound by living cells in plant roots, leading to low chromium content in aboveground parts of the plants. Similarly to this study, extreme concentrations of Cr, Ni, and Cu have been found in agricultural soils in Qatar [60] and other soils in different land-use types, that are affected by human activities, including roadside, urban and industrial areas [61].The higher concentrations of Cr and Ni found in the roots of plants in the agricultural land (POZ08) could be attributed to the application of fertilizers, which can contain heavy metals such as chromium and nickel. Several studies have reported the occurrence of heavy metals in fertilizers, and their potential impacts on soil quality and crop productivity [62–64].

The highest values of the translocation factor for Cr and Ni were observed in the industrial area (POZ05) and the old town (POZ07), which could be linked to the presence of manufacturing plants and intensive traffic in these areas. Previous studies have also reported similar findings, highlighting the role of industrial activities and traffic in the emission of heavy metals into the environment and their subsequent accumulation in plants [14,65]. The main sources of Ni can also be related to the use of cadmium–nickel batteries and local industry, which have been shown to contribute to the contamination of soil and water with heavy metals [66]. A similar correlation between the translocation factor and land use was also noted by Angelova and Ivanov [67], who found higher values of this index in areas with heavy traffic. Cadmium and copper were found to accumulate mainly in the roots. In the present study, the highest concentration of Cu and Cd was found in the roots of plants in the park (POZ06), which is located in close proximity to the highway and has undergone urbanization.

Furthermore, in the leaves, the industrial area (POZ05) showed a high concentration of Cr, Ni, and Pb, compared to other research sites, indicating poor air quality and proximity to pollution sources [68]. The concentration of heavy metals in leaves can be attributed to their uptake through the stomata or the adsorption of atmospheric deposition [14,69]. The elevated concentrations of Cr, Ni, and Pb detected in the leaves of the industrial area (POZ05) are in agreement with the emissions from industrial activities and transportation, recognized as significant contributors to heavy metal pollution in urban areas. Additionally, the close proximity of the industrial area to other potential sources of heavy metals in the surrounding environment may also have contributed to the observed levels of heavy metals in the leaves. These findings are consistent with the results of Farooq et al. [70], who conducted a study on heavy metal pollution in the vicinity of industrial areas. Their study showed that the concentration of heavy metals was significantly higher in the leaves of various vegetables grown in areas close to industrial zones compared to areas further away, which provides further support for the idea that industrial activities and transportation are significant sources of heavy metal pollution in urban areas. In contrast, the leaves of agricultural land (POZ08) showed a higher concentration of Cd, which may be related to agricultural activities, especially the use of fertilizers. The occurrence of cadmium in soil is predominantly influenced by the application of phosphate fertilizers [71]. The deposition of Cd in crops can pose health risks to humans; hence, it is crucial to monitor and control its levels in food crops [72].

5. Conclusions

Our research revealed that the order of heavy metal concentration in soil was Pb > Cu > Cr > Ni > Cd. Specifically, the industrial area had the highest contamination levels of Pb, Ni, and Cu. In roots and leaves, the order of heavy metal concentration was Pb > Cu > Cr > Ni > Cd, where the roots of *T. pratense* in agricultural land had the highest concentration of Cr, Ni, and Cu. In addition, the leaves of *T. pratense* had the highest concentration of Cr, Ni, and Pb in the industrial area. Furthermore, the consistent pattern of heavy metal concentration observed in the roots and leaves of *T. pratense* may be due to the specific physiological and biochemical mechanisms involved in heavy metal uptake, translocation, and concentration within the plant. Our study demonstrated the potential of *T. pratense* as a bioindicator of heavy metal contamination in urban areas. However, further research is necessary to validate the effectiveness of *T. pratense* as a bioindicator in controlled conditions as well as in other urban areas and throughout various seasons.

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Declaration of the author of the doctoral dissertation

I hereby declare that the submitted Doctoral dissertation entitled "Alternative heavy metals garden – evaluation of perspectives to use new bioindicators for air pollution" has been written by me personally, i.e.

- I have not commissioned the preparation of this dissertation or its part,
- I have not copied the dissertation or its part from other studies and works, which topic is related to my work,
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