dr hab. inż. Joanna Podlasińska, prof. ndzw. ZUT
Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
Wydział Kształtowania Środowiska i Rolnictwa
Katedra Kształtowania Środowiska
e-mail: Joanna.Podlasinska@zut.edu.pl

Szczecin, 26th of August 2024

DOCTORAL DISSERTATION REVIEW
of MSc. Ing. Arlinda Cakaj
entitled
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

The review was conducted based on the decision of the Scientific Council of the Discipline of Environmental Engineering, Mining, and Energy at the Poznań University of Life Sciences on July 20, 2024.

The reviewed doctoral dissertation of MSc. Ing. Arlinda Cakaj was conducted at Department of Ecology and Environmental Protection on Faculty of Environmental and Mechanical Engineering in Poznan University of Life Sciences under the scientific supervision of Prof. UPP dr hab. Maria Drapikowska and auxiliary supervisor of Dr inż. Marta Lisiak-Zielińska. Doctoral dissertation has been made in the field of engineering and technology in the discipline of environmental engineering, mining and energy.

I present the following review after familiarizing myself with the received doctoral dissertation along with the attachments and the files on a USB drive.

The doctoral dissertation submitted for review by MSc. Ing. Arlinda Cakaj was written in the form of a commentary based on a series of three thematically related original scientific articles published between 2023 and 2024 in peer-reviewed international journals indexed in the JCR database. These works present the research results conducted by the Ph.D. candidate, both in field conditions and in the laboratory:

1. 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/s41598-023-34019-9 140 points; IF=4.6

2. 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. DOI: https://doi.org/10.1016/j.envres.2024.119222 100 points; IF=8.3

3. 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 100 points; IF=3.9

The number of points constituting a series of publications, as per the Communication from the Minister of Science dated January 5, 2024, regarding the list of scientific journals and peer reviewed proceedings of international conferences is 340 points and IF of 17.8.

It should be emphasized that the Ph.D. candidate is the first author in all the articles, which indicates her leading role and her contribution to the creation of each work, according to the attached statements, was significant. Her involvement included the creation of the work's concept, selection of research sites, collection of plant and soil materials, preparation and processing of research materials, execution of research biochemical analysis, data analysis, literature review, formulation of conclusions and manuscript preparation for publication.

The submitted dissertation has been structured according to the conventional format typical of scientific works. The main body of the dissertation consists of a concise and comprehensive commentary spanning 31 pages. The commentary begins with acknowledgments, followed by a list of publications included in the series, a table of contents, and a list of abbreviations. Abstracts are provided in both Polish and English. In the subsequent sections of the commentary, the candidate, M.Sc. Eng. Arlinda Cakaj, delineates the scientific foundations and research challenges, outlines the assumptions and objectives of the dissertation, describes the materials and methods employed, and presents the results, which are discussed in a synthesized manner. The discussion is divided into two sections corresponding to the thematic scope of the works in the series. This discussion demonstrates that the author has acquired the ability to critically assess the results obtained. The results allowed the candidate to formulate four concise conclusions. The commentary concludes with a bibliography containing 74 references written in English, including seven by Polish authors. The references have been carefully selected and are cited correctly. The dissertation is complemented by the articles forming the basis of the dissertation, included as appendices, as well as statements regarding the contributions of the candidate and co-authors.

The primary aim of this research was clearly and logically articulated: 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. - in detecting and monitoring heavy metal contamination. The overarching goal was achieved by accomplishing specific sub-objectives that considered 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.

The research was conducted using seeds and experimental material collected at selected sites in the Wielkopolska region, as well as with the seed material for *Lolium multiflorum* var. Ponto sourced from Norddeutsche Pfanzenzucht Hans-Georg Lembke KG (Germany). The experimental material comprised five species of herbaceous plants - *Trifolium pratense* L., *Rumex acetosa* L., *Alcea rosea* L., *Amaranthus retroflexus* L., and *Plantago lanceolata* L. - which served as bioindicators of heavy metal pollution, as well as the well-known heavy metal accumulator *Lolium multiflorum* var. Ponto, used as a reference. Soil samples were also collected from the research sites. The degree of bioaccumulation and translocation was assessed using appropriate indices.

The Ph.D. candidate employed suitable research methods necessary to achieve the study's objectives. She undertook the analysis of extensive experimental material, and the results were processed using several multivariate statistical methods. The selection of statistical tests applied in the analysis of the obtained results is appropriate and raises no concerns.

It should be mentioned that the research conducted during the doctoral dissertation received financial support from the Faculty of Environmental and Mechanical Engineering and the discipline of environmental engineering, mining, and energy at Poznań University of Life Sciences (Poland), through the 'Innovator' program, grant number 02/2022/INN. Moreover the 3rd publication was financed by the Polish Minister of Science and Higher Education as part of the Strategy of the Poznan University of Life Sciences for 2024-2026 in the field of improving scientific research and development work in priority research area.

The first two original research papers ("Common weeds as heavy metal bioindicators: a new approach in biomonitoring" and "Plants as effective bioindicators for heavy metal pollution monitoring.") present the findings from experimental studies. In the first study, the plant material was initially grown in a greenhouse and then exposed to three designated areas in Poznań, each differing in land use and anthropogenic load. After six weeks of exposure, the levels of four heavy metals (Cd, Pb, Cu and Zn) were analyzed in both the plants (leaves and roots) and the soil in which the plants were grown. Based on these results, bioconcentration (BCF) and translocation (TF) factors were calculated to assess heavy metal uptake. Additionally, physiological measurements were conducted, including cell membrane stability, dry mass, relative water content, chlorophyll content, and photosynthetic activity. The Ph.D. candidate also performed analyses to determine oxidative stress and antioxidative enzyme activities, including hydrogen peroxide content, lipid peroxidation, protein quantification, and in situ detection of hydrogen peroxide. The doctoral candidate outlined 8 important characteristics basing on the obtained results in this experiment, describing aspects of the research such as species-specific variation, spatial variation, metal accumulation trends, bioconcentration and translocation factors, and finally physiological responses. It was found that: 1. Different plant species exhibited varying capacities for accumulating heavy metals, showing species-specific responses to contamination. 2. Differences in heavy metal concentrations were observed across study sites, indicating spatial variability in soil and plant tissue contamination. 3. The accumulation of heavy metals followed the order Zn > Cu > Pb > Cd across all species and sites. 4. Zn and Cd had the highest bioconcentration factors (BCF), with *L. multiforum*, *A. rosea*, and *P. lanceolata* showing the highest values. Translocation factors (TF) varied by species, with specific plants showing higher mobility for particular metals. 5. The species demonstrated adequate physiological responses to metal stress. Notably, *P. lanceolata* showed strong cell membrane stability and chlorophyll content, while *A. retroflexus* had high dry mass content. The plants also exhibited increased antioxidant enzyme activity, indicating enhanced detoxification mechanisms against oxidative stress.

In the second original study, the plants were subjected to controlled heavy metal contamination through irrigation with metal nitrate solutions at two levels: low and high. The plants received two treatment rounds, initially after 77 days and then again after 10 days, to achieve doubled contamination levels. Nitrogen compensation was provided using ammonium nitrate in N equivalents to the metal spiking, ensuring corresponding control treatments. The experiment included four variants (low contamination, high contamination, and their respective nitrogen controls) which with three replicates each, gave a total number of 60 pots. Similar to the first experiment, the content of heavy metals (Cd, Cu, Ni, and Zn) was examined in the studied plants as well as in the substrate, and physiological characteristics were measured. The outlined 8 important characteristics of the results in this experiment, describing aspects of the research such as heavy metals contamination of plants (roots, leaves) and soil; bioconcentration and translocation factors and physiological responses. Which in a compact version could be summarized as 1. At low contamination levels, the trend of metal accumulation in soil was Zn > Pb > Ni > Cd, with *T. pratense* showing the lowest and *L. multiflorum* the highest concentrations. At high levels, the trend was Pb > Zn > Ni > Cd, with *R. acetosa* showing the lowest and *T. pratense* and *L. multiflorum* the highest concentrations. 2. At low contamination, the trend of heavy metals in roots was Zn > Ni > Pb > Cd, with *A. retroflexus* showing the lowest and *T. pratense* and *P. lanceolata* the highest concentrations. At high levels, the trend was Zn > Pb > Ni > Cd, with *A. retroflexus* showing the lowest and *P. lanceolata* the highest concentrations. 3 . Across both contamination levels, the trend of heavy metals content in leaves was Zn > Ni > Pb > Cd. *T. pratense* consistently had the lowest concentrations, while *R. acetosa* and *P. lanceolata* showed the highest levels depending on the metal. 5. Zn had the highest BCF, exceeding 2 in *T. pratense* and *P. lanceolata*. Ni and Cd also had BCF values greater than 1, particularly in *T. pratense, L. multiflorum*, and *P. lanceolata*. *R. acetosa, A. retroflexus,* and P. lanceolata exhibited the highest translocation rates for Ni and Pb, with TF values exceeding 4 for Pb at low contamination levels. *T. pratense* generally showed the lowest TF ratios.6. *P. lanceolata* showed the lowest dry mass at low contamination, while *T. pratense* had the highest. MSI and RWC values varied, with species like *R. acetosa* and *A. retroflexus* showing the highest stability and water content at different contamination levels. 7. Significant variations in physiological responses to heavy metal stress were observed, with *R. acetosa* showing the highest CAT and APOX activities. Elevated MDA and H_2O_2 levels were noted in *T. pratense, A. retroflexus,* and *P. lanceolata*, indicating higher stress responses.

The third original study presented in the scientific publication by Cakaj, A. et al. (2023. *Trifolium pratense* and the heavy metal content in various urban areas. Sustainability, 15(9), 7325. DOI: https://doi.org/10.3390/su15097325) focuses on in-situ bioindication investigation, with *Trifolium pratense* L., as a potential bioidicator of heavy metals pollution. Soil samples with *Trifolium pratense* L. samples (leaves, roots) 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 helped to evaluate the concentration and translocation of the metals Cd, Cu, Cr, Ni and Pb in the organs of *T. pratense* plants, based on soil content. The studies confirmed that metal concentrations in soil samples varied by location, with Cr highest near the river, Cd in parks, and Ni, Cu, and Pb in industrial areas, the lowest in residential areas. In case of *T. pratense* grooving on agricultural land had the highest Cr and Ni concentrations in roots, plants growing in parks had elevated Cu and Cd, and Pb was highest in plants from agricultural areas and lowest near the lake. Industrial areas showed the highest levels of Cr, Ni, and Pb in leaves. Cu was more concentrated in leaves in parks, while Cd was higher in agricultural land and lower near houses. *T. pratense* was identified as a promising bioindicator of heavy metal contamination in urban environments.

In line with the theme of the “Alternative heavy metals garden – evaluation of perspectives to use new bioindicators for air pollution” this study carefully selected five plant species known for their wide distribution, frequent occurrence, and prevalence as weeds in urbanized environments. Although all examined plants have been individually tested in previous studies, they have not been collectively analyzed or comprehensively evaluated, making this approach particularly innovative. Moreover, the physiological responses of these plants to heavy metal exposure have not been extensively studied, further contributing to the originality of this research.

The original scientific papers comprising the doctoral dissertation have been published in prestigious scientific journals, as evidenced by their impact factor and the points awarded by the Ministry of Science and Higher Education (MNiSW). The editorial process ensures that the manuscripts undergo a thorough evaluation by competent reviewers, making it difficult to find significant shortcomings. However, the assessment of the doctoral dissertation would not be complete without raising questions and requesting clarification on certain issues. The remarks primarily concern the dissertation outline and relate to certain inconsistencies, as well as potential omissions:

1. Is it appropriate to use the term "soil" and "soil samples" in the context of the experimental studies described in the first two publications, where a mixture of peat and sand was used for plant growth? Shouldn't the term "substrate" be used instead?
2. How were the pots with the examined species placed at each site in the first study? Were they embedded in the ground level with the soil surface? Did they have drainage holes? Could placing the containers in the shade of natural vegetation have significantly limited the deposition of heavy metals from rainfall and airborne particles?
3. Why were the seeds of the studied plants used in the second experiment purchased from two medium-sized plant breeding companies in Poland and Germany? Did seeds collected from the Greater Poland region, from plants adapted to the local environment, exhibit poor germination, growth, etc.?
4. What does the phrase "From each plant, a small fraction of leaves or roots were placed in Teflon vessels with nitric acid and hydrogen peroxide (H_2O_2)" (page 3, publication 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) mean? Were the samples of uniform size? If so, how large or small were they?

5. In the same publication, also on page 3, there is a reference to the work of Yu and Zhou (2009), which is not listed in the references. These authors are cited in the context of explaining the significance of the Translocation Factor. Shouldn't the original creators of such concepts be cited when explaining formulas? One of the earliest and notable implementations of the translocation factor (TF) in assessing heavy metal mobility in plants can be traced to the work of Baker and Brooks in the late 1980s, who explored the concept of metal hyperaccumulation in plants. They identified that certain plant species could absorb and translocate heavy metals from the soil to their aerial parts, thus establishing a foundation for future research on translocation indices.
6. In the third publication describing the in-situ experiment, the methodology uses the term "soil materials and plant samples." What exactly is meant by "soil materials," and why wasn't the term "soil samples" used? What was the method of soil sample collection—were they cut out in blocks as clods of earth from around the plants?
7. My next note on the work is not a criticism but rather a suggestion for future research. In this study, the only soil properties examined were pH, conductivity, and heavy metal content. Perhaps it would be beneficial to expand this scope to include organic matter content, which likely varies significantly in soil samples collected from such diverse locations. As it is well known organic matter plays a crucial role in the dynamics of heavy metal contamination in soils, influencing the availability, mobility, and toxicity of these metals.
8. In the outline on page 16, six heavy metals were listed as being studied. However, in the third publication of the series, only five of them were described, with zinc being omitted. Why the measurement of zinc contamination in *Trifolium pratense* in the final study has been omitted?

Minor editorial comments include the following:

1. The unnecessary repetition of the research aim in Chapter 1 (Introduction) and Chapter 2 (Research Hypotheses, Aim...).
2. The omission of listing all three publications that comprise the doctoral dissertation in the reference list. Although these works are mentioned at the very beginning of the document before the table of contents, the doctoral candidate cites them in the chapter describing the research results.

3. The placement of the table of contents after the list of publications that constitute the dissertation cycle.
4. The absence of the abbreviation "ROS" used on page 23 in the list of abbreviations, as well as the inclusion of "TCF" instead of the commonly used abbreviation "TF" throughout the dissertation.
5. The word “efficiency” is misspelled in the 3^{ed} study finding on page 25.
6. The omission of the following references in the References chapter: Sawidis et al., 2011 (mentioned on p. 9); Liu et al., 2008 and Parmar and Thakur, 2013 (mentioned on p. 21); Nadgórska-Socha et al., 2017 (mentioned on p. 22); Dada, 2019 (mentioned on p. 23); Moustakas, 2023, and Gechev et al., 2005 (mentioned on p. 24).
7. The incorrect citation of Galal et al., 2015, which is listed as item 23 in the reference list, but incorrectly cited as Galal, T. M., & Shehata, H. S. (2015), and Ghori et al., 2019, which is listed as item 10 in the reference list but cited as a two-author publication.
8. The duplication of scanned documents, including the author's and co-authors' declarations, attached to the dissertation.

The presented work demonstrates the Author's significant commitment to solving the research problem and achieving the stated objective. It represents substantial scientific value and contributes to expanding knowledge in the field of biomonitoring. The scope of the planned research reflects the Doctoral Candidate's high level of competence in planning, data interpretation, and conducting scholarly discussions. Minor shortcomings do not diminish the substantive value of the work nor undermine its scientific merit, as they are purely editorial in nature. Furthermore, the research topic undertaken by M.Sc. Arlinda Cakaj aligns with current studies in field of eco-monitoring and bioindicators. As bioindicators are organisms or biological responses that provide information about environmental quality, particularly regarding pollution levels, including heavy metals the presentented dissertation covers the most important features of a new found bioindicators of heavy metals. The experiments described in scientific publication by Cakaj, A. et al. (2023. *Trifolium pratense* and the heavy metal content in various urban areas. Sustainability, 15(9), 7325) showed that plant such as *Trifolium pratens* has shown significant capacity for heavy metal accumulation, making it suitable for monitoring pollution levels in urban areas and other environments This accumulation capacity is essential for bioindicators to effectively reflect the degree of contamination in their surroundings. The search for new bioindicators, particularly for heavy metals contamination, is of paramount importance for several reasons. The importance of finding new bioindicators for heavy metals contamination lies in their ability to provide sensitive, cost-effective, and ecologically relevant assessments of environmental health. By expanding the range of organisms used as bioindicators, researchers can enhance monitoring efforts, inform policy decisions, and promote conservation initiatives.

In conclusion, I affirm that the doctoral dissertation of M.Sc. Arlinda Cakaj titled "Alternative heavy metals garden – evaluation of perspectives to use new bioindicators for air pollution" is original, demonstrates the ability to conduct independent scientific research, and deserves high recognition. The doctoral dissertation of M.Sc. Arlinda Cakaj meets the requirements specified in the art. 187 of Act of 20 July 2018 The Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended).

In light of the above, I recommend that the Scientific Council of the Discipline of Environmental Engineering, Mining, and Power Engineering at the Poznań University of Life Sciences approve M.Sc. Arlinda Cakaj to proceed to the subsequent stages of the doctoral examination process.

Furthermore, I respectfully propose that the doctoral dissertation of M.Sc. Arlinda Cakaj be considered for distinction.

dr hab. inż. Joanna Podlasińska, prof. ZUT