THÈSE DE DOCTORAT EN COTUTELLE

UNIVERSITÈ DE LILLE

Ecole doctorale Science de la Matière, du Rayonnement et de l'Environnement (SMRE)

Laboratoire de Génie Civil et géo-Environnement (LGCgE)

AND

CZESTOCHOWA UNIVERSITY OF TECHNOLOGY

Faculty of Infrastructure and Environment

Optimisation des processus de remédiation à l'aide de méthodes toxicologiques avancées

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Présenté par: **Marta JASKULAK**

Soutenue le 21.06.2021 devant le jury composé de:

Co-directors:

Professor Franck VANDENBULCKE Université de Lille, Sciences et Technologies, Laboratoire de Génie Civil et géo-Environnement, LGCgE

Professor Małgorzata KACPRZAK Czestochowa University of Technology, Faculty of Infrastructure and Environment

President: Maciej MROWIEC: Czestochowa University of Technology, Faculty of Infrastructure and Environment

Rapporteur: Magdalena GAJEWSKA: Faculty of Civil and Environmental Engineering, Gdańsk University of Technology

Rapporteur: Manuel BLOUIN : Institut national supérieur des sciences gronomiques, de l'alimentation et de l'environnement | AgroSup Dijon · Agroecology laboratory (UMR INRA, AgroSup Dijon, Univeristy of Burgundi

Examinateur: Iwona ZAWIEJA Czestochowa University of Technology, Faculty of Infrastructure and Environment

Examinateur: Annabelle DERAM : Université de Lille, Sciences et Technologies

Examinateur: Annette de VAUFLEURY: Université de Franche-Comté

DOCTORAL DISSERTATION IN COTUTELLE

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Optimization of remediation processes using advanced toxicological methods

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Examinator: Iwona ZAWIEJA Czestochowa University of Technology, Faculty of Infrastructure and Environment

Examinator: Annabelle DERAM : Université de Lille, Sciences et Technologies

Examinator: Annette de VAUFLEURY: Université de Franche-Comté

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NATIONAL SCIENCE CENTRE
POLAND

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ABSTRACT

The contamination of soil with heavy metals becomes a fast-rising issue worldwide and is caused primarily by anthropogenic activities. Most heavy metals degrade slowly and cannot be decomposed naturally, resulting in their long-lasting presence in the environment. As a result, they pose a direct threat to all food chain members. Thus, technologies involved in the effective remediation of contaminated areas are increasingly gaining importance. Assisted phytoremediation is a wildly accepted technology for the remediation of heavy metals from soil. It includes the use of hyperaccumulating plants and soil fertilization. In order to increase the efficiency of the assisted phytoremediation and at the same time deal with a constant rise in the quantity of produced wastewater worldwide, the application of sewage sludge to land is often seen as a viable option. Since large volumes of sewage sludge have to be disposed of or treated in some manner, its use as a fertilizer has become a more common practice in recent years. However, the safety of such action is still debated since sewage sludge is known to contain not only organic matter and macronutrients but also a vast scope of contaminants.

The identification of mechanisms by which plants respond to metal exposure is a prime objective in plant research. The main aim of the thesis was to identify the mechanisms by which plants respond to real, complex metal exposure and to soil supplementation with complex waste products (manures, sewage sludge). The designed studies aimed to evaluate the impact of complex metal contamination on the level of abiotic stress, the activity of antioxidative enzymes, genotoxicity, and the expression of selected genes in higher plant species suitable for phytoremediation. The project's practical purpose was mostly focused on proposing new physiological biomarkers as short-term toxicity tests, which will allow more precise planning of the application of waste products into soil remediation processes. The overall goal of the experiments was also to identify the ways in which sewage sludge application is influencing plants on gene expression level (including the expression of metal chelators and metal transporters), to broaden our understanding of the environmental impact and safety of such action. Moreover, since phytoremediation has already entered the stage of modeling, the presented work highlights the main advantages and limitations of existing models, explores their applicability in given circumstances, and proposes a simple model for the prediction of cadmium removal during assisted phytoremediation with sewage sludge.

RESUMÈ

La contamination des sols par des métaux lourds devient un problème en augmentation rapide dans le monde entier et est principalement causée par les activités anthropiques. La plupart des métaux lourds se dégradent lentement et ne peuvent pas être décomposés naturellement, ce qui entraîne leur présence durable dans l'environnement. En conséquence, ils constituent une menace directe pour tous les membres de la chaîne alimentaire. Ainsi, les technologies impliquées dans l'assainissement efficace des zones contaminées gagnent de plus en plus en importance. La phytoremédiation assistée est une technologie largement acceptée pour l'assainissement des métaux lourds du sol. Cela inclut l'utilisation de plantes hyperaccumulables et la fertilisation des sols. Afin d'augmenter l'efficacité de la phytoremédiation assistée et en même temps de faire face à une augmentation constante de la quantité d'eaux usées produites dans le monde, l'épandage de boues d'épuration sur le sol est souvent considéré comme une option viable. Étant donné que de grands volumes de boues d'épuration doivent être éliminés ou traités d'une manière ou d'une autre, leur utilisation comme engrais est devenue une pratique plus courante ces dernières années. Cependant, la sécurité d'une telle action est toujours débattue car les boues d'épuration sont connues pour contenir non seulement de la matière organique et des macronutriments, mais également une vaste gamme de contaminants.

L'identification des mécanismes par lesquels les plantes réagissent à l'exposition aux métaux est un objectif primordial de la recherche sur les plantes. L'objectif principal de la thèse était d'identifier les mécanismes par lesquels les plantes réagissent à une exposition réelle et complexe aux métaux et à la supplémentation du sol avec des déchets complexes (fumiers, boues d'épuration). Les études conçues visaient à évaluer l'impact d'une contamination métallique complexe sur le niveau de stress abiotique, l'activité des enzymes antioxydantes, la génotoxicité et l'expression de gènes sélectionnés dans des espèces végétales supérieures adaptées à la phytoremédiation. L'objectif pratique du projet était principalement de proposer de nouveaux biomarqueurs physiologiques comme tests de toxicité à court terme, ce qui permettra une planification plus précise de l'application des déchets dans les processus d'assainissement des sols. L'objectif général des expériences était également d'identifier les façons dont l'application des boues d'épuration influence les plantes au niveau de l'expression des gènes (y compris l'expression des chélateurs métalliques et des transporteurs de métaux), afin d'élargir

notre compréhension de l'impact environnemental et de la sécurité d'une telle action. De plus, la phytoremédiation étant déjà entrée dans la phase de modélisation, les travaux présentés mettent en évidence les principaux avantages et limites des modèles existants, explore leur applicabilité dans des circonstances données, et propose un modèle simple pour la prédiction de l'élimination du cadmium lors de la phytoremédiation assistée avec des boues d'épuration.

STRESZCZENIE

Zanieczyszczenie gleb metalami ciężkimi staje się szybko rosnącym problemem na całym świecie i jest wywołane przede wszystkim działalnością antropogeniczną. Większość metali ciężkich nie ulega rozkładowi, co powoduje ich długotrwałą obecność w środowisku. W rezultacie stanowią bezpośrednie zagrożenie dla wszystkich członków łańcucha pokarmowego. W związku z tym, coraz większe znaczenie zyskują technologie związane ze rekultywacją skażonych obszarów. Fitoremediacja wspomagana to powszechnie akceptowana technologia oczyszczania gleby z metali ciężkich. Obejmuje stosowanie roślin hiperakumulujących i nawożenie gleby. Aby zwiększyć efektywność takiego procesu, coraz częściej glebę nawozi się substancjami odpadowymi, w tym osadami ściekowymi. Ponieważ rosnące ilości osadów ściekowych muszą być w jakiś zagospodarowane, stosowanie ich jako nawozu stało się w ostatnich latach bardziej powszechną praktyką. Jednak bezpieczeństwo takiego działania jest nadal przedmiotem dyskusji ponieważ oprócz zawartości materii organicznej i makroskładników, osady ściekowe często zawierają także szeroką gamę zanieczyszczeń.

Głównym celem pracy była identyfikacja mechanizmów, za pomocą których rośliny reagują na złożoną ekspozycję na metale oraz na nawożenie gleby złożonymi produktami odpadowymi (w tym: obornikami i osadami ściekowymi). Zaprojektowane badania miały na celu ocenę wpływu zanieczyszczenia metalami na poziom stresu abiotycznego, w tym na: aktywność enzymów antyoksydacyjnych, genotoksyczność oraz ekspresję wybranych genów u gatunków roślin przydatnych do procesu fitoremediacji. Praktyczny cel pracy koncentrował się głównie na zaproponowaniu nowych biomarkerów fizjologicznych jako krótkoterminowych testów toksyczności, które pozwolą na bardziej precyzyjne planowanie stosowania produktów odpadowych w procesach rekultywacji gleby. Ogólnym celem eksperymentów było również zidentyfikowanie sposobów, w jakie aplikacja osadów ściekowych wpływa na rośliny na poziomie ekspresji genów (w tym ekspresji chelatorów i transporterów metali), aby poszerzyć naszą wiedzę na temat bezpieczeństwa takiego działania. Ponadto, ponieważ fitoremediacja weszła już w fazę modelowania, w przedstawionej pracy zwrócono uwagę na główne zalety i ograniczenia istniejących modeli, zbadano ich przydatność w danych okolicznościach oraz zaproponowano prosty model do prognozowania usuwania kadmu ze skażonej gleby podczas prowadzenia procesu fitoremediacji z użyciem osadów ściekowych jako nawozu.

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2. Jaskulak, M., Rorat, A., & Grobelak, A. (2019). Enzymatic assays confirm the toxicity reduction after manure treatment of heavy metals contaminated soil. South African Journal of Botany, 124, 47–53. doi:10.1016/j.sajb.2019.04.035

3. Jaskulak, M., Rorat, A., Grobelak, A., & Kacprzak, M. (2018). Antioxidative enzymes and expression of *rbcL* gene as tools to monitor heavy metal-related stress in plants. Journal of Environmental Management, 218, 71–78. doi:10.1016/j.jenvman.2018.04.052

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4. Jaskulak, M. (2018). Implementation of Omics Research to Enhance Phytoremediation Efficiency - a Review. Engineering and Protection of Environment, 21(4), 361-373. doi:10.17512/ios.2018.4.4

5. Jaskulak, M., Rorat, A., Grobelak, A., Chaabene, Z., Kacprzak, M., & Vandenbulcke, F. (2019). Bioaccumulation, antioxidative response, and metallothionein expression in *Lupinus luteus* L. exposed to heavy metals and silver nanoparticles. Environmental Science and Pollution Research. doi:10.1007/s11356-019-04972-y

6. Jaskulak, M., Grobelak, A., Grosser, A., & Vandenbulcke, F. (2019). Gene expression, DNA damage and other stress markers in *Sinapis alba* L. exposed to heavy metals with special reference to sewage sludge application on contaminated

sites. Ecotoxicology and Environmental Safety, 181, 508–517. doi:10.1016/j.ecoenv.2019.06.025

7. Jaskulak, M., Grobelak, A., & Vandenbulcke, F. (2020). Effects of sewage sludge supplementation on heavy metal accumulation and the expression of ABC transporters in *Sinapis alba* L. during assisted phytoremediation of contaminated sites. Ecotoxicology and Environmental Safety, 197, 110606. doi:10.1016/j.ecoenv.2020.110606

CHAPTER IV

8. Jaskulak, M., Grobelak, A., & Vandenbulcke, F. (2020). Modelling assisted phytoremediation of soils contaminated with heavy metals – main opportunities, limitations, decision making and future prospects. Chemosphere, 126196. doi:10.1016/j.chemosphere.2020.126196

9. Jaskulak, M., Grobelak, A., Vandenbulcke, F. (2020). Modeling and optimizing the removal of cadmium by *Sinapis alba* L. from contaminated soil via Response Surface Methodology and Artificial Neural Networks during assisted phytoremediation with sewage sludge. International Journal of Phytoremediation, 22(12), 1321-1330. doi:10.1080/15226514.2020.1768513

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Figure 3. Protein concentration. PS – control soil, CS – soil contaminated by HM from metallurgical industry, Zn360, Zn720, Ni360, Ni720, Pb360, Pb720, Cd360, Cd720 – organic peat soil contaminated artificially with zinc, nickel, lead or cadmium in two doses - 360 mg/kg of dry weight and 720 mg/kg of dry weight; $AgNO_3$ – organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nitrate, AgNP - organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nanoparticles, AgDis - organic peat soil contaminated artificially with dispersant without nanoparticles. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$)

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LIST OF ABREVIATIONS

18S	18S ribosomal RNA (abbreviated 18S rRNA, protein)
18S	gene encoding 18S ribosomal RNA (abbreviated 18S rRNA)
AAD	Average Absolute Deviation
ABA	Abscisic acid
ABC	ATP-binding cassette transporter (protein)
ABC	Gene encoding ATP-binding cassette transporter
Act/ACT	Actin (protein)
act/Act/ACT	Gene encoding actin
Ag	Silver
AgCl	Silver chloride
$AgNO_3$	Silver nitrate
AgNP/AgNPs	Silver nanoparticles
ANN	Artificial Neural Networks
ANOVA	Analysis of Variance
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BBD	Box–Behnken design
BCF	Bioconcentration factor
BLAST	Basic Local Alignment Search Tool
BOD	Biodegradable Organic Matter
bp – base pares	Base pares
BSA	Bovine Albumin Serum
β-tub	β-tubulin (protein)
β-tub	Gene encoding β-tubulin
C	Carbon
Ca	Calcium
$CaCl_2$	Calcium chloride
CAD	Cinnamyl-alcohol dehydrogenase
CAT	Catalase (protein)
cat	Gene encoding catalase
Cd	Cadmium

CdMT	Cadmium metalotionein (protein)
cdmt	Gene encoding cadmium metalotionein
cDNA	Complementary DNA
CEC	Cation Exchange Capacity
CESA	Plant Cellulose Synthase
Chl	Chlorophyll
cm	Centimeter
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
Ct	Chromium
CTSPAC	Model for coupled transport of water, heat and solutes in the soil-plant-atmosphere continuum
Cu	Copper
CW	Cell Wall
Dis	Dispersant
DNA	Deoxyribonucleic acid
DSS	Decision Support System
EDTA	Ethylenediaminetetraacetic acid
Ef /EF	Elongation factor (protein)
Ef	Gene encoding elongation factor
EPA	Environmental Protection Agency
EtBr	Ethidium bromide
Fe	Iron
FFBPNNS	Feed-forward backpropagation multilayer perceptron
g	Gram
GA	Gallic acid
GAE	Gallic acid equivalent
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPX	Guaiacol peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HKG/HGKs	House-keeping gene/House-keeping genes
HM/HMs	Heavy metal/heavy metals

ICP-OES	Inductively coupled plasma atomic emission spectroscopy
IRT	Iron regulated transporter (protein)
ISO	International Organization for Standardization
JRC	Joint Research Centre
K	Potassium
KCl	Potassium chloride
kDA	kilodaltons
kg	Kilogram
LMA	Low-melting-point agarose
Lx	Lux
M	Mole
mA - Milliampere	Milliampere
MAE	Mean Absolute Error
MANE	Mean Normalized Average Error
MDHA	Monodehydroascorbate
ME	Model Efficiency
mg	Milligram
$MgCl_2$	Magnesium chloride
Min	minute
miRNA	microRNA (abbreviated miRNA)
mL	Milliliter
mm	Millimeter
mM	Millimole
mm	micrometer
Mn	Manganese
mRNA	Messenger RNA
MSAE	methionine, S-adenosylmethionine, 1-aminocyclopropane-1-carboxylic acid, ethylene pathway
MSE	Mean Square Error
MT/Mt	Metallothionein (protein)
Mt/mt	Gene encoding metallothionein
N	Nitrogen
NaOH	Sodium hydroxide

NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencing
Ni	Nickel
nm	Nanometer
nM	Nanomole
NMA	Normal melting-point Agarose
NPK	Nitrogen-Phosphorus-Potassium
NPs	Nanoparticles
OECD	Organization for Economic Co-operation and Development
P	Potassium
PAE	Pectinase
PAHs	Polycyclic aromatic hydrocarbon
PAL	Phenylalanine ammonia lyase
Pb	Lead
PCR	Polymerase Chain Reaction
PCS	Phytochelatin (protein)
Pcs/pcs	Gene encoding phytochelatin
PG	Polygalacturonase
PGPR	Plant growth-promoting rhizobacteria
pH	potential of hydrogen
PME	Pectin methylesterase
POD	Peroxidase
ppb	Parts per billion
ppm	Parts per million
PPO	Polyphenol oxidase
PSI	Photosystem I
PSII	Photosystem II
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
rbcL	Ribulose bisphosphate carboxylase large chain (protein)
rbcL	Gene encoding Ribulose bisphosphate carboxylase large chain
RDX	Hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine
RMSE	Root Mean Square Error
RNA	Ribonucleic acid

ROS	Reactive Oxidative Species
RSM	Response Surface Methodology
RuBiSCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
s	Second
SCGE	Single Cell Gel Electrophoresis
SDA	System dynamic approach
SDH	Succinate dehydrogenase
SO_2	Sulphur dioxide
SOD	Superoxide dismutase
SS	Sewage Sludge
TBP	TATA-box-binding protein
TCE	Trichloroethylene
TM	Tail moment
TOC	Total organic carbon
TON	Total organic nitrogen
TPC	Total phenolic compounds
Tris	Tris(hydroxymethyl)aminomethane
Tub6	Tubulin6 (protein)
Tub6/tub6	Gene encoding Tubulin6
U	Unit
µg	Microgram
µl	Microliter
µm	Micrometer
UV-B	Ultraviolet B-rays
WRB	World Reference Base
WWTP	Waste Water Treatment Plants
XTH	Xyloglucan
Zn	Zinc
ZRT	Zinc transporter proteins
µA	Microamper

CHAPTER I

General introduction

1.1 Soil contamination with heavy metals & phytoremediation

Pollution of soil with heavy metals becomes a fast-rising issue in many places due to a vast scope of anthropogenic activities (Pant et al., 2014). The major contribution to this problem is often divided into two main sources. The first one includes industrial activities such as mining and manufacturing. The second one is connected to modern agriculture (Tóth et al., 2016).

Most heavy metals (HMs) degrade slowly and cannot be decomposed naturally, resulting in their long-lasting presence in the environment. As a result, they can seep into the deeper layers of the soil and pose threat to groundwater reservoirs (Motuzova et al., 2014; Pant et al., 2014). In addition, such soil pollution can increase oxidative stress in plants which can slowly and gradually decrease plant growth causing a significant decrease in photosynthesis efficiency (Chabukdhara et al., 2013). Overall, metal contamination of soil and water creates a direct obstruct in normal growth of plants reducing crop yields (Bolan et al., 2014).

Continually increasing soil contamination with heavy metals affects all links in the food chain - from soil microorganisms through plants, animals, and humans. Heavy metals are one of the most prevalent abiotic stress factors affecting the growth, development, and productivity of plants, and are a direct threat not only to the soil environment but also groundwater and human health, causing damage by oxidative stress and inducing cancer in cells (Mahar et al., 2016). High availability and toxicity of heavy metals in the soil, sometimes lasting over a few hundred years, makes it impossible to use contaminated areas for agricultural purposes (Liu et al., 2014). Accumulation of metals such as Lead (Pb), Chromium (Cr), Nickel (Ni) can seep through food chain and cause DNA damage via their mutagenic properties which can lead to carcinogenic changes in bodies of animals and humans.

Since long-term persistence of heavy metals in the environment causes a threat to human health and crop production, technologies involved in effective remediation of contaminated areas are increasingly gaining importance (Pant et al., 2014; Tóth et al., 2016). Moreover, due to the growing health concerns associated with heavy metals and their broad distribution in soil and water, more attention has been directed to identification

of the sources of such pollution and the remediation of contaminated sites (Duan et al., 2017). The use of physical and chemical methods to deal with heavy metal contaminated areas includes the use of ion exchange, reverse osmosis, chemical reduction, precipitation and evaporation (Gong et al., 2018). Modern techniques that allow for a reduction in the concentrations of hazardous substances include processes that promote the desorption and subsequent removal in the liquid phase. Such actions include soil washing techniques, as well as electrokinetic methods and heat treatments (Habibul et al., 2019). All of them can be successfully applied to the process of decontamination, but they require a lot of external resources and in most cases are too expensive for large-scale use (Sharma et al., 2014).

Due to those reasons, in recent decades, more and more attention has been given to phytoremediation, in which plants absorb and transform contaminants in order to detoxify the site and clean-up polluted environments (Yadava et al., 2018). It is worth mentioning that phytoremediation can also be applied to other contaminants - not solely to heavy metals but also to pesticides, explosives and crude oil. Overall, phytoremediation can takes advantage of the natural processes and requires less equipment and labor than other technologies (Burges et al., 2017, Jaskulak et al., 2019). In general, phytoremediation in accepted as a sufficient and cost-effective approach to clean up metal-contaminated soils.

Assisted phytoremediation is a wildly accepted technology used in remediation of heavy metals by use of hyperaccumulating plants and soil fertilization. Hyperaccumulators are able to store extremely high levels of heavy metals in their above-ground tissues without suffering its toxic effects. Such plants are characterized by their remarkable biochemical mechanisms which allow them to accumulate and translocate metals in their cells. Therefore, they show promise for the use in large-scale phytoremediation (Cortés-Eslava et al., 2018). **Understanding the molecular mechanisms of hyperaccumulation may, consequently, help in enhancing the performance of hyperaccumulators for phytoremediation.** During the process of phytoremediation, hyperaccumulators extract and accumulate heavy metals from soils, which is followed by harvesting biomass until the concentration of specific contaminant decreases to acceptable level (Chowdhary et al., 2018).

The identification of mechanisms by which plant respond to metal exposure is thus, a prime objective in plant research (Hattab et al., 2015). However, genes involved in the protection against metal toxicity and the molecular mechanisms underlying this protection are still largely undefined in plants. Especially in species other than model plants. Thus, understanding the gene expression and its regulation involved in metal toxicity is essential for understanding the genetic and molecular mechanisms involved in effective phytoremediation (Cortés-Eslava et al., 2018). To this day over 450 heavy metal accumulators have been identified, but overwhelmingly (80%) of them are tolerant to nickel. Hyperaccumulation of cadmium was noticed in two plant families: *Brassicaceae* and *Crassulaceae*. Hence, a number of physiological studies had been carried out to understand the hyper-tolerance and its mechanisms within these families. The development of high-throughput deep sequencing technology has enabled the large-scale RNA-seq of dynamic transcriptomes, even without fully sequenced reference genomes (Chowdhary et al., 2018).

1.2 Soil fertilization with sewage sludge and other waste products

In order to increase the efficiency of the assisted phytoremediation and at the same time deal with a constant rise in the quantity of produced wastewater worldwide, the application of sewage sludge to land is often seen as a viable option (Alvarenga et al., 2016). Since large volumes of sewage sludge have to be disposed or treated in some manner, the use of it as a fertilizer has become a more common practice in recent years. It was shown before that this kind of action improves soil properties and increases crop productivity (Bourioug et al., 2015). Beneficial application of organic waste like sewage sludge or animal manure in agricultural soils can allow to maintain and restore the quality of previously degraded soils, as well as reduce the need for application of synthetic fertilizers (Chowdhary et al., 2018). In recent decades, more and more lands have been chronically suffering from a severe decrease in organic matter caused mostly by overexploitation, and droughts (Peng et al., 2016). Therefore, the reuse of biosolids like manures and sewage sludge is proposed as an alternative solution for improvement in organic matter content and soil quality at low costs (Saveyn et al., 2014). In the past 20 years, the idea of reusing nutrients by applying sewage sludge to agricultural soil has become a more common practice and it is currently considered as a leading alternative to other disposal methods such as incineration or storing and processing at landfills. The application of organic waste such as sewage sludge as soil amendments is also a crucial strategy to the "end of waste" policy in Europe, that will contribute to an increase in the levels of soil organic matter content that tends to lower across almost all Europe mostly as a result of overproduction, industrialization, contamination with heavy metals (HMs) and droughts (Chowdhary et al., 2018).

On the contrary, the idea of using sewage sludge as a fertilizer has also a couple of important drawbacks that must be taken into consideration. Inadequately processed sewage sludge can have a wide variety of undesired traits and in consequence, adverse effects on given environment (Saveyn et al., 2014). Due to this risk, it is crucial to gather knowledge about reducing the hazards associated with using organic wastes such as sewage sludge as a soil amendments (Alvarenga et al., 2016). **Thus, further knowledge on biochemical and physiological responses of plants to stress helps develop new strategies for purification of contaminated areas and overall improvement of the environment.**

1.3 Aims of the work

The main aim of the thesis concerns the identification of mechanisms by which plants respond to metal exposure and to soil supplementation with waste products (manures, sewage sludge). The designed studies aimed to evaluate the impact of complex metal contamination, on the level of abiotic stress, activity of antioxidative enzymes, efficiency of photosynthesis, genotoxicity and expression of selected genes in higher plants and mutual correlation of these variables.

The practical purpose of the project was focused mostly on proposing new physiological biomarkers as short-term toxicity tests which will allow for a more precise application of waste products into soil during remediation processes. The overall goal of the project is also to identify the ways in which sewage sludge application is influencing plants on gene expression level (including metal chelators and transporters), to broaden our understanding on the environmental impact and safety of such supplementation of both agricultural and degraded soil (Chapters 2 & 3).

Moreover, since phytoremediation, despite still being a novel technology, has already entered the stage of modeling, the presented work highlights the main advantages, and limitations of existing models, to explore their applicability in given circumstances and proposes a simple model for the prediction of cadmium removal during assisted phytoremediation with sewage sludge supplementation (Chapter 4).

Current research gaps:

1. The need to identify and validate the usability of new biomarkers with high sensitivity and selectivity in order to increase the efficiency of the assisted phytoremediation process and precisely determine the degree of risk resulting from the presence of contaminants;

2. Lack of known sequences coding key genes associated with heavy metal detoxification processes in plants exposed in natural conditions with complex contamination;

3. Lack of information on potential reference genes for plants other than model species, especially in the case of complex contamination of natural soil;

4. Scarce information on the impact of soil additives, including sewage sludge, on changes in the level of gene expression in plants;

Specific goals:

1. Identification of key metal responsive genes in species other than model plants and important for the remediation purpose;

2. Assessment of the sensitivity of selected stress markers, along with the identification of the genes encoding them in higher plants;

3. Creation of an information gateway about metal detoxification and defense pathways, which will help to develop strategies for environmental clean-up and rehabilitation of contaminated soils;

4. Assessment of the impact of soil supplementation with sewage sludge and manures on genotoxicity and expression of selected genes in higher plants and the mutual correlation of these variables;

1.4 Research hypotheses:

1. Heavy metal contamination can affect plants on a cellular and transcriptomic level. Some biological parameters that vary in response to changing environmental conditions during the assisted phytoremediation can be considered as sensitive biomarkers:

    a. the level of activity of antioxidative enzymes;
    b. the state of the photosystems (the content of different fractions of chlorophyll, expression of genes encoding RuBiSCO protein);
    c. the expression level of genes encoding metal chelators and transporters (metallothioneins, phytochelatins, ABC transporters);
    d. the level of DNA damage;

2. Some of those biomarkers are sensitive enough that their measurement after a short time can be used as a tool for the prediction of phytoremediation efficiency, as well as for the choice of the dose of fertilizer required to ensure the plant survival on the contaminated site.

3. Soil supplementation with sewage sludge causes changes in plants gene expression involved in detoxification of heavy metals and other metabolic pathways;

4. **Such differentially expressed genes can be used to quickly and accurately assess the long-term effects and overall, the efficiency of large-scale phytoremediation processes via qRT-PCR;**

5. **Identification of mechanisms in which sewage sludge ich influencing specific gene expression in plants can be used to assess potential risks or safety of soil supplementation with sewage sludge;**

CHAPTER II

Biomarkers of abiotic stress as tools for planning and monitoring of phytoremediation efficiency

Heavy metals have been broadly spread into our environment due to mining, smelting, the use of synthetic fertilizers, and various other industrial activities. The impact of heavy metals on plants is a significant environmental concern.

The first publication in this chapter is a critical overview of the changes that occur in plant physiology during exposure to one of the most toxic heavy metals – cadmium. The primary objective was to showcase potential markers of metal toxicity, which will be used in the experimental work. Performed review showcased that research concerning the use of specific plant biomarkers for cadmium toxicity is still in an initial stage. Moreover, to this date, research on plant biomarkers mainly consisted of short-term exposures, usually in *in vitro* conditions on model species, which are often not suitable for phytoremediation.

The second publication presents the results of the first experiment where the chosen biomarkers were tested for their usefulness during the phytoremediation of metal contaminated soil on two plant species relevant for phytoremediation – *Sinapis alba* (a member of *Brassicaceae*), and *Robinia pseudoacacia* (a member of *Fabaceae*). The study showed that the standard toxicity tests are not always suitable for plant species other than model species, whereas the changes in the activity of guaiacol peroxidase could differentiate the changes caused by a 5% change in the dose of animal manure by which the contaminated soil was fertilized.

The third publication presents the second experiment, which added to the first one. This time three species relevant for phytoremediation were chosen – *Sinapis alba*, *Robinia pseudoacacia*, and *Lupinus luteus*. The aim of the study was to evaluate the sensitivity and potential applications of selected biomarkers (guaiacol peroxidase activity, chlorophyll and protein content, the amount of total phenolic compounds, and level of expression of one of the ribulose-bisphosphate carboxylase genes - *rbcL*), as potential tools an effective phytoremediation management. Moreover, the influence of organic additives: cattle, horse manure, and vermicompost on lowering plant abiotic stress caused by complex heavy metal contamination was studied to assess the possible use of selected stress markers in planning large-scale phytoremediation.

In this part, results are presented in the following three publications:

1. Jaskulak, M., & Grobelak, A. (2019). Cadmium Phytotoxicity — Biomarkers. Cadmium Tolerance in Plants, 177–191. doi:10.1016/b978-0-12-815794-7.00006-0

2. Jaskulak, M., Rorat, A., & Grobelak, A. (2019). Enzymatic assays confirm the toxicity reduction after manure treatment of heavy metals contaminated soil. South African Journal of Botany, 124, 47–53. doi:10.1016/j.sajb.2019.04.035

3. Jaskulak, M., Rorat, A., Grobelak, A., & Kacprzak, M. (2018). Antioxidative enzymes and expression of rbcL gene as tools to monitor heavy metal-related stress in plants. Journal of Environmental Management, 218, 71–78. doi:10.1016/j.jenvman.2018.04.052

Cadmium phytotoxicity – biomarkers*

Marta Jaskulak[1], Anna Grobelak[1]

[1] Czestochowa University of Technology, Faculty of Infrastructure and Environment, Department of Environmental Engineering, Czestochowa, Poland

Abstract

Cadmium has been broadly spread into the environment due to mining and smelting, application of phosphate fertilizers and its various industrial uses. The accumulation of cadmium in plants is an important environmental concern. There are many standard cadmium phytotoxicity biomarkers, mostly connected with germination rate, embryo growth and distribution of biomass, as well as of the activities of specific enzymes. Some studies on plants have demonstrated the presence of specific proteins - metallothioneins (MTs) as potential biomarkers, but the physiological roles of MTs are not completely understood. Recent studies are focusing on the on the identification and expression analysis of *mts* and *pcs* (phytochelatin) genes as a potential tool in the applicability of phytoremediation process. The challenge is identification and determination of expression level for metallothioneins and phytochelatins as potential toxicity biomarkers. Understanding the reaction of plants to Cd stress and applying appropriate strategies may reduce the Cd toxicity for plants and improve remediation efficiency on contaminated soil.

KEYWORDS: cadmium, metallothioneins, phytochelatins, phytotoxicity biomarkers

[*] *Jaskulak, M., & Grobelak, A. (2019). Cadmium Phytotoxicity — Biomarkers. Cadmium Tolerance in Plants, 177–191. doi:10.1016/b978-0-12-815794-7.00006-0*

1. PHYTOTOXICITY OF CADMIUM

Cadmium is commonly recognized as one of the most hazardous environmental contaminants which can affect the growth and development of plants at all levels of biological organization – including subcellular and even up to ecosystem level (Qadir et al. 2014). The interaction between cadmium ions and cellular components, initiates in a matter of seconds with a vast number of metabolic responses and the generation of reactive oxygen species (Lukacová et al. 2013). These disturbances can lead to permanent disruptions in plants development (Girish et al. 2014). Research at various molecular biomarkers can improve our understanding of toxic actions and exposure assessments as long as those markers will be directly linked to visible damage at the high level of organization – including changes in plants biomass (Konlechner et al. 2013). Despite consistently growing information on biomarkers, the relationships and quantitive sensitivity between those endpoints and overall plants development are receiving only little attention (Gichner et al. 2008). It is worth mentioning that cadmium element does not have any known metabolic significance to living organisms (Verma et al. 2008). It is commonly used in different branches of industry, including the production of pigments, batteries, stabilizers, and electroplating (Smith 2009). In soils, the use of phosphate fertilizers has been found to contain high levels of cadmium (Gove et al. 2001).

One of the essential traits of cadmium is its extremely long biological half-life – over 30 years, which makes Cd a cumulative contaminant and to this date there is no treatment for chronic cadmium toxicity in living organisms (Placek et al. 2016). In consequence, cadmium contamination of the environment is seen as a serious threat (Lukacová et al. 2013, Qadir et al. 2014, Placek et al. 2016). In plants, Cd is relatively easy up-taken and transported within the tissues. The increase in the bioavailability of Cd decreases plant biomass mostly due to the inhibition of photosynthesis (Grobelak et al. 2015). After cadmium is taken up through plant roots it is accumulated in all plants parts including fruits, shoots, roots, and grain (Verma et al. 2008). The accumulation of cadmium through the trophic levels of food chain constitutes a risk for animals and humans (Bolan et al. 2013). The contamination and level of phytotoxicity of soils contaminated by cadmium are usually established by chemical analysis and germination tests (Girish et al. 2014).

The mechanisms of cadmium phytotoxicity like the interference with metabolic processes in plants must be considered in order to properly asses the severity of contamination (Dong et al. 2006). The commonly available dose-response relationships in plants bioassays for the assessment of contaminated soils are based on only visual symptoms such as necrosis, chlorosis, the reduction of biomass, discoloration or leaf epinasty (Bolan et al. 2013). However, this kind of effects are occurring only at a high level of phytotoxicity and thus are insufficient to evaluate the soil quality (Dias et al. 2013). A better understanding of the Cd related symptoms needs to include more sensitive parameters such as markers that reflect cellular metabolic compounds and both, physiological and biochemical state of the plant (Qadir et al. 2014).

Overall, cadmium directly or indirectly inhibits crucial metabolic processes such as photosynthesis, respiration, water relation or gas exchange and can be also accumulated in chloroplasts (Balen et al. 2011). The photosynthesis is inhibited at several levels: the biosynthesis of chlorophyll, CO_2- fixation, transport of electron, stomatal conductance or enzymes of Calvin cycle (Siliang et al. 2013). These changes in the metabolism can be observed quicker and at low levels of cadmium contamination before the visual symptoms become evident (Gill et al. 2012, Siliang et al. 2013). The enzyme activity has been used in several research as a first diagnostic criteria to evaluate the phytotoxicity of soils contaminated by metals since one of the main toxic effects of trace metals is a generation of free radicals and oxidative stress linked with peroxidation of cellular membranes (Farinati et al. 2010).

2. PLANTS RESPONSE TO CADMIUM EXPOUSURE

Most of the time, cadmium enters the plant tissues due to soil contamination through roots and consequently, roots are most likely to experience damage and Cd toxicity (Cherif et al. 2011). In previous studies concerning Cd contamination of soils, roots of *Allium cepa* had damaged nucleoli, and in *Oryza* Cd changed the synthesis of RNA and inhibited the activity of ribonucleases (Benavides et al. 2005). Moreover, cadmium in well-known to reduce the nitrate absorption and thus its transport to different tissues (Farinati et al. 2010). Overall, the inhibition of plant growth is mostly caused by the suppression effects of the elongation cells (Farinati et al. 2010, Gill et al. 2012).

In another study, the Cd was shown to interact with plants water balance and damage photosynthetic apparatus, specifically the light-harvesting complex II (Hasan et al. 2011). In *Brassica napus*, Cd contamination lowered the content of chlorophyll, carotenoids and inhibited the oxidative mitochondrial phosphorylation (Tsakou et al. 2003). It was also shown to reduce the regular proton exchange and the activity of several crucial for plants development enzymes including glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, malic enzyme, RuBiSCO enzymes and carbonic anhydrase (Tsakou et al. 2003, Hasan et al. 2011).

In Dixit et al. 2011 studies, the embryos od pea plants, had significant chromatin abbreviations. It is hypothesized that the contamination of Cd can replace Zn ions and this is the reason for the interference with transcription mechanisms after Cd exposure (Dixit et al. 2011, Sawidis 2008). Cd like other metal ions induces oxidative stress in cells, and can cause inhibition or stimulation of various antioxidative enzymes depending on its dose and other environmental factors (Polle et al. 2003).

It was shown before that in leaves of *Asteraceae* plants, cadmium enhanced the activity of lipoxygenase and lipid peroxidation at the same time decreasing the activity of antioxidative enzymes including catalase, peroxidases, glutathione reductase, superoxide dismutase (Yadav 2010). On the other hand, a higher dose of Cd in *Phaseolus* caused a significant increase in the activity of guaiacol and ascorbate peroxidase (Dat et al. 2000). Such variation between plant responses are related both to the dose of Cd and the amount of thiolic groups both present before or induced after the cadmium exposure (Astolfi et

al. 2011). Thiols are well known for their strong antioxidative properties able to counteract oxidative stress (Dixit et al. 2011). Overall, the general response to Cd stress can be divided into 6 main groups of plants responses: (1) immobilization and/or exclusion, (2) synthesis of phytochelatins and metallothioneins; (3) induction of antioxidative enzymes; (4) compartmentalization, (5) synthesis of other stress-related proteins (6) production of ethylene (DalCorso et al. 2008).

2.1. Immobilization and exclusion

Immobilization is the first barrier against toxicity of metal ions including cadmium. It occurs mostly on a root level (Dheri et al. 2007). Exclusion generally means all the mechanisms that can prevent Cd ions from entering the cytosol through the membranes (Kranner et al. 2011). Cd can be immobilized by means of the cell wall or for example extracellular carbohydrates (Fusconi et al 2006). The importance of this processes varies significantly among different concentrations of Cd, plant species and other environmental factors involved (Fusconi et al. 2006, Dheri et al. 2007). In early stages of seed germination in several species, the entrance of Cd to cells was noticed through the Ca channels in the cell membrane (Kranner et al. 2011).

2.2. Compartmentalization

Compartmentalization in vacuoles plays one of the crucial roles in Cd detoxification and tolerance by preventing further free circulation of metal ions in cell cytosol (Lang et al. 2011). Cd exposure causes a rapid synthesis of phytochelatins and metallothionein which in their way create complexes with Cd ions and are successfully transported to the vacuole (Bolan et al. 2013). After that, due to the acidic pH in cells vacuole, formed complex dissociates, and metal can be further complexed by organic acids and possibly amino acids (Dias et al. 2013). Hydrolases can degrade used phytochelatins in vacuoles, or they can return to the cytosol to continue with their role (Clemens et al. 2006).

2.3. Stress ethylene

Exposure to cadmium can stimulate the ethylene biosynthesis mostly via MSAE pathway (methionine, S-adenosylmethionine, 1-aminocyclopropane-1-carboxylic acid, ethylene) (Iqbal et al. 2015). Production of ethylene causes an increase in the activity of some

antioxidant enzymes including guaiacol peroxidase and greater accumulation of insoluble and soluble phenolics (Masood et al 2012). In most species, the stimulation of ethylene production after Cd exposure peaks within only 5-10 hours after the initial exposure and declines after that (Iqbal et al. 2015). It is the hypothesis that in *in vitro* conditions the decrease in ethylene production is related to Cd-Sequestration and thus diminished stress. Due to the scarcity of available data about this response, it is impossible to assess at a molecular and cellular level the interactions between synthesis of ethylene and Cd stress (Masood et al 2012, Iqbal et al. 2015).

3. TOXICITY BIOMARKERS

Several processes including germination rate, growth, mortality and biochemical response have the potential to be used for quality evaluation of terrestrial environment (Sawidis 2008). Generally, the adverse effects of soil metal contamination on plants are assessed based on only chemical soil analyses (Dong et al. 2006). However, it needs to be taken into consideration that the bioavailability of metals depends on several different parameters like the content of organic matter, pH, carbon exchange capacity (Dheri et al. 2007, Placek et al. 2016). Since in most cases, soils are contaminated by a mixture of different metals – each of them can be phytotoxic in a different way or can interact with other contaminants (Cherif et al. 2011).

Biomarkers are used as an early warning for biotic or abiotic stress. Since 1994, biomarkers are defined as any physiological biochemical or morphological parameter used to quantify the exposure to stress (Dheri et al. 2007). Toxicity tests are the most frequently used tools for the assessment of the effects of soil contamination on plants growth and development (Aravind et al. 2009). Among them, the most famous test for acute toxicity on terrestrial plants is the seed germination test (Sawidis 2008). However, this test although sensitive to model plants can be somewhat insensitive to many contaminants including heavy metals (Cabral et al. 2018). Moreover, plant species other than model, including *Fabaceae* plants show that germination test is also insensitive among them since the metal uptake in seedlings is sharply limited due to the nutrients present in seed reserves (Malinowski et al. 2005). Second most wildly performed toxicity test in terrestrial plants is root elongation test which is often performed in *in vitro* conditions or with soil solutions and not as direct exposure (Aravind et al. 2009). Both, germination index and root elongation also face the same problem that the first stages of seedlings development, due to the content of micro and macronutrients in seeds is actually the most independent from the environment (Sawidis 2008, Aravind et al. 2009).

The effects of exposure to heavy metals are divided into acute or chronic toxicity (Cabral et al. 2018). Biomass, the number of leaves, shoot length, and other parameters that can be measured during the growth in controlled conditions of growth chamber must be long enough so its effects will be measurable, therefore limiting the advantages of using such tests as diagnostic tools (Cherif et al. 2011).

4. BIOMARKERS SPECIFIC FOR METAL STRESS

4.1. Phytochelatins (PCs)

Pollution by heavy metals is often considered as a critical threat to soil and water resources and by extension to human health (Hirata et al. 2005). Chelation and metal sequestration by specific ligands are the key mechanisms in plants to deal with toxic effects of metal ions (Guo et al. 2008). Today, two metal binding ligands are best characterized – phytochelatins and metallothioneins (Gonzalez-Mendoza et al. 2007). In most plant species the exposure to high level of metals induced the production of phytochelatins – metal binding, cysteine-rich polypeptides that are thought to play a central role in metal homeostasis in plants (Yadav 2010). Their general structure is (γ-Glu-Cys) (n = 2–11), the n ranges from 2 to 11 but typically is no more than 5 (Hirata et al. 2005). Previous studies also indicate their involvement in mechanisms of metal tolerance (Malinowski et al. 2005). Their synthesis is catalyzed by phytochelatin synthase (Yadav 2010). After the exposure to heavy metals including cadmium, PCs forms complexes with metal ions in the cytosol and transports them into the vacuole protecting plant from further toxic effects of heavy metal (Figure 1.) (Cobbett et al. 2000). Many previous biochemical, physiological and genetic studies have already confirmed that glutathione (GSH) is the substrate for PCs biosynthesis (Figure 2.) (Almaroai et al. 2013). In those studies, a vast variety of plant species was tested, mostly in *in vitro* cell cultures (Clemens et al. 2006). Early studies of this subject have demonstrated that the synthesis of PCs after the exposure to cadmium coincided with a significant decrease in glutathione levels (Cobbett et al. 2000). Further studies demonstrated that once more sibce after the exposure to a glutathione inhibitor (in this case buthionine sulfoximine) plants showed increased sensitivity to cadmium and inhibition of PC biosynthesis (Almaroai et al. 2013).

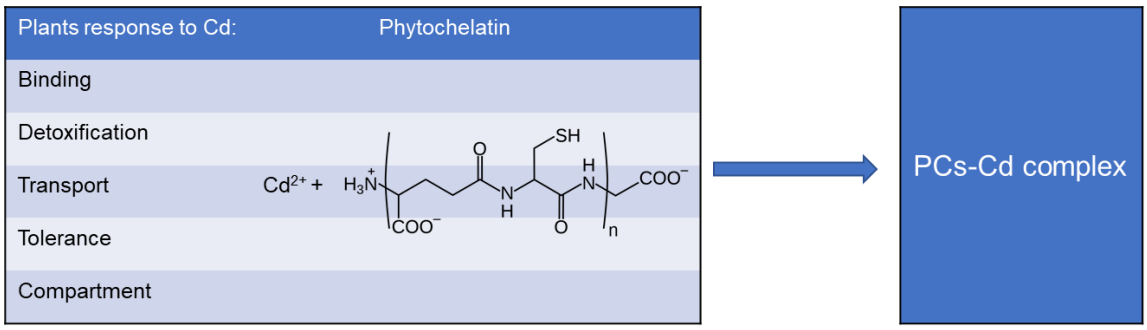

Figure 1. Functions of phytochelatins in plants (Cobbett et al. 2000, Clemens et al. 2006, Almaroai et al. 2013).

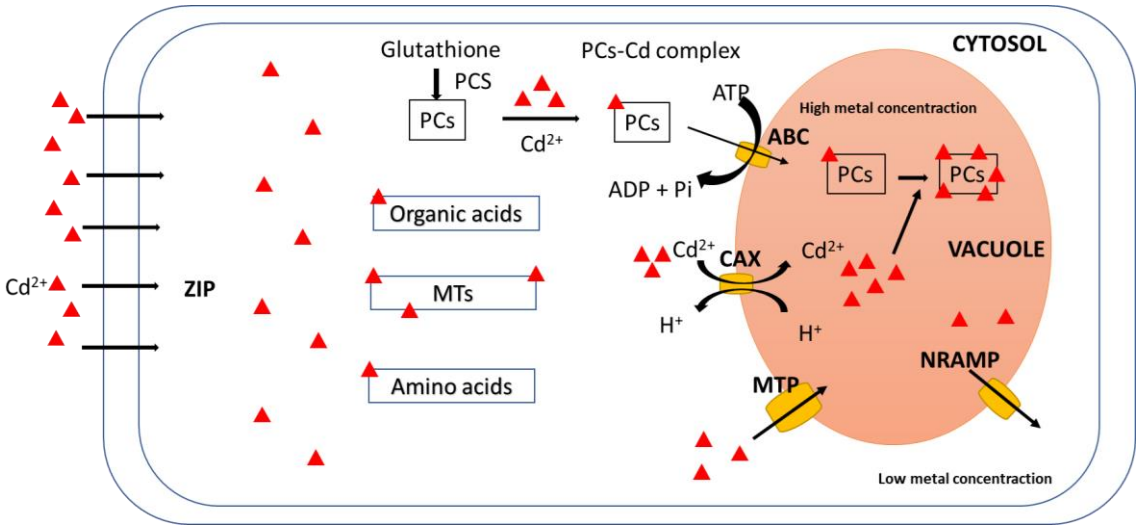

Figure 2. Sequestration of cadmium in plants cells. ZIP, ABC, CAX, NRAMP – metal transporters, PCs – phytochelatins, MTs – metallothioneins (Guo et al. 2008, Yadav 2010).

4.2. Metallothioneins (MTs)

Metallothioneins like previously mentioned phytochelatins are cysteine-rich metal chelators (Zimeri et al. 2005). Unlike PCs, MTs were initially discovered in animals in 1957 due to their ability to protect cells against the toxicity of cadmium (Gonzalez-Mendoza et al. 2007). MTs are small proteins that usually contain between 45 and 85 amino acids (Ren et al. 2012). In plants, they were discovered for the first time over 30 years ago in wheat embryos (Zimeri et al. 2005). Similarly to PCs they are synthesized from glutathione (Guo et al. 2005). Overall, their roles in plants metabolism are still being determined but most importantly, they are thought to sequester large amounts of different metal ions (Figure 2) (Cabral et al. 2018). To this date, MTs have been reported to play a part in processes like the regulation of cell growth, proliferation, scavenging of ROS and DNA damage repair (Ghoshal et al. 2013).

MTs can bind to different metal ions by formatting mercaptide bonds between their cysteine residues and the metal (Ren et al. 2012). Structurally, MTs are grouped into four main groups depending on their Cys arrangement (Ghoshal et al. 2013). Although most

of the time they are expressed throughout the plant, new research shows that in some cases they can be expressed only in specific tissues (Guo et al. 2005). The specific metals sequestered by MTs also vary for different structural types of metallothioneins (Gonzalez-Mendoza et al. 2007). It is worth mentioning, that not all structural types of metallothioneins exist among all plants taxonomical groups (Figure 3.). For example for *Pterophyte* plants, sequences were found only for the second type of metallothionein – MT2, and for *Lycophyte* plants, only MT1 and MT3 (Figure 3). In recent years, more and more of MT sequences are becoming available in databases, primarily due to genome sequencing projects and increasing interest in transcript profiling (Ren et al. 2012). In NCBI database currently, there are over 1000 metallothionein sequences found in plants (Cabral et al. 2018).

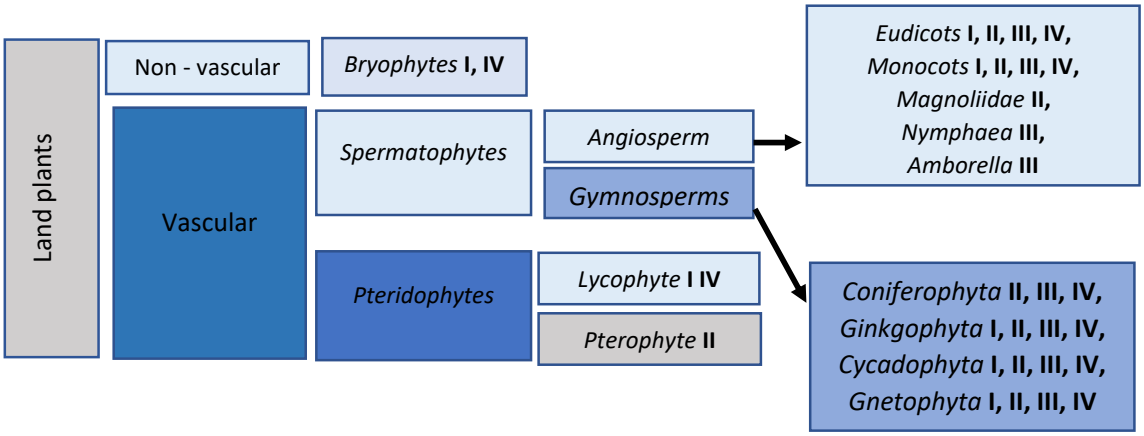

Figure 3. Different types of metallothioneins found in taxonomical groups of plants (Based on sequences available in NCBI – Genbank database)

5. BIOMARKERS NONSPECIFIC FOR METAL STRESS

General biomarkers, which can respond to a vast variety of environmental stressors are often used in toxicity assessments after soil contamination with heavy metals including cadmium (Gomes et al. 2013). These markers became undoubtedly useful to assess if something in plants environment is hazardous to its growth and development (Liu et al. 2008). Despite their lack of specificity to metals, they are successfully used to evaluate the metal toxicity of contaminated soils on the condition that other stressors are none existent or constant (Yadav 2010). The reason for that is the fact that phytotoxicity response of plants grown on contaminated soil under controlled conditions (growth chamber or *in vitro*) should reflect only the influence and interference of metals (Metwally et al. 2005). The level of activity of some antioxidative enzymes, membrane composition and permeability, the ratio of sugar in roots, fluorescence and level of polyamine are only some of the markers that can be used in toxicity tests after cadmium exposure (Chen et al. 2011, Gomes et al. 2013).

5.1. Enzymatic changes

As stress factors, cadmium ions induce oxidative stress in plants cells by disrupting their metabolic chains (Maksymiec et al. 2006). This leads to a significant imbalance between the production of reactive oxygen species (ROS) and their neutralization (Metwally et al. 2005). Reactive oxygen species such as hydrogen peroxide, superoxide radicals, singlet oxygen and hydroxyl radicals are removed by specific antioxidants including glutathione, carotenoids, proline, ascorbate, α-Tocopherol or antioxidative enzymes that play one of key roles in metal detoxification including: superoxide dismutase (SOD), peroxidases (guaiacol and ascorbate peroxidase – GPX and APX), catalases (CAT) (Dixit et al. 2001, Liu et al. 2008, Gill et al. 2011). Overall, plant cells possess numerous defense mechanisms that allow for minimizing damage caused by ROS overproduction after expousure to toxic metal ions (Chen et al. 2011). These systems include antioxidant enzymes and non-enzymatic antioxidants. Antioxidative enzymes participate directly in the removal of ROS and in redox reactions of ascorbic acid and glutathione (Maksymiec et al. 2006).

There are three main groups of antioxidative enzymes in plants:

(1): Superoxide dismutase (SOD) - catalyze the reactions of the dismutation of two superoxide anion radicals to the hydrogen peroxide molecule and oxygen (Dixit et al. 2001). This reaction takes place in two stages and includes the reduction and oxidation of the metal enzyme which is contained in the catalytic center (Gill et al. 2011). Depending on the type of cofactor, we distinguish iron dismissals: FeSOD, occurring mainly in plastids, manganese dismutases: MnSOD, occurring in the mitochondria and copper-zinc acceptors: Cu / ZnSOD, present in the cytosol, peroxisomes, apoplasts, chloroplasts, and mitochondria (Dixit et al. 2001, Gill et al. 2011);

(2) Peroxidases (POD) - directly involved in the removal of hydrogen peroxide (Verma et al. 2008). Peroxidase activity is essential in the functioning of plant cells by taking part in sprout germination, aging of cells, leaf and fruit fall, or reactions to emerging adverse environmental factors and pathogens (Gill et al. 2011). These include ascorbate peroxidase (APX), which is a crucial enzyme in the course of the ascorbate-glutathione cycle, where attaching two particles of ascorbate reduces H_2O_2 to water molecules to form monodehydroascorbate (MDHA) (Meng et al. 2012). Glutathione peroxidase plays a vital role in the removal of ROS, and its main function is to protect cellular structures against oxidation. In addition, peroxidases are characterized by tolerance to high temperatures while retaining partial activity even at 100°C (Verma et al. 2008). Among the peroxidases we also distinguish guaiacol peroxidase present in the cytosol, intercellular spaces, vacuoles and cell walls that play an essential role in the process of lignification, ethylene biosynthesis and protection against pathogens (Meng et al. 2012);

(3) Catalases (CAT) - carry out reactions of hydrogen peroxide dismutation to the oxygen and water molecule (Dong et al. 2006). They occur in peroxisomes and glyoxysomes, as well as cytosol and mitochondria. In all eukaryotes, catalases are homotetramers composed of four subunits with NADPH molecules and heme groups in active cents (Lukacová et al. 2013). They respond to hydrogen peroxide up to 10,000 times faster than peroxidases (Zhang et al. 2009). Among the catalases, there are three primary isomeric forms: CAT1, CAT2, and CAT3 (Dong et al. 2006). In places of significant production of hydrogen peroxide - cytosol, peroxisomes, and glyoxysome the CAT2 form significantly dominates (Gill et al. 2012). The third isomeric form, CAT3 predominates in young tissues of plants and seeds, catalyzing the decomposition of H_2O_2 resulting from

the degradation of fatty acids (Zhang et al. 2009). The concentration of H_2O_2 in the cells determines the type of catalase activity. At high concentrations of hydrogen peroxide, catalases exhibit catalase activity allowing removal of H_2O_2, while at low levels the principal activity of catalase is peroxidase activity (Zhang et al. 2009, Gill et al. 2012) . Another crucial role in the removal of excess ROS is also played by non-enzymatic proteins and small molecule antioxidants, including: (1) Ascorbate - a powerful antioxidant that reacts with singlet oxygen, organic peroxide radicals, hydrogen peroxide, superoxide and hydroxyl radicals. Occurs in the cytosol, vacuoles, chloroplasts, mitochondria, and peroxisomes (Wahid et al. 2007, López-Millán et al. 2009); (2): Tocopherol - a hydrophobic antioxidant present in biological membranes, mainly reducing lipid peroxide radicals and singlet oxygen (Redondo-Gmez et al. 2010). During the reaction of tocopherol with radicals, less reactive tocopherol radicals are formed, removed by other antioxidants - glutathione and ascorbate (Meng et al. 2012); (3) Carotenoids - by quenching triplet chlorophyll prevent excessive production of ROS. They also react with lipid radicals (Verma et al. 2008); (4) Flavonoids - they inhibit the activity of lipoxygenases, they are a substrate for other enzymes including peroxidases for the decomposition of hydrogen peroxide (Wahid et al. 2007).

The substances such as proline, mannitol, and sorbitol also have antioxidant functions in plant cells (Gomes et al. 2013). In addition, the mitochondrial inner membrane contains a decoupling protein (UCP) that has the ability to reduce ROS production by scattering the proton electrochemical gradient (Lukacová et al. 2013).

5.3. Metabolites

In addition to antioxidative enzymes, plants metabolites are often used as stress biomarkers and had been shown to be sensitive in regards to metal stress (Hayat et al. 2011). For example, a significant increase in putrescine was noticed after the exposure of *Hordeum* to toxic concentrations of different metals (Bergmann et al. 2001). Similar results were also observed in *Vigna radiata* plants treated with cadmium, after which substantial increase in the putrescine was observed (Nahar et al. 2016). For both plants, presented changes were even more significant in plants roots. Moreover, in *Vigna radiata* the ratio between sugars – glucose / saccharose / fructose was also elevated after the

exposure to cadmium and was shown to be a sensitive biomarker (Nahar et al 2016). In other studies, putrescine was observed to be potential stress marker after toxic exposures to SO_2 and even UV-B (Meng et al. 2012).

5.4. Membrane parameters

Membrane degradation products such as the thiobarbituric acid reactive compounds and the membrane permeability were shown to increase under metal contamination and other environmental stresses (Figure 4) (Hasan et al. 2011). The membrane permeability which is most of the time quantified by the potassium leakage was proved to be a sensitive biomarker for several plants species and ecological stressors including cadmium toxicity (Redondo-Gmez et al. 2010).

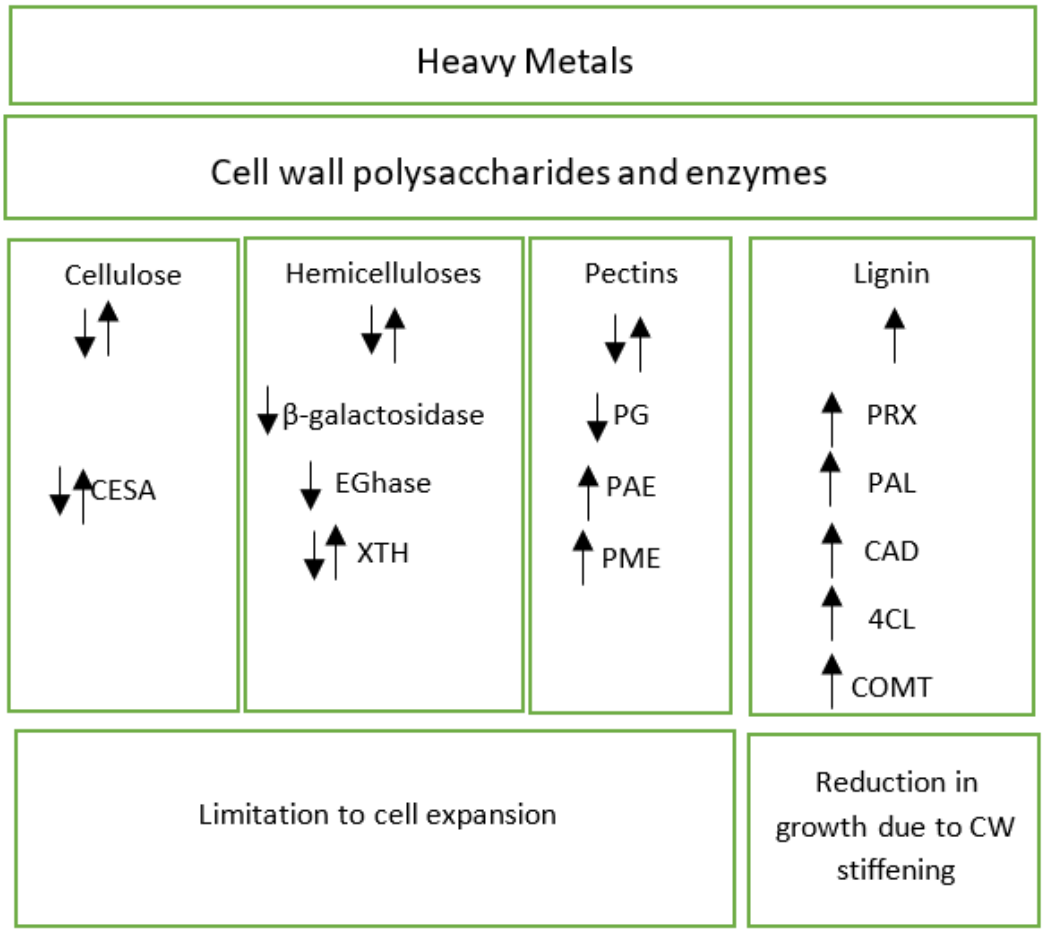

Figure 4. Changes in cell wall after the exposure to heavy metals in plants. CW – cell wall, CESA - Plant Cellulose Synthase, XTH – Xyloglucan, PG – Polygalacturonase,

PAE – Pectinase, PME - Pectin methylesterase, PRX – peroxidase, PAL - Phenylalanine ammonia lyase, CAD - Cinnamyl-alcohol dehydrogenase, 4-Coumarate-CoA ligase, COMT - Catechol-O-methyltransferase. Presented arrows show an increase or a decrease in activity of selected enzymes after the exposure to heavy metals (Hasan et al. 2011).

5.5. Fluorescence

In recent years, the evaluation of the biochemical and physiological condition of plants based on fluorometric analysis of photosynthesis process is gaining general acceptance (Baker 2008). Since toxicological data specific to plants photosynthetic systems has been collected for thousands of chemicals and a vast number of plant species, it makes the chlorophyll fluorescence one of the most commonly used and sensitive biomarkers for environmental stressors (Buryński et al. 2004). The fluorescence of chlorophyll was already shown to be a useful biomarker for soil contamination with heavy metals (Siliang et al. 2013). Toxicity tests based on that mechanisms are sensitive and feasible (Baker 2008). One of the most important advantages of this analysis is the facts which it is non-destructive and easy to use in a field. It also requires a lot less time than other technics (Siliang et al. 2013).

6. CONCLUSIONS

Research concerning the use of specific plant biomarkers for cadmium toxicity are still in an initial stage (DalCorso et al. 2008, Almaroai et al. 2013). For quantification of cadmium toxicity additional research needs to be done especially on higher-plants species (Gill et al. 2012). Standardization of sampling, the preparation of the material and quantitive algorithms indicating relationships between cadmium concentration and its biological effects need to be established (Ghoshal et al. 2013). To this date, research on plants biomarkers mainly consisted of short-term exposures, usually in *in vitro* conditions (Siliang et al. 2013).

Enzymatic assays confirm the toxicity reduction after manure treatment of heavy metals contaminated soil*

Jaskulak Marta[a*], Agnieszka Rorat[b]; Anna Grobelak[a]

[a]*Department of Infrastructure and Environment, Institute of Environmental Engineering, Czestochowa University of Technology, Czestochowa, Poland;*

[b]*Université Lille Nord de France, LGCgE-Lille 1, Ecologie Numérique et Ecotoxicologie, F-59650 Villeneuve d'Ascq, France*

Abstract

Soil contamination with heavy metals is a growing issue that results in adverse effects on plant production. In this study, the influence of manures on heavy metals toxicity reduction in *Sinapis alba* L. and *Robinia pseudoacacia* L. was tested in order to plan phytoremediation process The primary objective was to assess the effects of manure fertilization on plants grown on highly degraded soil contaminated by a range of heavy metals. The measurement endpoints were seed germination, plants biomass, roots length, guaiacol peroxidase activity (GPX) and protein concentration. The study showed that among other stress markers, GPX activity showed statistically significant changes ($\alpha < 0.05$) between different types of manures and between the 5% different in applied doses. Therefore performed tests could be used as efficient protocols to assess the required doses of organic amendments in planning of phytoremediation process.

KEYWORDS: phytoremediation, phytotoxicity, pollution, stress, biomarkers

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Highlights

- Germination, and biomass were adversely affected by contamination
- Guaiacol peroxidase activity (GPX) shown significant sensitivity between 5% change of the dose
- Supplementation with manure reduced the toxic effects of heavy metals
- Presented plant growth test can be suitable protocol to assess toxicity

1. INTRODUCTION

Heavy metals (HMs) have a negative impact on plant growth and overall the quality of crops. Even a slightly contaminated environment should not be regarded as safe to use to produce food as biomass may contain heavy metals and amassed in organisms further down the food chain (Pal et al. 2010). One example of such adverse impact is in Chinese agriculture, where about 15% of all rice field areas contain heavy metals. In such regions, rice productivity has declined considerably (Lone et al. 2008, Marques et al. 2009). The problem with heavy metal-polluted soils is increasing significantly worldwide as a result of industrialization, mining operations and improper waste and water treatment (Placek et al. 2016). Heavy metals cause abiotic stress that results in poor development of plants, lower yields and finally, at high concentrations, it causes plant death (An, 2004). Plants possess a wide variety of defense strategies whenever confronted with the presence of HMs. The first step toward dealing with this threat is avoidance strategy mostly via restricting metal uptake. When this strategy fails, tolerance mechanisms for detoxification are activated including metal sequestration, compartmentalization, and various antioxidative systems. Soon after exposure to heavy metals, metabolism of reactive oxidative species (ROS) is altered and oxidative stress is induced (Fernández et al. 2005).

As with most other stress factors, heavy metals induce oxidative stress by disrupting metabolic chains which lead to the imbalance in ROS generation and neutralization (Xin et al. 2017). In normal conditions ROS species such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals are removed by antioxidants (ascorbate, glutathione, α-Tocopherol, carotenoids) or enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD) including guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) (Hooda, 2007). An overflow of ROS that cannot be adequately neutralized by the antioxidative system causes damage to proteins and DNA. Toxic effects of that imbalance accumulate in cells and leads to the death of plants (Murtaza et al. 2010). The activity of these enzymes can be used to determine stress levels and to determine environment toxicity before any visible impact on growth appears. Such assays may be useful tools in environmental engineering, for example as an indicator of the severity of soil pollution in phytoremediation efficiency. Artificial increase in the rate of ROS neutralization may led to an increase in yield quality and enable some species to be cultivated outside their native climates (Remans et al. 2012).

High input of chemical fertilizers in recent years has led to a decrease in the quality of soil due to a reduction in soil organic matter content (Adiloglu et al. 2017). Therefore, the use of organic fertilizers such as compost or manure is an alternative practice for sustaining economically viable crop production with less environmental pollution (Grobelak et al. 2013). Moreover, the use of *Fabaceae* plants, such as *Robinia pseudoacacia* L. in processes like phytoremediation can improve soil quality progressively over time since adequately supplemented soil will allow *Fabaceae* to create a symbiosis with nitrogen binding bacteria, which leads to a significant improvement in soil quality over time.

Fuentes et al. (2004) and others studies have shown that many plant species, including Fabaceae, would not produce conclusive results in standard toxicity tests such as germination index due to their ability to concentrate nutrients in their seeds (Fuentes et al., 2004, Araujo et al. 2005, Chabukdhara et al. 2013). Moreover, large variations among standard toxicity bioassays and plant species were shown in previous studies. Seed germination tests are regarded as an insensitive method in comparison to root length, when used as an assay for the evaluation of environmental toxicity (Fuentes et al. 2004; Kapanen et al. 2001). Seed germination was also shown to be relatively insensitive to a vast range of toxic substances, since many chemicals and other toxic substances may not be absorbed by seeds. Such effect is created as the embryonic plant draws its nutritional requirements internally from seed stored materials and is effectively isolated from the environment (Chabukdhara et al. 2013).

Beneficial effects of using manure have been reported in the past to improve tolerance of drought stress in plants (Qiao et al. 2010) as well as enhancing tolerance to heavy metals such as lead (Plociniczak et al. 2013). Among organic amendments, manure can have potential applications in phytoremediation of degraded soils, because of its abundance, accessibility, low price and easy technology know-how.

The objective of this study was to determine the stress response of *R. pseudoacacia* L. (black locust) and *Sinapis alba* L. (white mustard) under the influence of heavy metals and supplementation of manures. The aim of the study was to evaluate sensitivity and

potentially large-scale applications of including GPX activity measurement as a sensitive biomarker in phytoremediation under complex heavy metal contamination. The main criteria used for selecting plants for phytoremediation were their tolerance to heavy metals, high biomass production and resistance to climate observed in the chosen area including cold weather and seasonal drought. *S. alba* is an important crop plant and can be used in phytoremediation due to its ability to absorb heavy metals such as cadmium. It has a substantial amount of desirable agronomic traits such as resistance to drought, some diseases, and pests (Zha et al. 2016). *R. pseudoacacia* as a member of *Fabaceae* also possesses desirable characteristics such as high biomass production and the ability to hyperaccumulate metals. Moreover, it can promote microbial activity in soil and is resistant to cold weather and drought (Seneviratne et al. 2016).

2. MATERIALS AND METHODS

2.1. Substrates characterization

The experimental soil used in this research was collected from highly degraded areas of Poland (GPS: 50°30'N 18°56'E). According to WRB classification, studied soil belongs to podzols type and can be also described as sandy loan with approximately 65% sand, 20% slit and 15% clay. Soil was characterized by high content of heavy metals, low sorption capacity, low fertility, and low biological activity. In addition, soil was slightly acidic (Table 1) (Table 2). For illustrative purposes, the experiment was also carried out on organic garden soil in order to assess the levels of selected markers in plants grown on optimal conditions.

Table 1. Physical and chemical properties of used soil – PS – uncontaminated garden soil, CS – contaminated soil without any amendments, C – cattle manure, H – horse manure. Results shown as mean ± standard deviation (n = 3). Different letters "a", "b" "c" "d" in columns indicate a significant difference according to Kruskal–Wallis test ($\alpha < 0.05$).

Treatment	pH		N (%)	P_2O_5 [mg/100g]	K [mg kg^{-1}]	TOC [g kg-1]	CEC [cmol/kg]
	H_20	KCl					
PS	7.1 ± 0.4a	6.6 ± 0.3a	8.3 ± 0.2a	82.7 ± 0.5a	233.4 ± 0.6a	26.8 ± 0.2a	30.1 ± 0.3a
CS	5.5 ± 0.1b	4.5 ± 0.1b	3.6 ± 0.1b	79.4 ± 0.2b	75.2 ± 0.8b	12.6 ± 0.4b	25.2 ± 0.1b
15% C	6.9 ± 0.3a	5.9 ± 0.3c	7.6 ± 0.1c	73.2 ± 0.2c	140.5 ± 1.7c	23.2 ± 1.1c	27.7 ± 0.1c
20% C	7.2 ± 0.1a	6.5 ± 0.2a	8.1 ± 0.2a	69.1± 0.1d	190.4 ± 1.2d	24.1 ± 1.4a	30.6 ± 0.1a
15% H	7.0 ± 0.2a	6.4 ± 0.1a	6.5 ± 0.2d	76.4 ± 0.1^{e}	211.7 ± 3.5^{e}	21.5 ± 0.3d	27.9 ± 0.2c
20% H	6.9 ± 0.1a	6.1 ± 0.3a	7.1 ± 0.1^{e}	72.0 ± 0.1f	253.1 ± 2.7f	25.3 ± 0.7a	28.7 ± 0.2d

Samples were collected from the surface layer (up to 30 cm deep). Prior to the experiment, experimental soil was dried at room temperature for 14 days, after which it was mixed and sieved. Manures used in the experiment were obtained from commercially available sources and were tested for physical and chemical properties (FLORMIX, Poland). Cattle manure was characterized by NPK content of approximately 5.3 g N kg^{-1} dry weight, 1.1 g P kg^{-1} dry weight and 4.1 g K kg^{-1} dry weight. Horse manure contained a higher content of NPK, including approximately 6.4 g N kg^{-1} dry weight, 2.3 g P kg^{-1} dry weight of

phosphorus and 4.9 g K kg^{-1} dry weight. The doses of manure used in the experiment were selected on the basis of values never exceeding the nitrogen content of the Council Directive 91/676/EEC standards (170 kg of nitrogen/year/ha)).

Table 2. Content of cadmium, lead and zinc in experimental soil. PS – uncontaminated garden soil, CS – contaminated soil without any amendments, C – cattle manure, H – horse manure. Results shown as mean ± standard deviation (n = 3). Different letters "a", "b" "c" "d" in columns indicate a significant difference according to Kruskal–Wallis test ($\alpha < 0.05$).

Treatment	Cd [mg kg^{-1}]	Pb [mg kg^{-1}]	Zn [mg kg^{-1}]
PS	0.02 ± 0.01a	1.1 ± 0.7a	154.5 ± 10.3a
CS	19.5 ± 1.2b	1133.8 ± 3.5b	733.2 ± 1.4b
15% C	19.7 ± 2.1b	1106.4 ± 13.8b	700.1 ± 3.4c
20% C	20.4 ± 1.4b	1135.2 ± 24.8b	689.4 ± 8.1d
15% H	18.5 ± 1.2b	1020.8 ± 29.2c	632.3 ± 6.3e
20% H	17.7 ± 0.9b	969.3 ± 19.5c	591.4 ± 11.4f

The manure was applied at 15% and 20% in order to estimate the sensitivity of the selected stress markers to slight differences in dosages of soil additives. In order to adequately assess the physiological development of plants grown on highly contaminated soil, standard peat soil was used as a control group to provide optimal conditions to growth without any abiotic stressors (Table 1). The contaminated soil (without any organic fertilizer amendments) was supplemented with sand in the same ratio as the treatment to avoid "dilution" effect. We chose sand due to the physical composition of contaminated soil which is sandy loam with approximately 65% of sand. Before planting seeds, soil mixtures with different concentrations of additives were prepared, homogenized in a mortar and sieved again. After preparation, samples were collected and physical and chemical assays were performed. For pH determination of the soil, samples were measured in distilled water and 1M solution of KCl according to ISO 10390:2005 using a standard laboratory pH-meter (Cole Parmer Model No. 59002–00, Illinois, USA). Cation-exchange capacity (CEC) was determined by Kappen's method (Kappen et al. 1995). The content of total organic nitrogen in samples was established by Kjeldahl's

method (Distillation Unit K-350 / K-355, Buschi, Switzerland), while the content of phosphorus and potassium by the Egner's method (Egnér et al. 1960). Concentration of heavy metals that occur in our soil – cadmium, lead, and zinc was determined by ICP-OES (ICP-OES; Thermo apparatus, Illinois, USA) after sample microwave mineralization in nitric acid. Samples were digested in a microwave digestion system according to the EPA method 3051. This digestion method provided a pseudo-total metals concentration, in manuscript referred as "total" metal concentration. As an environmental matrix, the following reference materials were used: soil material (LGCQC3004) and sewage amended soil (RTC-CRM005-50GBCR-146R). For ICP analysis the Quality Control Standard (QCS) was used and individual samples were measured in triplicates for 30 s integration time. For soil samples the determination limits were: Cd (5 ppb), Zn (12 ppb), Pb (35 ppb).

2.2. Pot experiment

Two plant species: *S. alba*, and *R. pseudoacacia* were grown separately in different soil mixtures. Commercially available, high-quality seeds were used (Dary Podlasia, Poland). Seeds vitality was measured prior to the experiment on petri dishes following ISO 11269-2 and germination index was established at approximately 96% for *S. alba* and 90% for *R. pseudoacacia* (data not shown). Each soil mix was prepared in three replicates in pots with square base (a = 12 cm, H = 15 cm). Clean peat soil without any metal contamination was used as a control. Our main experimental condition consisted of soil contaminated with a vast range of heavy metals with or without the addition of selected organic fertilizer. All measurements were performed in three biological replicates and two technical replicates. Samples were harvested after 14, 21 and 28 days. Experiment and the sampling of plants was carried out between march and april of 2017. For the incubation, plants were placed in growth chamber (model FS360, Biogenet, Poland) under controlled conditions: (ISO 11269-2): i) temperature of 21°C and 18°C during the day and the night, respectively; ii) photoperiod of 16 h of light and 8 h of dark; iii) light intensity of 4000 lx (photosynthetic LED light). Germination index was measured and calculated according to ISO 11269-2. After incubation, plants were harvest, washed in distilled water and stored at -80°C for further analyses. In addition, plants were uprooted carefully, washed with distilled water and root length (cm) for each treatment was measured. Fresh weight (g) of shoots after harvest was also estimated. Metal

concentrations in roots and shoots were determined using ICP-OES (iCAP 6000 Series, Thermo apparatus, USA) after 28 days of incubation followed by harvest and dehydration at 40^{o}C. For ICP analysis as an environmental matrix, the reference material was used white clover (BCR-402). Guaiacol peroxidase (GPX) activity assay

GPX activity was measured using the method established by Asada with modifications (Asada et al. 2006. Caverzan et al. 2012) where the activity is estimated by the level of guaiacol oxidation in the presence of hydrogen peroxide in 2 min. Approximately 100 mg of plant material (shoots and roots) were mixed with 3 mL phosphate buffer (pH 6) and homogenized in a mortar. The homogenate was centrifuged at 11000 g for 5 minutes at a temperature of 4^{o}C. Guaiacol reaction mixture was prepared in cuvettes for spectrophotometric analysis containing 3 mL guaiacol reagent (100 mM potassium phosphate buffer, pH 7.4 and 0.35% guaiacol). For each sample, 10µL supernatant obtained after homogenization and 10 µL 30% hydrogen peroxide was added, mixed and immediately measured at 430 nm by a spectrometer (HACH spectrometer DR/4000V, HACH, USA) and then after 2 min from when oxidation of guaiacol started. Peroxidase activity was defined as an amount of GPX that produces a change in absorbance at 430 nm of 0.021 min. Results were normalized to U mg of determined protein concentration.

2.3. Protein content

The analysis was performed using a modified Lowry assay (Lowry et al. 1951). Bovine serum albumin (BSA) was used as a standard and analysis was carried out by spectrometry (HACH spectrometer DR/4000V, HACH, USA)

2.5. Statistical analysis

For the statistical analyses, the nonparametric Kruskal-Wallis test was used. Level of significance $\alpha = 0.05$. Different letters in figures and tables show a significant effect ($\alpha < 0.05$), whereas the same letters indicate the non-significant difference ($\alpha > 0.05$). The results are expressed as mean ± standard deviation (three pots for each soil mix conditions). Descriptive statistics and statistical tests were produced using the OriginPro 2015 software.

3. RESULTS

3.1. Germination index, plants development and metal accumulation

Germination index was highly reduced by soil contamination, whereas the addition of organic fertilizers at both concentrations strongly enhanced the germination of both plant species in contaminated soil (Figure 1). The highest germination percentage occurred in samples grown in control soil for both plant species. Overall, both species presented similar tendency to germinate on prepared soil mixes. The number of shoots in samples with any fertilizer (Figure 1) was at least doubled in comparison to contaminated soil without any fertilizers. Shoots biomass and roots length were also severely affected by soil contamination and significantly ($\alpha < 0.05$) improved after fertilization with both manures with the lack of statistically significant changes among different doses of selected manures. Over the selected time points, both root length and shoot biomass increased with time (Figure 2 and Figure 3). For *S. alba* the highest increase in shoot biomass and roots length was noticed between 14 and 21 days of incubation (Figure 2a and Figure 2b) whereas for *R. pseudoacacia* between 21 and 28 days of incubation (Figure 3a and Figure 3b).

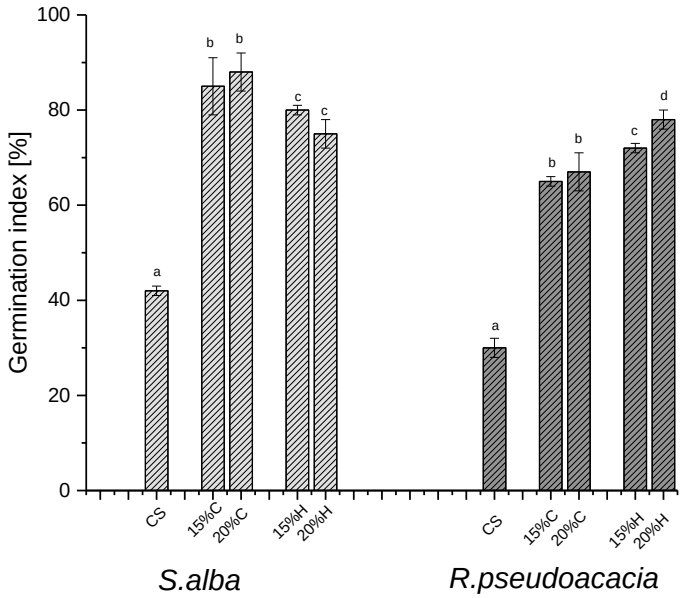

Figure 1. Germination index of *Sinapis alba* L. and *Robinia pseud*oacacia L. grown on different mediums: CS – contaminated soil without any fertilizers, C – cattle manure, H – horse manure. Results shown as mean ± standard deviation (n = 3). Different letters “a”, “b” “c” "d" on top of bars indicate a significant difference according to Kruskal–Wallis test ($\alpha < 0.05$).

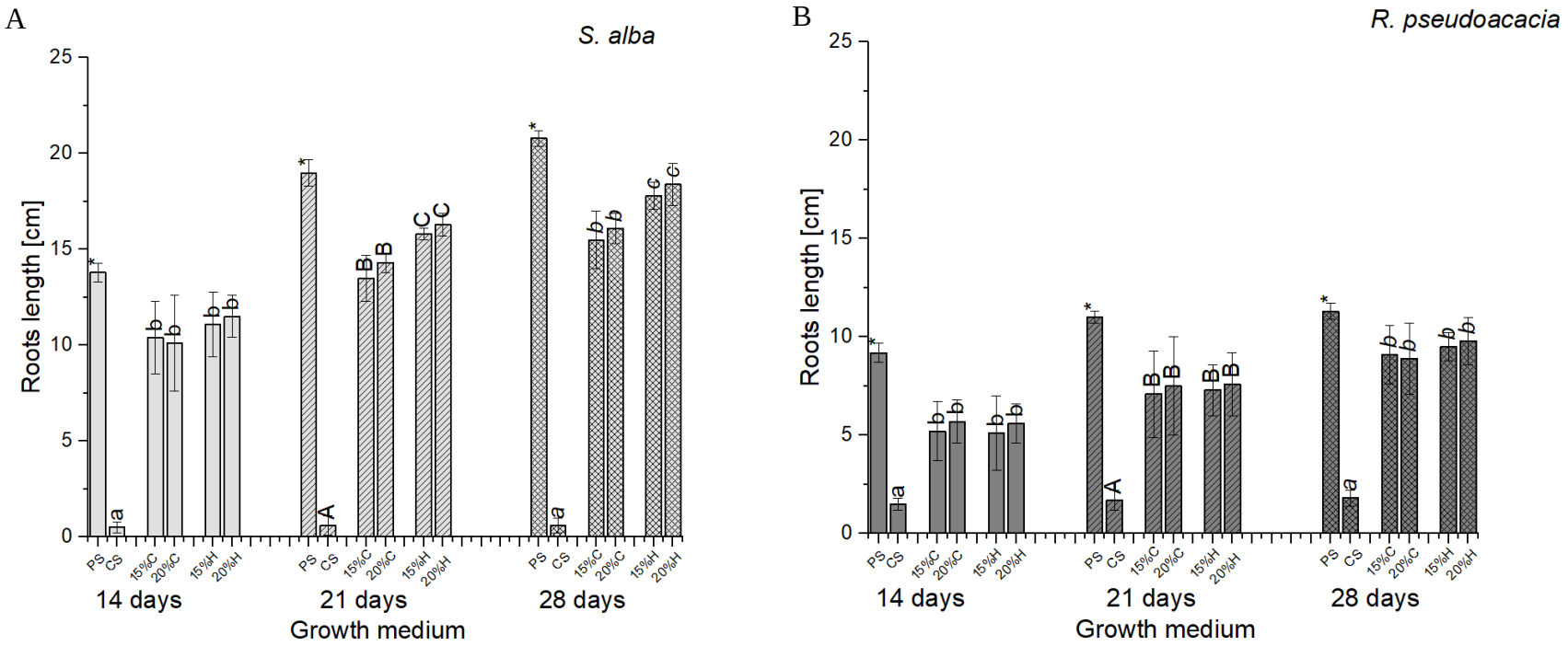

Figure 2. Roots length in *S. alba* (A) and *R. pseudoacacia* (B) after 14, 21 and 28 days of incubation (PS – control soil without any contamination, CS - contaminated soil without any fertilizers, 15%C / 20%C - CS amended with 15% / 20% cattle manure; 15% / 20% H – CS amended with 20% horse manure]. Results shown as mean ± standard deviation (n = 3). Different letters “a”, “b” “c” "d" on top of bars indicate a significant difference according to Kruskal–Wallis test ($\alpha < 0.05$).

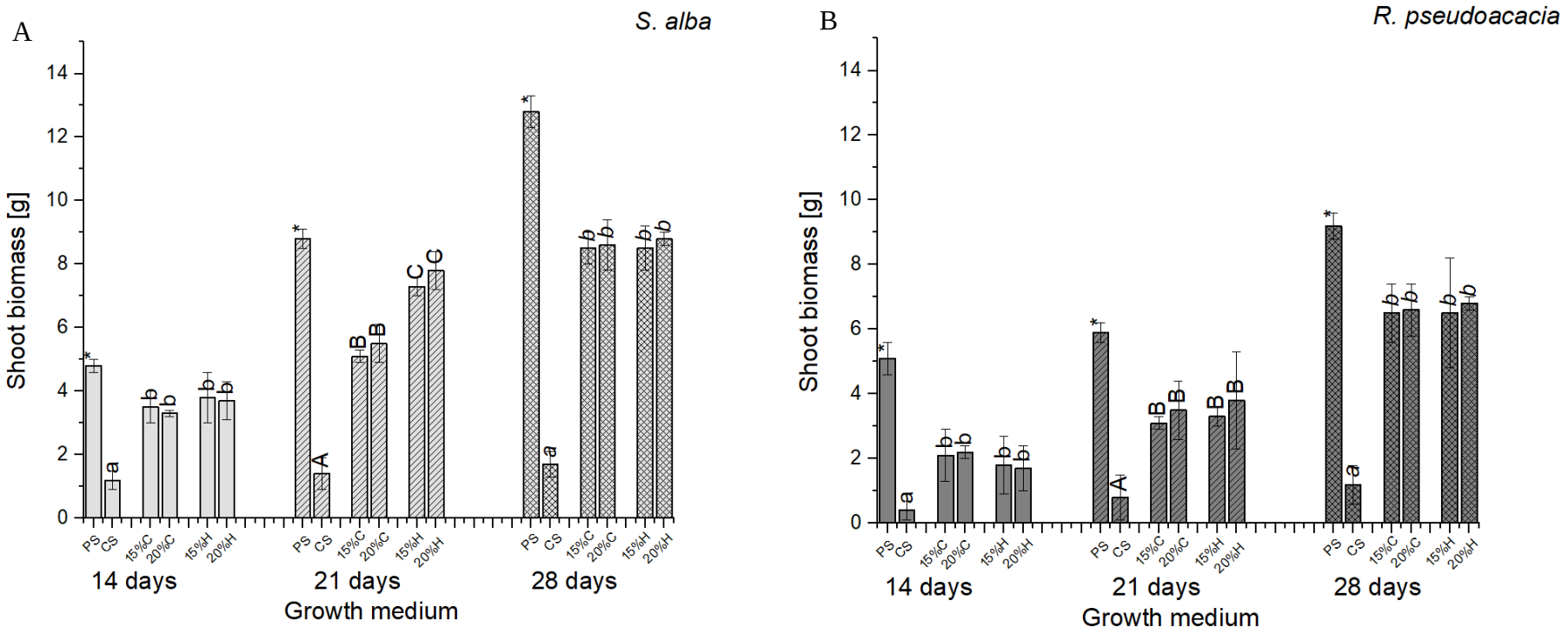

Figure 3. Shoot biomass [g] in *S. alba* (A) and *R. pseudoacacia* (B) after 14, 21 and 28 days of incubation (PS – control soil without any contamination, CS - contaminated soil without any fertilizers, 15%C / 20%C - CS amended with 15% / 20% cattle manure; 15% / 20% H – CS amended with 20% horse manure]. Results shown as mean ± standard deviation (n = 3). Different letters “a”, “b” “c” "d" on top of bars indicate a significant difference according to Kruskal–Wallis test ($\alpha < 0.05$).

Metal accumulation in both species was significantly reduced after supplementation with manure (Table 3). Overall in most cases, *S. alba* accumulated a higher concentration of lead, cadmium, and zinc in both its shoots and roots in comparison to *R. pseudoacacia*. For both tested species highest accumulation was observed in contaminated soil without any fertilizers and was reduced by both types of manure. Moreover, there was almost no statistically significant changes between the two tested types of manures and their concentrations.

Table 3. Metal accumulation in plants after 28 days of incubation. 'A' – accumulation of Pb in plants shoots an roots, 'B' – accumulation of Cd, 'C' – accumulation of Zn. PS – uncontaminated garden soil, CS - contaminated soil without any amendments, C – cattle manure, H – horse manure. Results shown as mean ± standard deviation (n = 3). Different letters "a", "b" "c" "d" in columns indicate a significant difference according to Kruskal–Wallis test ($\alpha < 0.05$).

A	**Pb [mg kg^{-1}]**			
	S. alba		*R. pseudoacacia*	
	Shoots	Roots	Shoots	Roots
PS	1.2 ± 0.2a	2.6 ± 0.5a	0.8 ± 0.3a	1.3 ± 0.5a
CS	104.5 ± 7.8b	211.3 ± 11.2b	57.4 ± 4.1b	127.8 ± 10.5b
15%C	42.8 ± 2.8c	97.5 ± 6.1c	33.1 ± 2.2c	64.5 ± 7.1c
20%C	38.1 ± 1.1d	104.8 ± 10.2c	26.5 ± 4.3d	65.1 ± 5.6c
15%H	44.3 ± 3.1c	94.7 ± 5.1c	40.9 ± 3.7e	50.1 ± 6.2d
20%H	47.1 ± 4.3c	88.4 ± 4.2d	42.4 ± 4.5e	49.7 ± 6.6d

B	**Cd [mg kg^{-1}]**			
	S. alba		*R. pseudoacacia*	
	Shoots	Roots	Shoots	Roots
PS	0.02 ± 0.01a	0.03 ± 0.02a	0.03 ± 0.02a	0.02 ± 0.02a
CS	11.9 ± 4.6b	26.8 ± 3.1b	5.9 ± 0.7b	17.4 ± 1.3b
15%C	2.5 ± 1.5c	17.7 ± 1.1c	3.1 ± 0.4c	6.5 ± 0.6c
20%C	3.2 ± 0.6c	14.2 ± 1.6d	2.4 ± 0.6d	7.2 ± 0.8c
15%H	2.7 ± 0.8c	15.1 ± 0.4d	1.9 ± 0.7d	5.1 ± 0.6d
20%H	2.2 ± 0.4c	14.5 ± 0.7d	2.5 ± 0.5d	4.4 ± 0.9d

C	Zn [mg kg^{-1}]			
	S. alba		*R. pseudoacacia*	
	Shoots	Roots	Shoots	Roots
PS	23.9 ± 1.9a	35.6 ± 2.3a	30.1 ± 7.3a	42.2 ± 2.4a
CS	135.1 ± 12.2b	182.4 ± 13.8b	109.5 ± 10.5b	130.8 ± 12.6b
15%C	62.5 ± 6.5c	97.6 ± 9.1c	48.3 ± 3.6c	76.5 ± 6.6c
20%C	53.7 ± 2.4d	95.3 ± 7.5c	50.1 ± 2.8c	79.1 ± 8.1c
15%H	52.3 ± 3.8d	82.4 ± 6.2d	49.8 ± 4.7c	82.3 ± 8.5c
20%H	55.9 ± 4.1d	85.7 ± 5.4d	40.5 ± 1.3d	74.3 ± 6.8c

3.2. GPX activity

In shoots and roots of both plants, the activity of GPX was the lowest in the biomass grown on control soil (Figures 3 - 6), whereas the highest values were observed in plants grown on contaminated soil without any additives. All additives resulted in decreased activity of GPX in time and lower oxidative stress in relation to contaminated soil without amendments (significantly) (Figure 4).

Activity of GPX in roots was notably higher than in shoots (Figure 3, 4). In roots of *R. pseudoacacia* L. GPX activity in roots was even 10 times higher than in shoots. In roots of *S. alba* L., activity in other treatments was around twice higher than in the control with the lowest value at 28th day of the experiment for 20% horse manure treatment (Figure 5, 6).

Supplementation with both: horse and cattle manure caused a significant decrease in GPX activity in all samples. Moreover, there were significant differences between the doses of amendments and their type.

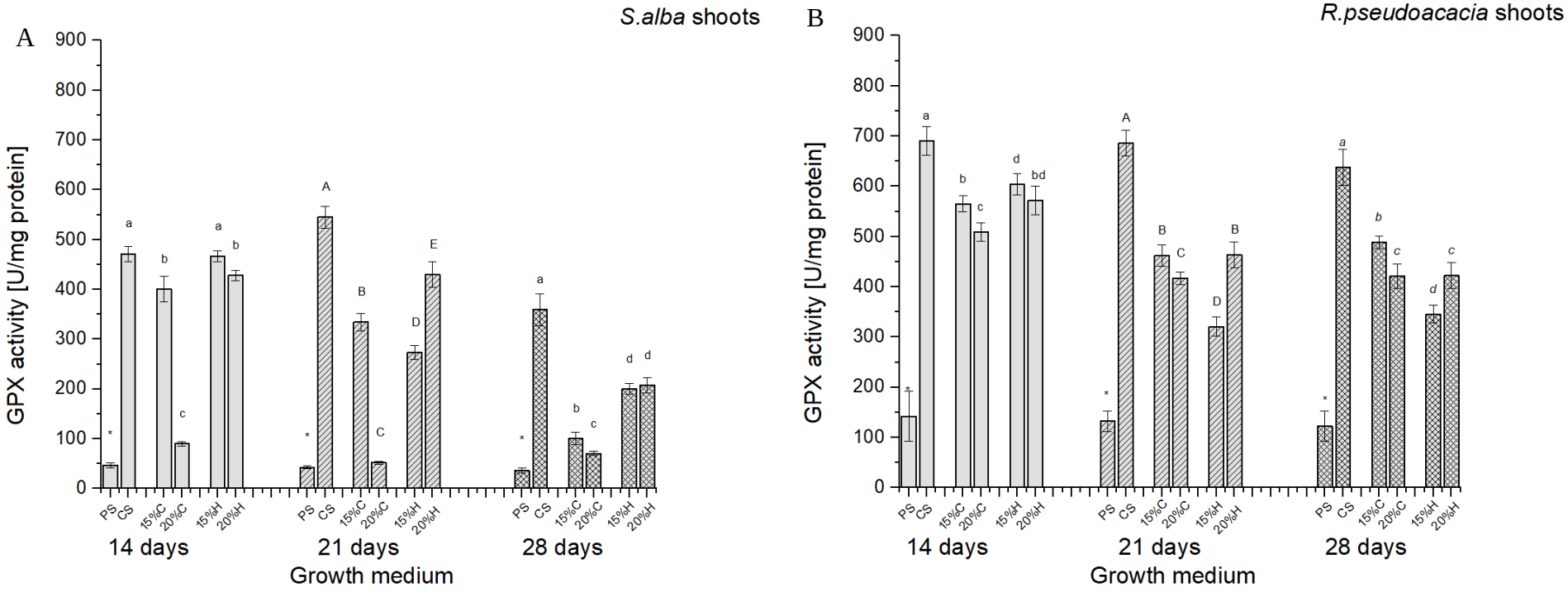

Figure 4. GPX activity in shoots of *S. alba* (A) and *R. pseudoacacia* (B) after 14, 21 and 28 days of incubation (PS – control soil without any contamination, CS - contaminated soil without any fertilizers, 15%C / 20%C - CS amended with 15% / 20% cattle manure; 15% / 20% H – CS amended with 20% horse manure]. Results shown as mean ± standard deviation (n = 3). Different letters “a”, “b” “c” "d" on top of bars indicate a significant difference according to Kruskal–Wallis test ($\alpha < 0.05$).

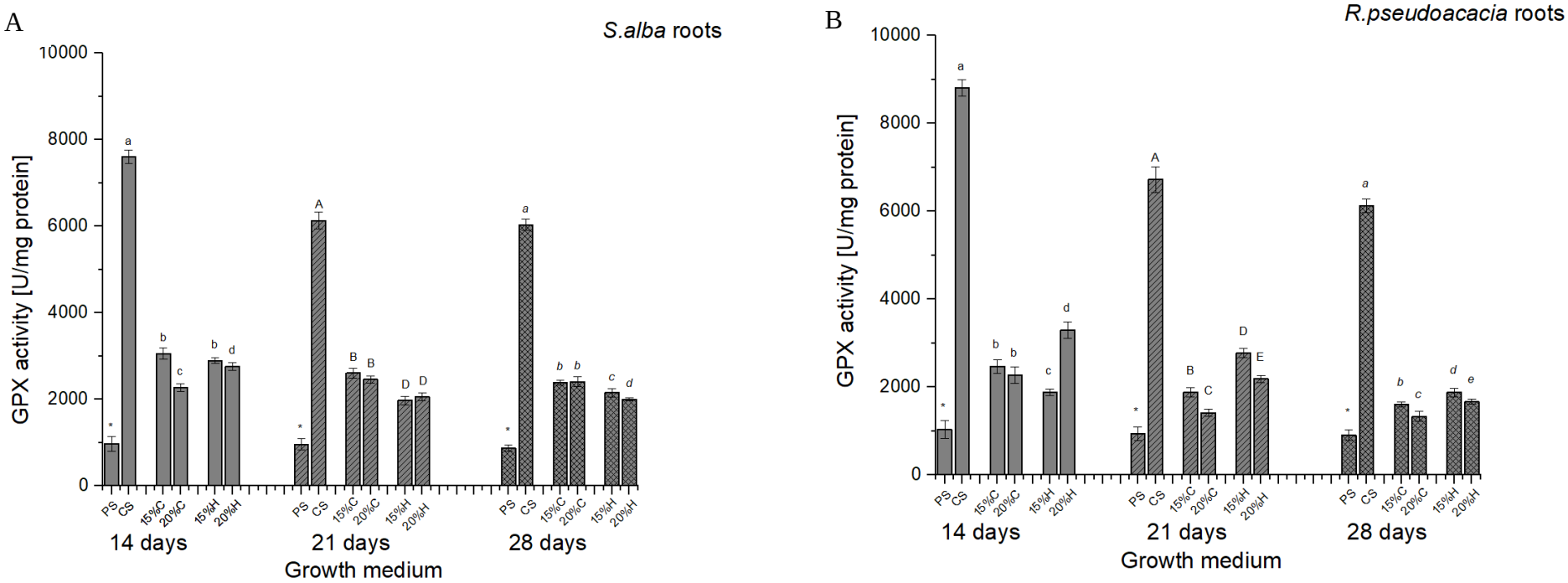

Figure 5. GPX activity in roots of *S. alba* (A) and *R. pseudoacacia* (B) after 14, 21 and 28 days of incubation (PS – control soil without any contamination, CS - contaminated soil without any fertilizers, 15%C / 20%C - CS amended with 15% / 20% cattle manure; 15% / 20% H – CS amended with 20% horse manure]. Results shown as mean ± standard deviation (n = 3). Different letters “a”, “b” “c” "d" on top of bars indicate a significant difference according to Kruskal–Wallis test ($p < 0.05$)

3.3. Protein content

Protein concentration in both shoots and roots was highly affected by soil conditions (Table 4). For both investigated species, the lowest protein content was noted for plants grown without any amendments on soil highly contaminated with heavy metals. Additions of cattle and horse manure at least doubled protein concentration in both plants. In shoots and roots of *S. alba* L. the highest protein content was observed after addition of cattle manure – approximately 3800µg/g in shoots and 300µg/g in roots, whereas in shoots and roots of *R. pseudoacacia* L. the highest protein content was observed after the addition of horse manure. In comparison to contaminated soil without any fertilizers samples grown with fertilizers shown a significant increase in protein content.

Table 4. Protein concentration in shoots and roots of *Sinapis alba* L. and *Robinia pseudoacacia* L. after 28 days incubation on various media. PS – uncontaminated garden soil, CS – contaminated soil without any fertilizers, C - cattle manure, H – horse manure. Results shown as mean ± standard deviation (n = 3). Different letters “a”, “b” “c” "d" in columns indicate a significant difference according to Kruskal–Wallis test ($\alpha < 0.05$).

Growth medium	**Protein concentration [µg/g]**			
	Shoots		Roots	
	S. alba	*R. pseudoacacia*	*S. alba*	*R. pseudoacacia*
PS	4051 ± 214.2a	2543 ± 187.4a	408 ± 32.5a	211.7 ± 24.5a
CS	1255.0 ± 74.5b	206.8 ± 14.8b	120.9 ± 54.1b	69.1 ± 5.1b
15%C	3827.3 ± 154.8c	483.9 ± 24.9c	323.3 ± 17.7c	133.7 ± 28.4c
20%C	2907.6 ± 137.1d	801.2 ± 46.1d	333.7 ± 14.8c	118.9 ± 6.8c
15%H	2138.5 ± 108.7e	1273.1 ± 62.3e	206.1 ± 11.7d	138.2 ± 38.4c
20%H	1915.7 ± 96.2f	1451.8 ± 78.2f	260.6 ± 64.5d	113.3± 6.1c

4. DISCUSSION

Organic amendments such as animal manure are applied to land mostly as a means to provide crop nutrients. It was shown before that such supplementation can also bring positive effects on highly degraded and contaminated soils (Gajewska et al. 2006., Mohammadzadeh et al. 2017). Highly sensitive stress markers can provide information about the phytoremediation efficiency and to adequately chose the dose of fertilizer in order to achieve high efficiency with lowest possible cost (Alaraidh et al., 2018). In our study, the influence of manures on plants grown on soil contaminated by HMs was assessed to check its effects as well as the sensitivity of selected stress markers in two plant species relevant for use in phytoremediation process. All chosen endpoints showed adverse effects of contamination and some level of improvement after supplementation with manure. However, GPX activity more often than other markers showed statistically significant differences between both – two different types of manure and between the 5% difference in selected doses. Germination index, shoots biomass, and roots length all showed significant effects of soil contamination in comparison to optimal growth conditions of garden soil but did not provide a considerable outcome about optimal doses of fertilizers. Therefore, in our study GPX activity turned out to be a sensitive marker for planning the process of phytoremediation of contaminated sites.

Since an increase in the activity of guaiacol peroxidase under heavy metal stress could indicate increased production of ROS and a build-up of protective mechanisms to reduce oxidative damage triggered by abiotic stress (Allen et al. 2000). In the present work, the responses of GPX activity to heavy metal stress and supplementation with manure suggest that soil contamination with heavy metals leads to a significant increase in ROS production in comparison to plants grown on peat soil without any contamination. In the present study, the addition of manure, even in the sub-optimal concentration of 15%, resulted in lowered stress levels and enabled plants to develop proper roots and shoots.

Shoots of *R. pseudoacacia* L. exhibit higher GPX activity then shoots of *S. alba* L. which is shown in control groups of plants. Nevertheless, GPX activity in roots of both plant species is approximately at the same level. Roots of plants exhibit higher vulnerability to oxidative stress due to their direct contact with soil contaminants (Alaraidh et al., 2018). In our experiment, GPX activity in roots was much greater than in shoots which could

suggest that most stresses occurred in plant roots or since plants roots are the first line of defense during any soil-related stress, such results could also mean that plants antioxidative response is more prominent in roots.

GPX activity changed significantly on different soil mixes ($\alpha > 0.05$) which suggests that presented assessment could potentially be useful assay in choosing proper additives and their concentrations for phytoremediation of degraded soils. Statistically significant ($\alpha > 0.05$) decrease in GPX activity in all selected species and all additives suggests that plants' reaction to heavy metal contamination caused the same response in overproducing of ROS and that organic additives such as manure can lower the toxic effects of heavy metals on plant's development.

In tolerant plant species like *Arabidopsis halleri*, GPX activity was found to be higher, enabling plants to protect themselves against the oxidative stress (Peters et al. 1989), whereas such activity was not observed in sensitive plants like a member of *Fabaceae* - *Vicia faba* L. (Kacprzak et al. 2014). In this experiment, the GPX activity significantly increased in both plant species – *S. alba* L. and *R. pseudoacacia* L. Significant changes in GPX activity were observed both in shoots and roots of studied plants as a result of heavy metals contamination and supplementation of degraded soil with manure. Therefore, increased GPX activity could be used to monitor the heavy metal-induced damage in both studied species.

For all tested species, the highest guaiacol peroxidase activity was reported in roots of the seedlings. This is related to their structure and function since they are the most vulnerable part of plant in regards to both biotic and abiotic stresses present in soil due to their direct contact with soil. Concerning GPX activity measured in shoots, levels in roots were on average over tenfold higher in case of both plant species. In our experiment, highest GPX activity was reported in contaminated soil without any fertilizers. This indicates a high level of abiotic stress in samples exposed to this conditions. GPX activity in plants grown on contaminated soil without any additives also decreased in time. In plants grown on contaminated soil with the addition of manure, a decrease in GPX activity was even more apparent due to a significant stress reduction.

In general, the addition of cattle or horse manure had positive results both in higher germination index and lower GPX activity compared to the plants grown on soil with no additives. This leads us to a conclusion that plants can cope with higher levels of stress in soil that is rich in nutrients. On fertile soil, plants have all the resources necessary to synthesize a wide array of enzymes and antioxidants that take part in active and passive ROS neutralization. When considering the macronutrients N and P, studies indicate that P is mainly responsible for proper growth of plants in conditions with heavy metals and in stress neutralization process (Seneviratne et al. 2016). Since the contaminated soil in this experiment did not lack in P levels and supplementation with manures influenced mostly levels of N and K, the amount of phosphorus stayed on a similar level between all used treatments (Table 1).

In most of the tests, GPX activity in both *S. alba* L. and *R. pseudoacacia* L. was lower at 28th day compared to 14th day of the experiment. GPX activity may be a viable way to estimate how the plant is coping with a difficult environment. This test can be used primarily for planning during phytoremediation processes, especially during the decision making regarding the type and the dose of soil additive.

In order to achieve high efficiency of phytoremediation, specific strategies that can optimize plants survival, growth, proper development and metal uptake must be identified. To slow down the process of desertification, further loss of organic carbon and biodiversity in degraded sites the process of phytostabilization can be used as a cost-efficient and effective method. If the goal is to extract metals from the topsoil in the process called phytoextraction, adjusting soil pH is the most efficient way to enhance metal uptake. It is well established that lowering pH levels causes an increase in metal uptake. In our study, the addition of manures induced a significant increase in soil pH values Table 1). Studied soil among high contamination with heavy metals was also characterized as acidic and with decreased levels of organic carbon. Therefore, supplementation with manure caused an increase in soil pH and significant ($\alpha < 0.05$) decrease in metal uptake. Moreover, manures have been shown to improve plants proper growth and development which can be seen in plants biomass, roots length, and GPX activity. Overall, such effects can lead to the stabilization of contaminated sites since it can slow down the process of carbon loss by creating plant cover on top of the

contaminated soil. An increase in pH and decrease in metal uptake is also a preventive method which decreases the transport of metal contamination through the food chain.

Total protein concentration was also highly affected by contamination with heavy metals (Table 3). In shoots and roots of both plant species, a significant decrease in total protein content was noticed in plants grown on contaminated soil without any additives. Highest reduction of total protein content was found in roots of plants – for *S. alba* L. total protein content in roots was decreased approximately 6 times in comparison to peat soil, and for *R. pseudoacacia* L. even 17 times. Supplementation of degraded soil with manure caused an increase in protein concentration in studied plants. The highest increase was noticed in shoots, where the concentration of proteins after supplementation even exited beyond a concentration on peat soil without contamination. However, in roots of plants, an increase in total proteins was also significant but much lower than in shoots. The roots remained in direct contact with toxic heavy metals so that the effects of metals on plant growth remained visible also after supplementation. Similar results were obtained in studies researching the impact of fertilizers on drought stress (Adiloglu et al. 2017).

The highest increase in protein concentrations in *Sinapis alba* L. roots was observed after application of cattle manure at a concentration of 20% (Table 4). The concentration of total proteins in samples grown on contaminated soil with 20% cattle manure was nearly 3 times higher in comparison to contaminated soil without any additives. In roots of *R. pseudoacacia* L., there was a 2-fold increase in protein concentration with 15% horse manure.

5. CONCLUSIONS

Two plant species: *S. alba* L. and *R. pseudoacacia* L. were tested to assess the toxicity of degraded soils contaminated with heavy metals. The measurement endpoints used were: seed germination, guaiacol peroxidase activity (GPX) and protein content for both shoots and roots of seedlings. Seed germination was reported to be a less sensitive endpoint, and it is often inconclusive as toxicity indicator. The study investigated the influence of manures on plants grown under abiotic stress caused by heavy metal contaminated soil. Heavy metals in soil contaminated by the metallurgical industry caused a decrease in plants roots length, germination and content of proteins whereas increased the activity of GPX. Results showed that determination of GPX activity can be the useful assessment during the process of large-scale phytoremediation planning. Overall, the GPX activity showed significantly different results for the majority of different treatments and chosen doses. More research, especially field studies are needed in the future in order to develop more sensitive biomarkers that could be used for precise remediation planning.

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Antioxidative enzymes and expression of *rbcL* gene as tools to monitor heavy metal-related stress in plants*

Marta Jaskulak[1*], Agnieszka Rorat[2], Anna Grobelak[1], Małgorzata Kacprzak[1]

[1]*Institute of Environmental Engineering, Faculty of Infrastructure and Environment, Czestochowa University of Technology, Czestochowa, Poland;*

[2]*Université Lille Nord de France, LGCgE-Lille 1, Ecologie Numérique et Ecotoxicologie, F-59650 Villeneuve d'Ascq, France;*

Abstract:

The aim of the study was to evaluate sensitivity and potential applications of selected biomarkers in phytoremediation under complex heavy metal contamination in *Sinapis alba* L., *Robinia pseudoacacia* L. and *Lupinus luteus* L., as a potential tools in effective phytoremediation management. The toxicity assessment was conducted using selected measurement endpoints, both classical and advanced, i.e., germination index, roots length, guaiacol peroxidase activity (GPX), chlorophyll and protein content, the amount of total phenolic compounds (TPC) and level of expression of one of the ribulose-bisphosphate carboxylase genes (*rbcL*). Moreover, the influence of organic additives: cattle, horse manure, and vermicompost on lowering plant abiotic stress caused by complex heavy metal contamination was studied to assess the possible applications of selected stress markers in large scale phytoremediation planning. The results demonstrated the beneficial effects of selected soil additives on plant development. The 5% difference in the quantity of applied amendment caused statistically significant differences in GPX, TPC, chlorophyll content and expression level of *rbcL*. Among all endpoints, GPX activity, chlorophyll, and phenolic compounds content, as well as the expression of *rbcL*, turned out to be the most reliable assays for determination of the type and dosage of selected soil amendments (fertilizers) in the assisted phytoremediation process. Selected markers can be used to achieve the desired level of plant abiotic stress and consequently photosynthesis efficiency and CO_2 sequestration. The results showed, that presented assays can be used in different taxonomical groups such as *Fabaceae* for planning effective phytoremediation process.

KEYWORDS: phytotoxicity, plant toxicology, bioremediation, heavy metals, soil toxicology, biomarkers, phytoremediation

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Highlights

- Phenolic compounds and ribulose-bisphosphate carboxylase are key stress markers
- GPX activity, chlorophyll, phenolic compounds content, expression of *rbcL*, can be used as a tool in phytoremediation planning
- Presented assays can be used not only for model plants in testing the effective phytoremediation

1. INTRODUCTION

The pollution of soil is becoming a fast-rising issue worldwide due to a vast presence of anthropogenic actions such as mining and manufacturing (Bolan et al., 2014; Pant and Tripathi, 2011). Industrial waste make soil useless for many years by lingering at its surface. Moreover, agriculture was recognized as the second largest contributor to soil degradation (Motuzova et al., 2014). Therefore, pollutants seep into the deeper layers of the ground and pose a threat to groundwater reservoirs (Tóth et al., 2016; Mahar et al., 2016; Tarek et al., 2015). In addition, soil pollution can increase oxidative stress in plants and thus, decrease plant growth gradually, causing a significant reduction in photosynthesis efficiency (Chabukdhara and Nema, 2013; Caverzan et al., 2014). Organic fertilizers, such as compost or manure, can provide a broad spectrum of nutrients, which prevent symptoms of deficiencies in plants. Moreover, such supplementation of soil can decrease the level of oxidative stress by increasing plant resistance to pollutants and also improving the efficiency of phytoremediation, sequestration and overall quality of yield and soil (Grobelak., 2016). Therefore, the use of organic fertilizers such as compost or manure is a common practice for sustaining economically viable crop production with minimal environmental pollution as an alternative to chemical fertilizers (Wloka et al., 2017). Organic fertilizers can also promote microbiological activity in soil, leading to the enhancement of soil quality since plants depend on microorganisms to mineralize organic matter (Grobelak et al., 2015). Beneficial effects of using manure were reported mainly concerning drought stress in plants (Kakoei and Salehi, 2013; Wang et al., 2012) but also to heavy metals exposure, e.g., lead (Qiao and Libault, 2013). Among organic amendments, manure can have potential applications in phytoremediation of degraded soils, because of its abundance, accessibility and low price.

Phytoextraction, one of the most commonly used phytoremediation methods, implies the use of hyperaccumulators to extract and accumulate heavy metals from soils, followed by harvesting plant biomass until the concentration of specific contaminant decreases to an acceptable level (Dimkpa et al., 2009; Ullah et al., 2015). Several hyperaccumulator plants have been identified, and many are still being investigated for continuous removal of heavy metals from soil and water (Ent et al., 2012; Chibuike and Obiora, 2014). For instance, *Fabaceae* plants such as *Robinia pseudoacacia* L. and *Lupinus luteus* L. create a symbiosis with nitrogen-fixing bacteria, leading to an improvement in soil quality and

allow other, less resistant species to sprout on such areas over time (Alavi et al., 2014). Other studies indicate the plant growth promoting rhizobacteria (PGPR) in improving plant resistance to abiotic factors (Ullah et al., 2015). Nevertheless, still little is known about the specificity of those stress biomarkers and their proper practical application. One of the most commonly used biomarkers of oxidative stress in plants can be divided into two major groups (Murtaza and Asghar, 2013). The first one uses biochemical markers that include measuring the activity of specific enzymes such as superoxide dismutase (SOD), catalases or peroxidases (POD) (Alscher et al., 2002). The content of chlorophyll, phenolic compounds, and overall proteins can also be used to assess the influence of different pollutants or fertilizers on plant metabolism since they are highly sensitive to changes in the environment. The second group uses a vast range of genetic markers to assay such effects on the growth and development of plants (Pal et al., 2010).

In the presented study, the biomarkers mentioned above functioned as tools to assess the possibility and predictability of organic soil amendments application for phytoremediation of areas highly polluted by metallic trace elements. In those terms, the main aim of the study was to: (1) assess the impact of particular organic additives (cow/horse manure, vermicompost) on selected parameters involved in immune defence (potential biomarker), namely content of phenolics, chlorophyll, proteins, expression of the *rbcL* gene in three selected plant species; (2) select the biomarkers of stress for reliable assay in determining the most effective methodology of large scale phytoremediation.

2. MATERIALS AND METHODS

2.1. Substrates characterization

Soil used in this experiment was derived from highly degraded and heavy metal contaminated areas in Poland (GPS: 50º30'N 18º56'E). Soil samples were collected from the surface layer (0 - 30 cm) of the landless area contaminated mostly from metallurgical industry.

Besides the very high content of heavy metals, mainly cadmium (Cd), lead (Pb) and zinc (Zn), the soil exhibited deficient microbiological activity, fertility, and sorption capacity (Table 1). Before planting seeds, the soil was dried, sieved and thoroughly mixed with selected organic fertilizers: cattle manure, horse manure and vermicompost in particular concentrations: 10, 15, 20% by dry weight. The doses of manure and vermicompost used in the experiment were selected to meet the EU nitrogen standards (170 kg of nitrogen/year/ha) (EUR-Lex - 31991L0676 – EN).

The difference between applied doses - 5% was intended to estimate the sensitivity of selected stress markers between slight differences in dosages of soil additives. The control group consisted of organic peat soil without any contamination providing optimum growth conditions to fully assess the influence of contaminated soil on plant development in comparison to their natural capabilities on clean soil without any abiotic stressors. Organic additives used in the experiment were derived from commercially available sources (FLORMIX, Poland) and were tested for their chemical and physical properties (Table 1).

Cattle manure used in the experiment was characterized by a nitrogen content of approximately 5.3 g kg^{-1} dry weight, phosphorus - P – 1.1 g kg^{-1} dry weight and potassium – 4.1 g kg^{-1} dry weight. Horse manure contained a higher content of NPK, including approximately 6.4 g kg^{-1} dry weight of nitrogen, 2.3 g kg^{-1} dry weight of phosphorus and 4.9 g kg^{-1} dry weight of potassium. In Vermicompost the content of nitrogen was approximately 6.9 g kg^{-1} dry weight, the content of phosphorus – 2.2 g kg^{-1} dry weight and 5.1 g kg^{-1} dry weight of potassium.

After preparation of soil mixtures, samples were collected to perform physical and chemical assays. The pH values of the soil samples were measured in distilled water and 1M solution of KCl according to ISO 10390:2005. For pH determination, standard laboratory pH-meter was used (Cole Parmer Model No. 59002–00). Cation-exchange capacity (CEC) was determined by the Kappen method (Kappen et al., 1995). The content of total organic nitrogen in samples was established by the Kjeldahl method (PN-ISO 11261:2002) (Bradstreet, 1954), while the content of phosphorus and potassium by the Egner method (Egner et al., 1960). A concentration of heavy metals was measured by ICP-OES (ICP-OES; Thermo apparatus, USA). Samples were digested in a microwave digestion system according to the EPA method 3051.

Table 1. Soil and soil + amendment mixture characterization: PS – peat soil without contamination, CS - contaminated soil without any additives, CM – soil + cattle manure, HM –soil + horse manure, W - soil + vermicompost. Results shown as mean ± standard deviation

Growth	**pH**		**CEC**	**N**	**P_2O_5**	**Cd**	**Pb**	**Zn**
medium	H_2O	KCl	**[cmol/kg]**	**[%]**	**[mg/100g]**	**[mg kg^{-1}]**	**[mg kg^{-1}]**	**[mg kg^{-1}]**
PS	5.61 ± 0.05	7.12 ± 0.11	33.1 ± 0.21	0.81 ± 0.07	1.05 ± 0.2	1.05 ± 0.2	7.39 ± 1.11	27.4 ± 2.2
CS	5.45 ± 0.04	3.60 ± 0.06	25.2 ± 0.09	0.25 ± 0.12	1.07 ± 1.1	**19.5 ± 1.2**	**1133.8 ± 3.5**	**733.2 ± 1.4**
10% CM	6.92 ± 0.28	7.56 ± 0.04	27.7 ± 0.14	0.64 ± 0.05	09.4 ± 1.5	19.7 ± 2.1	1106.41 ± 13.8	700.1 ± 3.41
15% CM	7.22 ± 0.02	8.08 ± 0.21	30.6 ± 0.04	0.71 ± 0.22	1.9 ± 1.5	20.4 ± 1.4	1135.2 ± 24.8	689.4 ± 8.1
20% CM	6.92 ± 0.28	6.50 ± 0.13	31.9 ± 0.17	0.75 ± 0.18	2.1 ± 1.2	19.9 ± 1.1	1153.1 ± 18.8	689.8 ± 6.1
10% HM	6.97 ± 0.11	7.13 ± 0.08	27.7 ± 0.11	0.71 ± 0.07	1.7 ± 1.5	18.5 ± 1.2	1020.8 ± 29.2	632.3 ± 6.3
15% HM	6.95 ± 0.02	7.02 ± 0.11	29.1 ± 0.21	0.75 ± 0.13	1.7 ± 0.8	18.7 ± 1.6	1042.1 ± 18.3	622.1 ± 7.8
20% HM	5.45 ± 0.04	3.62 ± 0.06	32.2 ± 0.09	0.76 ± 0.16	1.7 ± 0.6	17.7 ± 0.9	969.3 ± 19.5	591.4 ± 11.4
10% W	6.90 ± 0.11	7.33 ± 0.04	27.7 ± 0.14	0.76 ± 0.03	1.6 ± 0.4	18.7 ± 0.8	1116.2 ± 7.3	713.2 ± 5.2
15% W	7.41± 0.14	7.40 ± 0.21	30.6 ± 0.04	0.79 ± 0.07	1.2 ± 0.9	17.6 ± 0.4	1071.0 ± 16.3	621.1± 5.5
20% W	6.25 ± 0.02	6.20 ± 0.13	33.9 ± 0.17	0.81 ± 0.14	1.7 ± 1.1	17.2 ± 0.7	952.4 ± 17.8	617.4 ± 7.1

2.2. Experiment procedure

Three plant species: *S. alba*, *R. pseudoacacia* and *L. luteus*, were grown separately in 11 different soil mixtures, in three replicates. Commercially available, certified and high-quality seeds were used (Flormix, Poland). Each pot (H – 15 cm, a – 12 cm) contained 300 g soil and 20 seeds sown approximately two cm deep in the soil. Plants were then incubated for 28 days in a growth chamber (Biogenet FS360, Poland) under controlled conditions: photoperiod: 16 h light 8 h dark, temperature during the day 21^{o}C, night 18^{o}C, light intensity 4000 lx (photosynthetic LED light). After 28 days, plants were extracted from soil and biomass was stored in -80^{o}C for further analyses. Germination was recorded every 24 h for 14 days and was considered to occur when radicles were at least 2 mm long. All seedlings with short, deformed or spiral formed hypocotyls were considered as abnormally germinated. Germination index was after calculated as a percentage of properly developed seedlings. Plant growth parameters like biomass, foliar surface, chlorosis or signs of fungi infection have been assessed. No significant variation between conditions was observed (data not shown).

2.3. Guaiacol peroxidase activity assay

The activity of GPX was established by the Asada method (Asada, 2006) with modifications (Sharkey, 2005, Caverzan et al., 2012;). Briefly, GPX activity was estimated by a level of guaiacol oxidation in the presence of hydrogen peroxide within the 2-minute period. Approximately 100 mg of each plant shoot material was mixed with 3 mL of phosphate buffer (pH 6), and homogenized in a mortar. The homogenate was centrifuged at 11000 g for 5 minutes. A guaiacol reaction mixture prepared in plastic cuvettes for spectrophotometric analysis contained 3 mL of guaiacol reagent (100 mM potassium phosphate buffer, pH 7.4 and 0.35% guaiacol). For each sample, 10μL of the supernatant obtained after homogenization and 10μL of 30% hydrogen peroxide was added, mixed and immediately measured spectrophotometrically. Absorbance was measured at λ 430 nm after 2 minutes of guaiacol oxidation. Peroxidase activity was defined as an amount of GPX that produces a change in absorbance at 430 nm of 0.021/min (HACH DR/4000V, USA). Results were normalized to U/mg of determined protein concentration in plant biomass.

2.4. Protein concentration

The assay performed in test tubes is essentially a micro Lowry assay (Lowry et al., 1951). A standard curve was prepared as follows. Standard bovine serum albumin (BSA) (Sigma-Aldrich, USA) powder was dissolved in distilled water and diluted to a concentration of 1μg μL^{-1}. A series of dilutions (0.1, 0.2, 0.5, 1, 2.5, 5, 10, and 20μg/μL) were made in triplicates with a final volume of 100μL. Subsequently, standards and samples were diluted and transferred to the test tubes; 200μL of Lowry reagent was added to each well and mixed thoroughly with repeated pipetting and vortexing. A Lowry reagent was prepared by mixing 0.5 mL of 1% cupric sulphate with 0.5 mL of 2% sodium potassium tartrate, an addition of 50 mL of 2% sodium carbonate in 0.1M NaOH. The mixture was then incubated at room temperature in a dark area for 30 minutes prior to the addition of 20μL per sample of 0.1 mL Folin & Ciocalteu's reagent (Sigma-Aldrich, USA). Samples were mixed immediately with repeated pipetting and vortexing and then incubated for 30 minutes at room temperature in a dark place. Then, the absorbance was measured at 650 nm with a HATCH spectrometer (HACH DR/4000V, USA) (a blank sample consisted of reaction reagents without a sample).

2.5. Total chlorophyll content

Total leaf chlorophyll was extracted by homogenizing 100 mg of leaf fresh weight in 10 mL of 80% acetone. After centrifugation for 15 min at 6000 g, chlorophyll contents in supernatants were analysed spectrophotometrically (HACH DR/4000V, USA) following the method of Aaron (Aaron et al., 1949), at wavelengths 663 nm and 645 nm.

2.6. Total phenolic compounds content (TPC)

The total phenolic content was determined using the Folin-Ciocalteu method (Marinova et al., 2005). Briefly, 100 mg of plant shoots were mixed with 2.5 mL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, USA) and kept for 5 min at 37ºC. Then, 2 mL of saturated Na_2CO_3 (7,5%) was added, and the mixture was brought to 10 mL with the addition of deionized water. The mixture was maintained at room temperature in the dark for 120 minutes, and the absorbance of the mixture was measured at 765 nm against a reagent blank using a spectrometer (HACH DR/4000V, USA). Gallic acid equivalent (GAE) (Chempol, Poland) was used as the reference standard to perform a standard curve, and

the total phenolic content was expressed as mg of GA equivalents per gram of a plant's fresh weight.

2.7. Extraction of RNA and reverse transcription

Expression analyses were conducted on total RNA extracted from shoots of plants by using the Eur_x Universal DNA/RNA/Protein kit (Eur_x, Poland) according to the manufacturer's instructions. 100 mg of plant fresh shoots were used for each extraction. The quantity and purity of isolated RNA was measured using a spectrometer (Eppendorf BioPhotometer D30, USA) with a nanodrop. RNA integrity was also confirmed on 1.5% agarose gel stained with SimplySafe® (Eur_x, Poland). Reverse transcription of 1.5 μg total RNA was performed using NG dART RT-PCR kit (Eur_x, Poland) according to manufacturer instructions.

2.8. Real-time PCR

The mRNA levels of *rbLc* genes were determined by real-time quantitative PCR after reverse transcription and were performed using the Eppendorf MasterCycler® realplex (Eppendorf, USA). Levels of expression and amplification effectiveness of target *rbLc* gene was compared to the expression of constitutively expressed *actinβ* and *18S*.
The primers for *actin* were: (Chandna et al., 2012)
F: 5′-CGCCATCCAGGCTGTGCTTTC- 3′, R: 5′-GATGGTGTCAGCCATACGTG-3′, for *18S*: (Duclos and Björkman, 2008)
F: 5′GGAGTATAAGTCGTAACAAG- 3′, R: 5′ CCTATCAATTAGAGGAAGGAG- 3′, for *rbcL*: (Levin et al., 2003)
F: 5′-GTTGATTTACTGCGCGATGA- 3; R: 5′GAGCTACTCGGTTGGCTACG- 3;

All real-time PCR reactions were performed using SYBR Green Master Mix (Eur_x, Poland). The thermocycler program for all reactions included an initial denaturation step at 95 °C for 5 min, followed by 40 amplification cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72°C for 45 s followed by final extension at 72°C for 7 minutes. The relative expression level of *rbcL* gene was calculated according to the formula $R=(ETg)^{CPTg}/(Eref)^{CPref}$ and normalized to gene expression of plants grown in organic peat soil without any contamination. The induction factor was measured by quantitative real-time PCR and in all cases, it is the ratio of the relative *rbcL*

concentration in contaminated samples to that in a plant grown on clean, peat soil. Moreover, in each case, experiments were performed in three biological replications and two technical replicates.

2.9. Statistical analysis

The results are expressed as means ± standard deviation, n = 3. Tukey's test ($p < 0.05$), after ANOVA showed a significant effect, the same letters indicate the non-significant difference (ANOVA $p > 0.05$). Descriptive statistics and statistical tests were produced using the OriginPro 2015 software. A Horizontal line indicates plants grown in peat soil without any contamination. Different letters "a", "b" "c" "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey's test $p < 0.05$. For all tests, p values below 0.5 were considered statistically significant.

3. RESULTS

3.1. Germination index and roots length

Germination was recorded every 24 h for the whole incubation period of 28 days and was considered to occur when radicles were at least 2 mm long. Germination was highly influenced by contaminated soil and used additives in *S. alba*, but there was no influence in *Fabaceae* plants *(R. pseudoacacia* and *L. luteus)* due to their ability to store high concentrations of nutrients in seeds (Figure 1). The use of organic fertilizers has shown a positive effect on a number of seedlings only in *S. alba*. Moreover, no significant differences ($p < 0.05$) were observed between seedlings grown with different concentrations of specific fertilizer for all tested plant species.

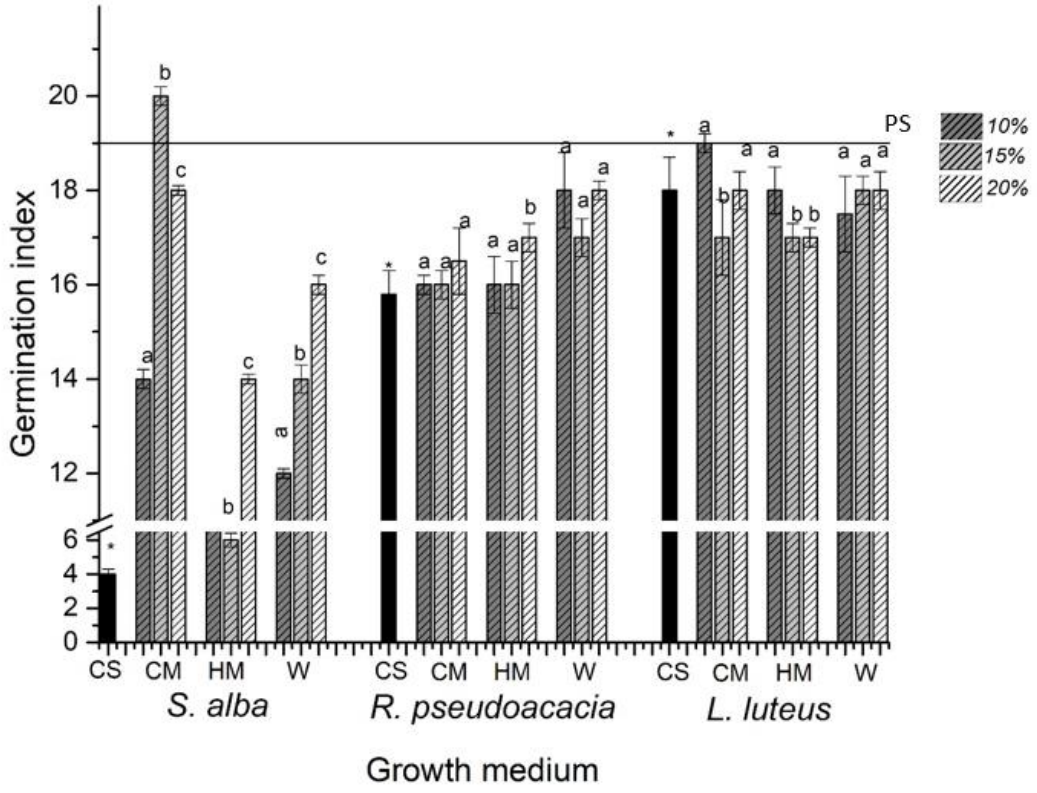

Figure 1. Germination index of *S. alba, R. pseudoacacia and L. luteus* grown on different mediums. (CS – contaminated soil without any fertilizers, CM – CS + cattle manure, HM – CS + horse manure, W – CS + vermicompost. The horizontal line (PS) indicates average root length in plants grown on clean peat soil without any contamination. Different letters "a", "b" "c" "d" on top of bars indicate a significant difference according to ANOVA with

post-hoc Tukey's test $p < 0.05$). The "*" indicates a significant difference between all groups according to ANOVA. Results shown as means ± standard deviation, n = 3.

Root development was strongly decreased by heavy metals in all plant species (Fig. 2). Addition of manure or vermicompost to contaminated soil caused the development of stronger and denser roots in all plants and all fertilizers but there was no significant difference ($p < 0.05$) between the doses of used additives.

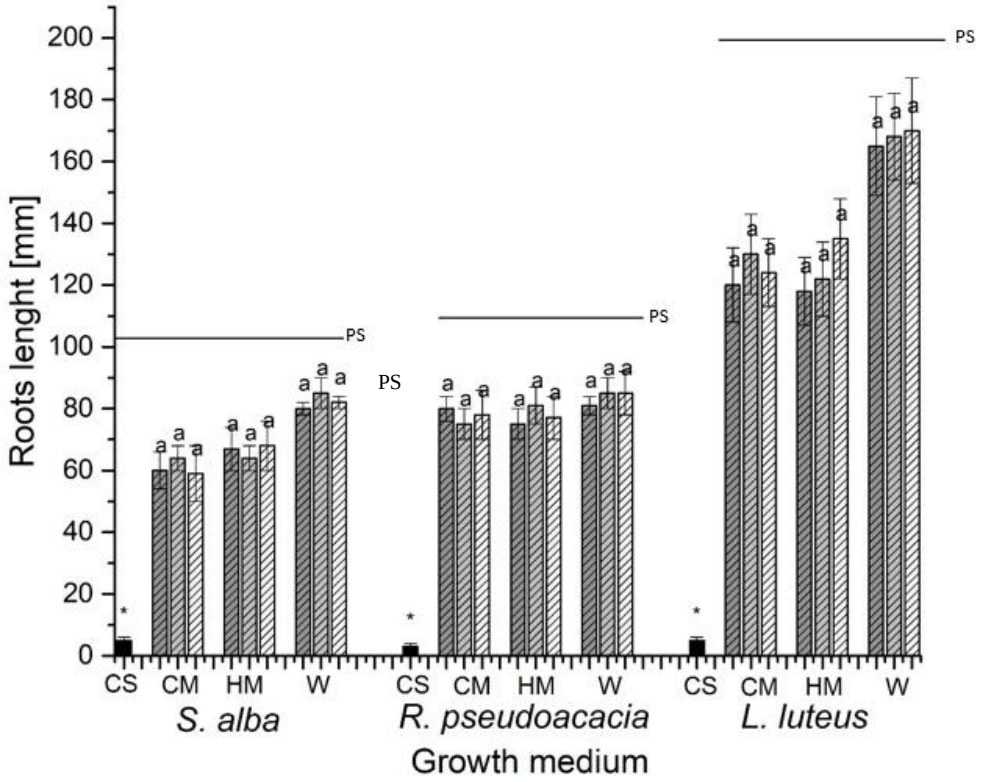

Figure 2. Roots length [mm] of *S. alba, R. pseudoacacia and L. luteus* grown on different mediums. (CS – contaminated soil without any fertilizers, CM – CS + cattle manure, HM – CS + horse manure, W – CS + vermicompost. The horizontal line (PS) indicates average root length in plants grown on clean peat soil without any contamination. Different letters "a", "b" "c" "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey's test $p < 0.05$). The "*" indicates a significant difference between all groups according to ANOVA. Results shown as means ± standard deviation, n = 3.

3.2. GPX activity

Exposure to heavy metals in contaminated soil significantly inhibited the growth of three tested species, which is confirmed by an increase in GPX activity. Total guaiacol peroxidase activity for all plants was at its highest point on contaminated soil without any organic additives (CS) and decreased progressively with increasing concentrations of manures and vermicompost (Fig. 3.). The largest decrease in GPX activity occurred in samples grown with 20% vermicompost (W). The activity of guaiacol peroxidase is species-specific: *Fabaceae* species revealed much higher GPX activity then *S. alba*. Supplementation of contaminated soil with cattle/horse manure or vermicompost in even the lowest concentration (10%) decreased the level of GPX activity by at least by half compared to degraded soil without any additives.

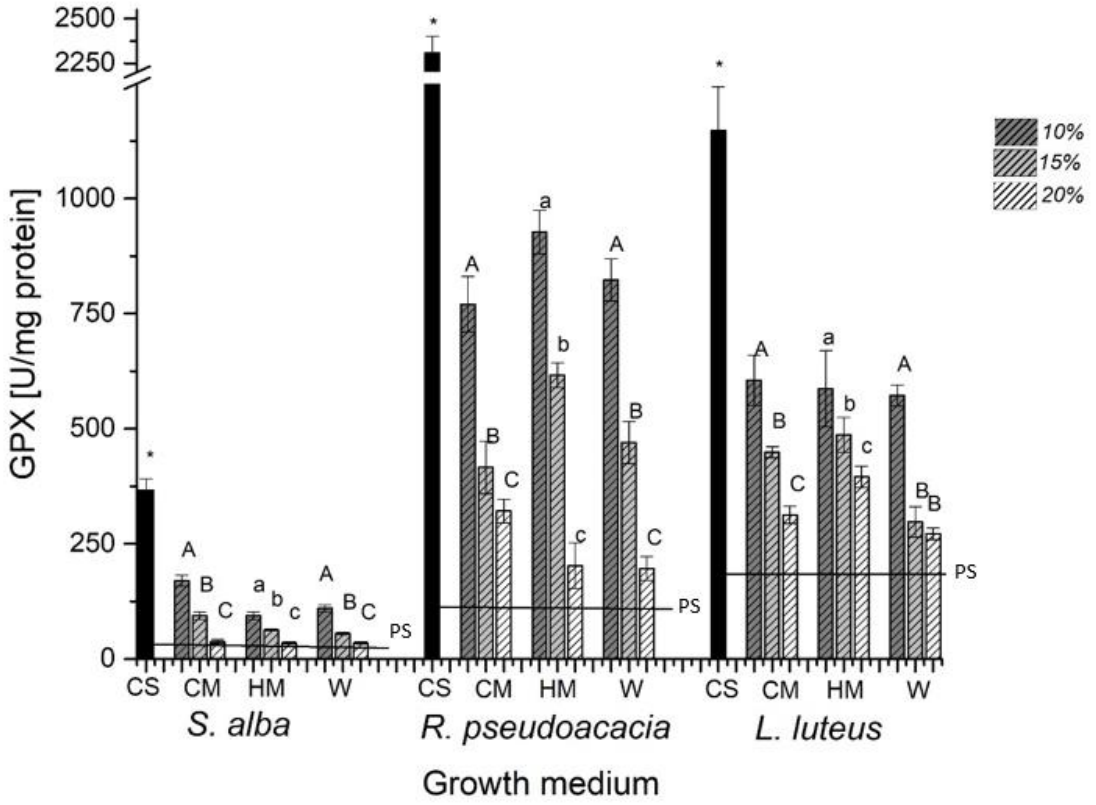

Figure 3. GPX activity [U/mg protein] in shoots of S. alba, R. pseudoacacia and L. luteus grown on different mediums. (CS – contaminated soil without any fertilizers, CM – CS + cattle manure, HM – CS + horse manure, W – CS + vermicompost. The horizontal line (PS) indicates average root length in plants grown on clean peat soil without any contamination. Different letters “a”, “b” “c” "d" on top of bars indicate a significant

difference according to ANOVA with post-hoc Tukey's test $p < 0.05$). The "*" indicates a significant difference between all groups according to ANOVA. Results shown as means ± standard deviation, n = 3.

3.3. Protein content

Heavy metals contamination and the use of organic additives had a significant impact on total protein content in shoots of all tested species (Fig. 4). The lowest protein concentration was noticed on contaminated soil without any additives (CS) and the highest on contaminated soil with 20% of vermicompost (W). Differences in protein content between a specific concentration of organic fertilizers were statistically significant ($p < 0.05$) in almost all samples with the exception of cattle manure between 10 and 15%.

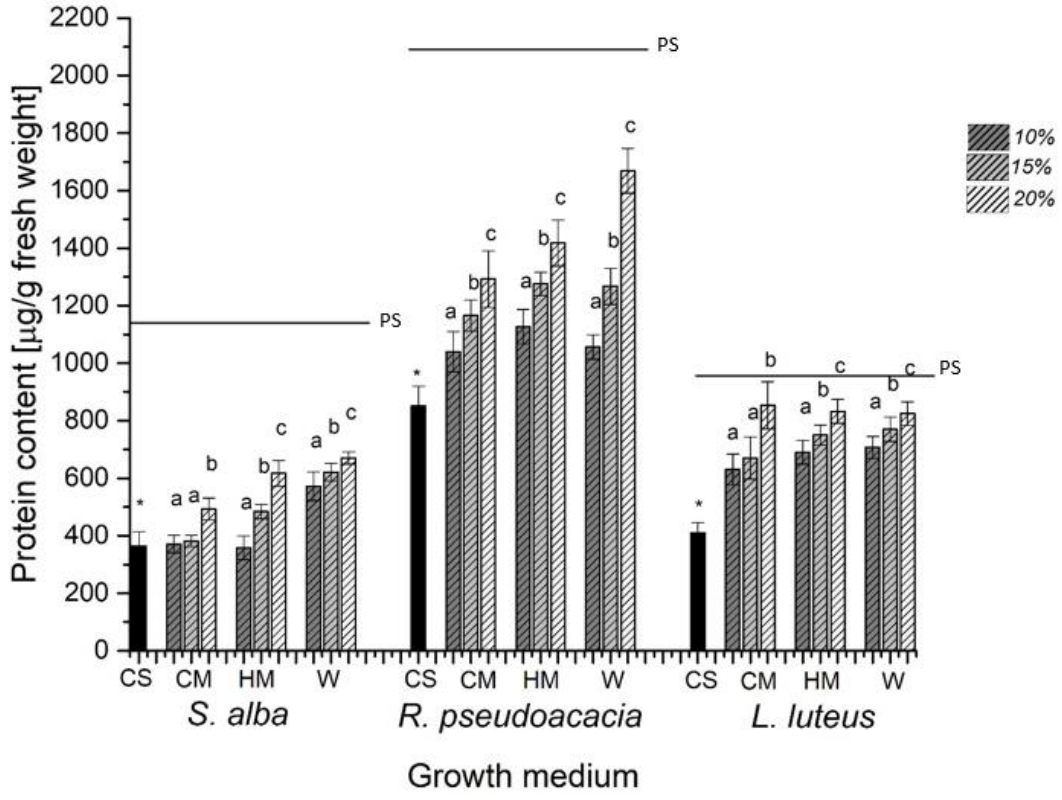

Figure 4. Total protein content [µg/g fresh weight] in shoots of *S. alba, R. pseudoacacia and L. luteus* grown on different mediums. (CS – contaminated soil without any fertilizers, CM – CS + cattle manure, HM – CS + horse manure, W – CS + vermicompost. The horizontal line (PS) indicates average root length in plants grown on clean peat soil

without any contamination. Different letters “a”, “b” “c” "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey’s test $p < 0.05$). The “*” indicates a significant difference between all groups according to ANOVA. Results shown as means ± standard deviation, n = 3.

3.4. Chlorophyll content

It was found that, that in plants grown in soil contaminated with heavy metals, especially Cd, Pb and Zn, the significant reduction of shoots chlorophyll content in *S. alba*, *R. pseudoacacia* and *L. luteus* (Fig. 5) was noted. In comparison to plants grown on organic peat soil without contamination, chlorophyll content was at least two times lower on degraded soil. The highest increase in chlorophyll content on the degraded soil was noticed after soil treatment with vermicompost for *S. alba*.

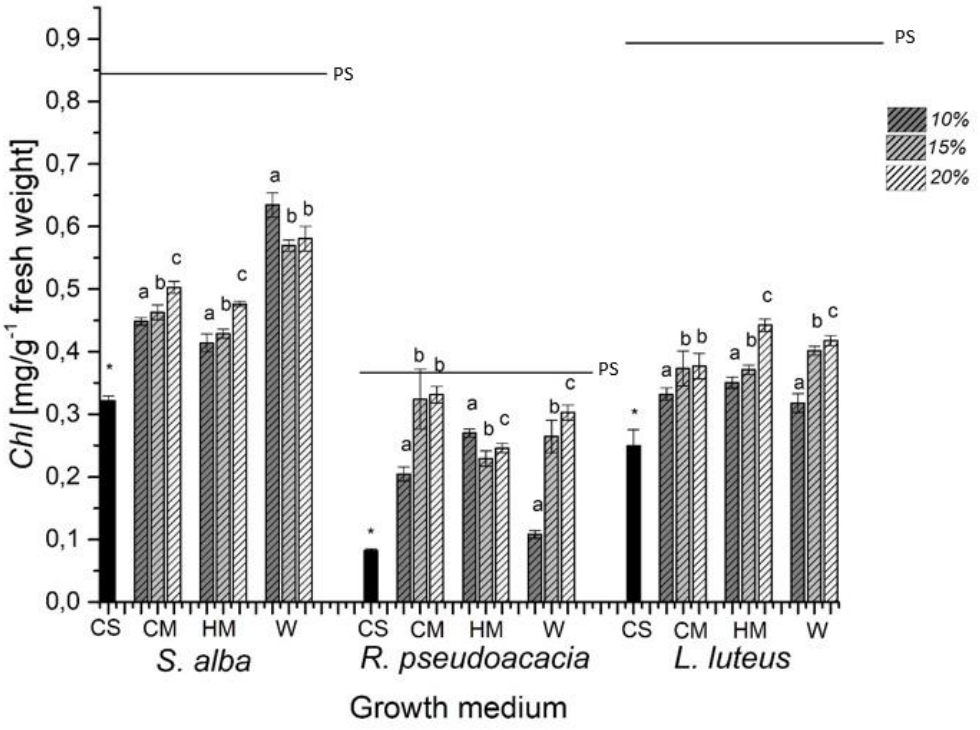

Figure 5. Total chlorophyll content [mg/g^{-1} fresh weight] in shoots of *S. alba, R. pseudoacacia and L. luteus* grown on different mediums. (CS – contaminated soil without any fertilizers, CM – CS + cattle manure, HM – CS + horse manure, W – CS + vermicompost. The horizontal line (PS) indicates average root length in plants grown on clean peat soil without any contamination. Different letters “a”, “b” “c” "d" on top of bars

indicate a significant difference according to ANOVA with post-hoc Tukey's test $p < 0.05$). The "*" indicates a significant difference between all groups according to ANOVA. Results shown as means ± standard deviation, n = 3.

3.5. Total phenolic compounds content

The content of total phenolics compounds (TPC) was observed at its highest point for plants grown in contaminated soil, and an addition of manure/vermicompost caused a significant decrease in TPC in all tested species and soil treatments (Fig. 6). Moreover, statistically significant differences in TPC were noted between different concentrations of each amendment. Among all biomarkers only TPC showed statistically significant differences between 10, 15 and 20% dosage for each organic fertilizer. This indicated that TPC could be applied in phytoremediation studies, and a toxicity test could be used as a proper assay to identify very precise and optimal type and concentration of fertilizer.

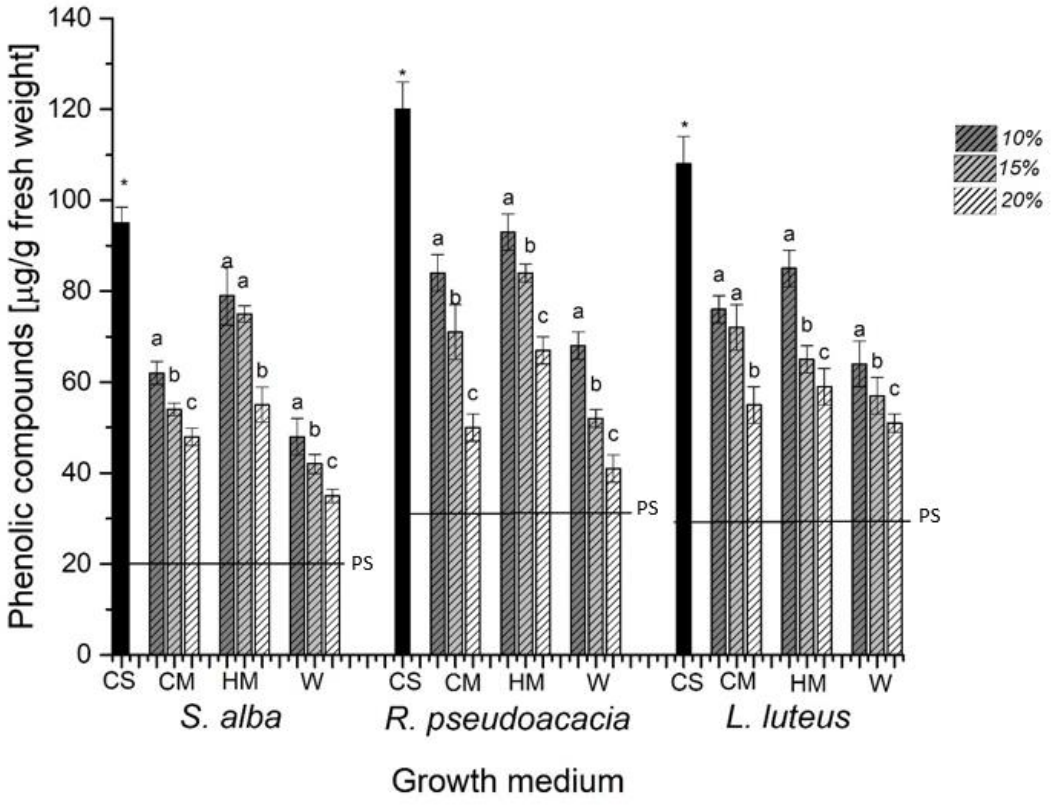

Figure 6. Total content of phenolic compounds (TPC) [µg/g fresh weight] in shoots of *S. alba, R. pseudoacacia and L. luteus* grown on different mediums. (CS – contaminated soil without any fertilizers, CM – CS + cattle manure, HM – CS + horse manure, W – CS

+ vermicompost. The horizontal line (PS) indicates average root length in plants grown on clean peat soil without any contamination. Different letters “a”, “b” “c” "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey’s test $p < 0.05$). The “*” indicates a significant difference between all groups according to ANOVA. Results shown as means ± standard deviation, n = 3.

3.6. Expression of *rbcL* gene

The level expression of the *rbcL* gene gradually increased with increasing concentrations of organic additives in all investigated plants: *S. alba*, *R. pseudoacacia* and *L. luteus* (Fig. 7). Presented results of the *rbcL* expression were normalized to the level of *rbcL* expression in plants grown on organic peat soil without any contamination (induction factor set as 0). Plants grown in contaminated soil without any fertilizers showed the highest decline in in *rbcL* expression and photosynthesis efficiency. For all three plant species, an addition of 15 or 20% of vermicompost caused the highest increase in *rbcL* expression approaching the level of *rbcL* in plants grown in soil without any contamination (organic peat soil).

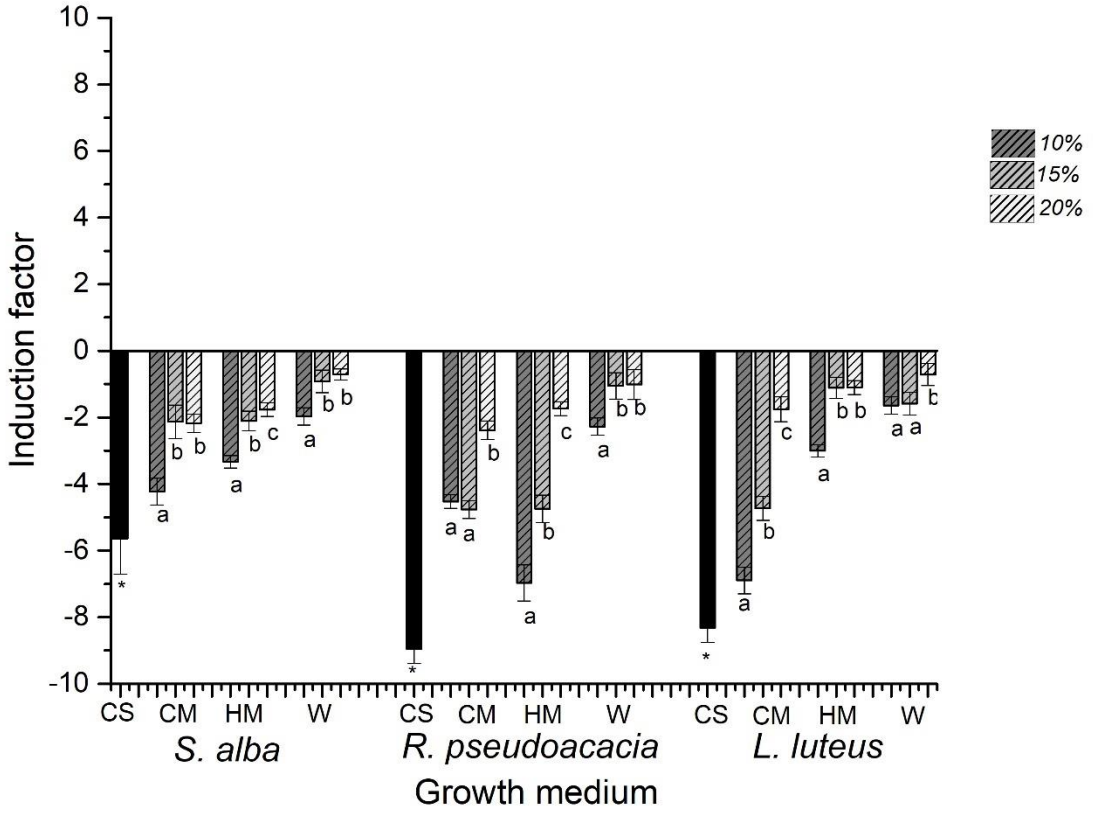

Figure 7. Induction factor of *rbcL* gene in shoots of *S. alba, R. pseudoacacia and L. luteus* grown on different mediums. (CS – contaminated soil without any fertilizers, CM – CS + cattle manure, HM – CS + horse manure, W – CS + vermicompost. The horizontal line (PS) indicates average root length in plants grown on clean peat soil without any contamination. Different letters “a”, “b” “c” "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey’s test $p < 0.05$). The “*” indicates a significant difference between all groups according to ANOVA. Results shown as means ± standard deviation, n = 3.. Results were normalized to level of *rbcL* expression in plants grown on clean peat soil without any contamination (horizontal line). Results shown as means ± standard deviation, n = 3.

4. DISCUSSION

The necessity for accurate and quick toxicity assessment will undoubtedly gain importance both as a research tool and as a means to assess the viability of soil to be used for crop production and phytoremediation, mostly for determination of the influence of different chemicals, fertilizers and organic additives on soil quality and effectiveness of conducted processes. Toxic and inhibitory effects of heavy metals, including Cd, Pb, and Zn on plant growth and development have been reported in a number of previous studies (Mortel et al., 2008; Gajewska et al., 2005; Gajewska et al., 2006; Mirzahosseini et al., 2014). Most metals cause increased formation of reactive oxygen species (ROS) within the plant tissues, inducing oxidative stress and causing a cellular redox imbalance (Sharma and Dubey, 2007). In mine lands, there are some significant constraints to plant survival, and the success of restoration is dependent upon overcoming these problems. In our study, the metal concentration in shoots of selected plant species was not affected significantly by the addition of organic fertilizers. As seen in tested biomarkers, fertilizers did lower the levels of oxidative stress, improved the quality of yield and the effectiveness of phytoremediation, overall rising the survival rate. Translocation of metals did not receive any significant changed caused by the fertilizer or its contamination. Low metal concentrations and the relatively high metal tolerance of used species could make them useful for the phytostabilization and as valuable plant species for the initial colonization of heavy metal contaminated sites.

R. pseudoacacia and *L. luteus* are highly resistant to heavy metals and other abiotic stressors (Mirzahosseini et al., 2014). Due to their symbiosis with nitrogen-binding bacteria and the ability to hyperaccumulate metals, *Fabaceae* plants have many potential applications in large-scale phytoremediation of highly degraded soils (Mitton et al., 2016). Nevertheless, the present study shows that standard toxicity tests such as a germination index can determine soil toxicity, or the effectiveness of phytoremediation process only on model plants such as *S. alba* but are highly inconclusive in *R. pseudoacacia* and *L. luteus*, which are commonly used in phytoremediation processes (Hattab et al., 2015). Seedlings that germinated on contaminated soil showed many developmental disorders including a decrease in root length, chlorophyll, and overall protein content as well as an increase in GPX activity and content of total phenolic compounds. The highest germination index occurred in samples grown on control peat

soil without contamination for all three studied species. Unlike *S. alba, R. pseudoacacia* and *L. luteus* would not produce true results in standard toxicity tests based only on two biomarkers: seed germination and root length. Seeds of this species contain a large stock of all necessary nutrients for a plant to germinate, even in a highly toxic environment. It was found, that overall root length is more sensitive to contamination than the germination index, especially in *Fabaceae* species, but cannot be used to assess the proper amount of selected fertilizer to achieve the desired level of improvement in plant development.

The first defensive mechanism of oxidative stress is the scavenging of activated oxygen species at the sites where they are generated, especially in chloroplasts, where heavy metals accumulate. Many authors noted the impact of heavy metals on the decrease of photosynthesis efficiency (Murtaza and Asghar, 2013; Dimkpa et al., 2009; Ullah et al., 2015). In general, *in vivo* photosystem, I (PS1) is only slightly inhibited by heavy metal concentrations, while the PSII inhibition is almost absolute (Liu et al., 2014; Singh et al. 2007). Our results suggest the possible use of chlorophyll measurement to provide a rapid method for detecting and quantifying damage to a leaf photosynthetic apparatus due to environmental stresses in toxicity studies. Such a marker can provide necessary information about the type and optimal concentration of a specific fertilizer that needs to be supplemented to contaminated soil in order to achieve the desired level of photosynthesis efficiency, CO_2 sequestration, and overall phytoremediation effectiveness. In this study, chlorophyll content was highly declined on contaminated soil without any fertilizers and indicated damage to the chloroplast functioning. The amount of total chlorophyll in plants under heavy metal stress decreases, causing a reduction in the efficiency of photosynthesis (Murtaza et al., 2013). Similarly, in this experiment, the soil supplementation with organic additives showed an increase in chlorophyll content in plants. The type and concentration of particular organic additives significantly influenced chlorophyll content, proving the effectiveness of measuring chlorophyll content as a marker in phytoremediation studies.

The assay of peroxidase activity showed that addition of organic fertilizers had a major impact on a decrease in plant stress. GPX activity changed significantly on different soil mixes ($p < 0.05$) which indicates that the presented assessment can be useful in choosing proper additives and their concentrations for phytoremediation of degraded soils. A

statistically significant ($p < 0.05$) decrease in GPX activity in all selected species and all additives suggests that plant reaction to heavy metal contamination caused the same response in overproducing of ROS, and that organic additives such as manure and vermicompost can lower the toxic effects of heavy metals on plant development. Detection of GPX activity may be a viable way to estimate how a plant is coping with a difficult environment. This test can be used primarily for monitoring plant's acclimatization phase during phytoremediation processes.

Phenols are oxidized by peroxidase (POD) and polyphenol oxidase (PPO), which leads to catalyzing the oxidation of the o-diphenols and hydroxylation of monophenols (Pal et al., 2010). These enzymatic activities can increase in response to different types of abiotic and biotic stress. Both enzymes have been linked to the occurrence of physiological injuries caused in plants by thermal stress. In plants, phenols can act as antioxidants by donating electrons to guaiacol-type peroxidases (GPX) for the detoxification of H_2O_2 produced under different kinds of stress (Naguib et al., 2012).

Naguib et al., (2012) reported a significant influence of temperature stress on total phenolic compounds in watermelon, although there are no published reports on the effects of organic additives such as manure or compost on TPC in plants grown under heavy metal stress. Our study showed a significant accumulation of TPC in plants grown on polluted soil without any additives and a decrease in TPC in samples grown with organic fertilizers, indicating lowering toxic effects of heavy metals, by proper supplementation of degraded soil. The TPC test turned out to be a highly sensitive stress marker for selected plants and conditions since changes between different concentrations of selected fertilizer were statistically significant ($p < 0.05$).

Another important enzyme, RuBiSCO, is a bifunctional enzyme located in the chloroplast stoma and catalyzes photosynthetic CO_2 fixation to form ribulose-1,5-bisphosphate (RuBP) (Mota et al., 2015, Palacio et al., 2007). Therefore, the transcriptional levels of its large subunit: *rbcL* genes in studied plants species may indicate the photosynthetic efficiency of plants. (Wang et al., 2012). Wang et al., 2012, reported *rbcL* gene as a potential biomarker in plants grown under drought stress. However, there are no published reports showing the effects of heavy metal stress and organic additives on the expression of *rbcL* and overall photosynthesis effectiveness, which can be used to assay the effectiveness of phytoremediation, CO_2 sequestration after supplementation of

degraded soil. In this study, heavy metals in investigated soil caused a high reduction in expression of *rbcL* in comparison to plants grown in soil without pollutants. Chosen organic additives caused a significant increase in expression of *rbcL*, which leads to an improvement in photosynthesis effectiveness. Estimation of *rbcL* expression may be used for proper selection of type and concentration of soil additive in order to achieve a desired effectiveness of photosynthesis or overall phytoremediation and CO_2 sequestration. The presented results are similar to those found in one species of algae*: Chlorella sorokiniana* grown under metal stress where the individual effects of metals on *rbcL* expression varied significantly (Gojkovic et al., 2014). Furthermore, the increased photosynthetic performance and higher expression level of key *rbcL* has clearly demonstrated the role of Fe and Mg in enhancing the growth of *Chlorella sorokiniana* (Gojkovic et al., 2014).

5. CONCLUSIONS

The study has shown that determination of GPX activity, concentration of proteins and chlorophyll, total phenolic compounds and levels of *rbcL* expression can give more precise information on the induction of heavy metals induced stress, and the influence of selected organic additives on effectiveness of photosynthesis, sequestration of CO_2 and overall phytoremediation of degraded soils, in comparison to standard germination testing. The presented assays can be used in designing an effective phytoremediation process on a large scale by providing necessary information about a type and minimal and optimal concentration of selected fertilizer in order to achieve the desired level of photosynthesis efficiency, CO_2 sequestration or desired level of plant's abiotic stress. The most reliable toxicity test, independent of the tested species of plants, is TPC measurement and expression level of *rbcL*, which turned out to be a highly sensitive stress markers, especially in the planning of desired and precise dosage of selected fertilizer.

In the presented study, organic soils amendments (cattle manure, horse manure, vermicompost) completely eliminated the adverse effects of heavy metals on plants, which were noted for untreated soil without any additives, like: (1) decreased germination and roots length; (2) accumulation of soluble phenolics; (3) high GPX activity; and (4) low chlorophyll content, total proteins, (5) and a reduction in *rbcL* gene expression. Results indicated that presented assays can be used in different taxonomical groups such as *Fabaceae* and not only on model plants for planning the effective phytoremediation process.

Acknowledgements:

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Appendix A. Supplementary data

Table 2. Plant tissue-borne heavy metal concentration: PS – peat soil without contamination, CS - contaminated soil without any additives, CM – soil + cattle manure, HM –soil + horse manure, W - soil + vermicompost. Results shown as mean ± standard deviation, n = 3, Different letters "a", "b" "c" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey's test p>0.05).

Species	Metal [mg kg^{-1}]	PS	CS	10% CM	15% CM	20% CM	10% HM	15% HM	20% HM	10% W	15% W	20% W
S. alba	Cd	<0.05	1.22 ± 0.47a	1.12 ± 0.22a	1.34 ± 0.31a	0.87 ± 0.18a	0.92 ± 0.15a	1.26 ± 0.27a	1.32 ± 0.41a	1.17 ± 0.34a	0.89 ± 0.19a	1.07 ± 0.35a
	Pb		16.42 ± 2.71a	16.37 ± 2.54a	15.87 ± 2.68a	17.01 ± 3.41a	19.45 ± 2.44a	16.02 ± 2.21a	17.25 ± 1.9a	16.98 ± 3.02a	17.54 ± 2.54a	18.01 ± 2.41a
	Zn		47.9 ± 4.74a	51.22 ± 4.28a	49.47 ± 6.4a	44.65 ± 5.75a	41.74 ± 6.72a	39.54 ± 5.51a	40.74 ± 4.59a	50.98 ± 4.73a	42.56 ± 5.47a	42.41 ± 6.01a
L. luteus	Cd	<0.05	3.49 ± 1.13a	3.27 ± 1.85a	3.41 ± 0.74a	3.33 ± 1.11a	3.52 ± 1.37a	3.39 ± 1.18a	3.7 ± 0.79a	3.42 ± 0.92a	3.56 ± 0.53a	3.47 ± 0.77a
	Pb		35.47 ± 3.75a	33.57 ± 2.32a	36.98 ± 1.47a	34.87 ± 3.41a	37.21 ± 2.88a	35.64 ± 3.02a	33.54 ± 3.45a	32.87 ± 4.07a	37.65 ± 3.92a	39.85 ± 3.45b
	Zn		73.2 ± 18.82a	54.7 ± 34.81a	68.1 ± 24.14a	89.4 ± 26.17a	63.7 ± 35.41a	58.1 ± 17.41a	74.4 ± 19.54a	59.7 ± 21.65a	65.4 ± 24.32a	61.4 ± 17.14a
R. pseudoacacia	Cd	<0.05	3.17 ± 0.62a	3.08 ± 0.81a	3.34 ± 0.58a	3.11 ± 0.62a	2.98 ± 0.52a	3.42 ± 0.55a	3.15 ± 0.61a	3.37 ± 0.65a	3.05 ± 0.47a	3.21 ± 0.45a
	Pb		38.54 ± 4.88a	40.05 ± 4.64a	36.07 ± 3.54a	37.94 ± 2.98a	38.41 ± 4.55a	40.12 ± 4.61a	36,74 ± 3.24a	38,56 ± 3.32a	39,47 ± 4.17a	38,64 ± 4.21a
	Zn		51.24 ± 5.87a	55.12 ± 5.47a	49.14 ± 6.12a	55.34 ± 5.92a	52.41 ± 5.87a	50.65 ± 5.54a	53.47 ± 6.12a	49.88 ± 4.98a	52.47 ± 5.42a	55.98 ± 6.31a

CHAPTER III

The influence of contaminants on the expression of genes encoding metal chelators & metal transporters in plants

Phytoremediation is recognized as a cost-effective and widely acceptable alternative to chemical and physical technologies of soil remediation but it requires an overall more prolonged time to achieve success. In the last decade, the vast progress in omics research had led to improvements in our understanding of plants' metabolism while being exposed to toxic substances.

In this chapter, the results of the experiments concerning the influence of metal contamination and soil supplementation of sewage sludge, on the expression of metal chelators and ABC transporters in plants, are presented.

In the first part, we provide an overview of how omics research, including transcriptomic, proteomic, genomic, and metagenomic approaches, might reduce the negative impact of toxic elements on plant growth and development in order to ultimately enhance phytoremediation efficiency.

In the second part, an experiment was performed on artificially contaminated soil with single metal contamination on which *Lupinus luteus* was grown. The study was conducted to assess plants' response to contamination by overexpression of metallothioneins (MTs). Overall, the study provided insights into the identification and validation of housekeeping genes (HKG) for *L. luteus* under exposure to metal stress and showed the effects of selected heavy metals on metallothioneins' expression.

In the third and the fourth part, experiments were designed to explore the use of genes encoding metal chelators and ABC transporters as biomarkers to evaluate the safety of sewage sludge application to soil. The experiments included kinetic analyses as well as the correlation between the expression of the selected genes and the metal accumulation in plant tissues. Moreover, four different sewage sludges (two industrial, two municipal) were used in the experiments. Bioindicators are promising tools used to detect the long-term effects of selected biosolids on plant development and should be implemented before large-scale supplementation of sewage sludge into the soil. The presented studies show the impact of sewage sludge application on metal-sensitive toxicity biological parameters (biomarkers) in *Sinapis alba*, including germination, root length, the activity of guaiacol

peroxidase, the chlorophyll content, the level of DNA damage, and the expression level of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*), metallothionein (mt), ABC types C, G, and B.

In this part, the results as presented in the following four publications:

1. Jaskulak, M. (2018). Implementation of Omics Research to Enhance Phytoremediation Efficiency - a Review. Engineering and Protection of Environment, 21(4), 361-373. doi:10.17512/ios.2018.4.4

2. Jaskulak, M., Rorat, A., Grobelak, A., Chaabene, Z., Kacprzak, M., & Vandenbulcke, F. (2019). Bioaccumulation, antioxidative response, and metallothionein expression in *Lupinus luteus* L. exposed to heavy metals and silver nanoparticles. Environmental Science and Pollution Research. doi:10.1007/s11356-019-04972-y

3. Jaskulak, M., Grobelak, A., Grosser, A., & Vandenbulcke, F. (2019). Gene expression, DNA damage and other stress markers in *Sinapis alba* L. exposed to heavy metals with special reference to sewage sludge application on contaminated sites. Ecotoxicology and Environmental Safety, 181, 508–517. doi:10.1016/j.ecoenv.2019.06.025

4. Jaskulak, M., Grobelak, A., & Vandenbulcke, F. (2020). Effects of sewage sludge supplementation on heavy metal accumulation and the expression of ABC transporters in *Sinapis alba* L. during assisted phytoremediation of contaminated sites. Ecotoxicology and Environmental Safety, 197, 110606. doi:10.1016/j.ecoenv.2020.110606

Marta Jaskulak[1,2]

[1]Institute of Environmental Engineering, Faculty of Infrastructure and Environment, Czestochowa University of Technology, Czestochowa, Poland;

[2]Université Lille Nord de France, LGCgE-Lille 1, Ecologie Numérique et Ecotoxicologie, F-59650 Villeneuve d'Ascq, France;

Implementation of omics research to enhance phytoremediation efficiency – an overview*

Abstract:

Phytoremediation is recognized as a cost-effective and widely acceptable alternative to chemical and physical technologies of soil remediation but requires an overall longer time to achieve success. In the last decade, the vast progress in omics research had led to improvements in our understanding of plants metabolism while being exposed to toxic substances and the interactions between microbial communities and plants. By merging available omics tools with new bioinformatic approaches, it is possible to understand and determinate the specific patterns of plants response to various stress factors. In this review, we provide an overview of how omics research including transcriptomic, proteomic, genomic and metagenomic approaches might be used to reduce the negative impact of toxic elements to plants growth and development in order to ultimately enhance the phytoremediation efficiency.

KEYWORDS: phytoremediation, heavy metals, phytotoxicity, omics, transcriptomics, genomics

**Jaskulak, M. (2018). Implementation of Omics Research to Enhance Phytoremediation Efficiency - a Review. Engineering and Protection of Environment, 21(4), 361-373. doi:10.17512/ios.2018.4.4*

1. INTRODUCTION

Rapid industrial development of past two centuries has caused a significant contribution to soil and water contamination, primarily with heavy metals and organic pollutants (Chaabene et al., 2018). Strategies for environmental clean-up technologies require not only reliable tools but also precise approaches and procedures that are cost-efficient and possible to use during large scale remediation programs subjected to continually changing conditions (Grobelak et al., 2019). The technologies of phytoremediation which involves the use of plants to treat polluted areas have been extensively examined in the past studies.

By definition, phytoremediation takes advantage of plants natural ability to extract various substances including contaminants from soil and water (Anjum et al., 2014). The primary benefit of this process is its low cost – it had been shown to be at least 10 times less expensive in comparison to chemical and physical methods of environmental clean-up. Moreover, it is also considered a safe practice since plants can also stabilize the contaminated area minimizing erosion and the amount of contaminated dust that could potentially move to another remote areas (Ceyher-Keskin et al., 2018).

In comparison to bioremediation with bacteria and fungi, phytoremediation is visible and thus can be monitored, and continuously controlled. Besides, plants can provide nutrients and stimulate rhizosphere bacteria which can also improve soil quality and aid the whole remediation process (Song et al., 2016). Finally, phytoremediation of degraded sites, especially after large-scale industrial activities can provide a new habitat for wildlife including insects, birds and small mammals (Chen et al., 2018).

In the past, phytoremediation has been used to treat multiple kinds of pollutants including heavy metals, polycyclic aromatic hydrocarbons (PAHs), petroleum, solvents, and other toxic organic substances (Ceyher-Keskin et al., 2018). Depending on the type of substance, phytoremediation can involve different processes and approaches. For example, phytoremediation of metals has a unique challenge due to the fact that, they cannot be metabolized and therefore have to be translocated into the above-ground parts of plants which are easily removable by conventional harvesting. This approach is often referred to as phytoextraction. It is an effective method of site remediation which reduces the total mass of contaminant (Ren et al., 2018). Moreover, in comparison to physical methods which in this case would remove the top, most fertile layer of soil,

phytoremediation does not reduce the fertility of the land (Chen et al., 2016). Yet, after years of extensive research and successful laboratory trials, phytoremediation still lack large-scale application (Yildirum et al., 2016).

The primary drawback of such actions in comparison to classic chemical and physical methods is that it is too slow. In many countries, the regulatory agencies require significant effects in site decontamination to be obtained after just a few years which can make the use of phytoremediation impossible. Due to that, recent research on the ways to enhance the efficiency of phytoremediation has gained a lot of interest (Nahar et al., 2017). In the past, technologies aimed for developing specific approaches to use plants as bioindicators and then to use such species for remediation were strictly focused on using standard toxicity tests in order to evaluate the level of phytotoxicity and the level of persistence of selected contaminant in plants tissues (Cox et al., 2017).

For future improvement and optimization of any phytoremediation activities, the precise identification of metabolic mechanisms involved in contaminant tolerance, uptake and detoxification is required (Chaabene et al., 2018). These consist of extremely complex cellular systems and pathways controlled by large families of genes. Thus, to precisely identify such traits for chosen species of plants and their symbiotic microbes, the application of new omics approaches can be a promising idea (Bramhachari et al., 2017).

To tackle that issues, the next-generation sequencing (NGS) technologies have started to appear around the year of 2005 and since were vastly used in plants research including environmental studies, broadening our understanding of plants metabolism and interactions between plants and symbiotic microbes (Liu et al., 2017). In the beginning, large omics data was translated into applicable technologies mostly exclusively in medicine and health sector, due to its extremely high costs at the time. However, the total cost of NGS techniques is rapidly decreasing in recent years and therefore it is being applied to all kinds of life sciences including the problems of soil and water contamination (Feng et al., 2017, Zhu et al., 2018).

This review explores recent advances in application of omics in research involved in remediation of contaminated soils. Study mostly focuses on the applicability of transcriptomic and proteomics studies that allow for the identification of genes related to specific pathways of detoxification after exposure to heavy metals and organic pollutants. Moreover, the study presents recent advances of using omics studies to design new,

genetically engineered plants for phytoremediation. We describe and discuss how the NGS approaches can be used to advance plants research and how it can be used in new phytoremediation strategies. Overall, we focused on phytoremediation; however, the high-throughput approaches are also massively studied for other environmental purposes such as managing plants pathogens and invasive species or optimizing the production of crops.

2. STANDARD APPROACHES FOR PHYTOREMEDIATION OF CONTAMINATED SITES

The cost of chemical and physical treatments of contaminated sites is exceptionally high and involves transport, soil washing, excavation, extraction, pumping and chemical treatments including the addition of potassium permanganate, hydrogen peroxide and finally incineration. Therefore, due to the high cost, contaminated lands owned by private companies are more often abandoned and not properly cleaned up (Ren et al., 2018). Hence, the implementation of more cost-efficient methods of soil remediation, such as phytoextraction of metals or phytodegradation of organic pollutants could potentially be a promising solution, especially in large areas contaminated in the past via various range of anthropogenic sources (Marchand et al., 2015). In order to achieve satisfactory results, research into the specific plant species best suitable for given process is a necessity (Azab et al., 2016).

Plant species that are the most useful for phytoextraction are termed as metal hyperaccumulators – meaning that they are able to concentrate high level of metals in its biomass (Jaskulak et al., 2018). For example, in recent years *Petris vittata* had been used to remove arsenic by its hyperaccumulation. Arsenic is an acute and lethal poison, and *P. vittata* was shown to extract it from a soil concentration of 97 ppm up to 894μg per gram of its biomass. Moreover, approximately 95% of arsenic was translocated to plants shoots which were easily harvested (Liu et al., 2017). Another example of successfully field-tested hyperaccumulator is *Thlaspi caerulescens* which is able to accumulate toxic cadmium in its shoots in a concentration exceeding the phytotoxic threshold by over a 1000 times (Baycu et al., 2016). The mechanisms of cadmium uptake in the whole *Brassicae* family has been studied and were shown to involve metal transporters which are extensively expressed during such exposure. At the same time, since *Brassicae* are quite small and light-weight plants, high-biomass species including poplar and willow trees are also vastly studied for phytoremediation purposes (Kumar et al., 2018).

For degradation of organic pollutants, phytodegradation seems to be an optimal solution which involves degradation of such contaminants by specific plants (Azab et al., 2016). During this approach, plants metabolism breaks down the organic substance through internal or secreted enzymes. This process is currently mostly used for breaking down polycyclic aromatic hydrocarbons (PAHs), chlorinated hydrocarbons and multiple

explosives (Ceyher-Keskin et al., 2018). As an example, hybrid poplars had been used to take up and degrade trichloroethylene (TCE) which is a common pollutant of groundwater that is both hepatoxic and carcinogenic for humans (Benisrael et al., 2019). Other example of victorious application of phytodegradation includes the use of multiple plant species to degrade carbon tetrachloride (CT) and perchloroethylene (Ma et al., 2015). Phytodegradation also had been shown to be successful for polycyclic aromatic hydrocarbons (PAHs) and explosives including trinitrotoluene (TNT) (Benisrael et al., 2019, Kumar et al., 2018). Through recent decades, the amount of PAHs in the environment is constantly increasing. One example of plant efficiency in PAHs degradation is *Populus nigra* which was shown to reduce the range of PAHs such as pyrene, benzopyrene, anthracene, phenanthrene, and chrysene (Marchand et al., 2015).

Another alternative for contaminant clean-up is augmented bioremediation with specific bacterial species and strains that are able to transform or degrade particular contaminants (Grobelak et al., 2019). On the other hand, this technology faces major drawback involving high sensitivity of microbial communities and thus, for successive remediation strict conditions must be kept on an optimal level. The primary conditions affecting the efficiency of such activities include the bioavailability and accessibility of specific microorganisms, the ability of selected microorganisms to survive and grow on a given environment, the presence and persistence of other dominant species, the presence of carbon as well as access to the source of all required micro and macronutrients (Bramhachari et al., 2017).

3. METAGENOMIC APPROACH TO ENHANCE EFFICIENCY OF PHYTOREMEDIATION

Introduction of plants to highly degraded and contaminated soils may augment both the quantity and quality of microbial populations occurring in bulk soil (Balcom et al., 2018). Although plant-microbe interactions are incredibly complex and sensitive to different environmental conditions, plants can favour certain species that promote its growth or inhibit the activity of pathogens. Moreover, microorganisms can also impact the potential uptake of pollutants including the uptake and translocation of heavy metals from roots to shoots of plants (Kumar et al., 2018). Overall, microbial communities that are able to create associations with plants can influence plants growth and development both positively and negatively which as a consequence will alter plants ability to remediate the soil (Ma et al., 2015). Contaminants such as heavy metals can be also either transformed by specific microorganisms in rhizosphere or be taken up and translocated by plants. Stimulation of microbial activities can be increased due to plants through the release of root exudate (Bramahachari et al., 2017).

A couple of recent studies in phytoremediation research including actual field trials used targeted amplicon sequencing in order to show that, the bacterial and fungi communities are shaped by two main factors: the concentration of the contaminant in the soil and the plant phylogeny (Phillips et al., 2012, Benisrael et al., 2019). Other studies also noticed that the composition of arbuscular mycorrhizal depends on the concentration of metal (Chen et al., 2018). Thus, studies suggest that plants abiotic stress can determinate the stimulation of microbial bioremediation. For example, experiment from 2012 showed, that exudate produced by wild rye exposed to hydrocarbons were not as repressive as exudate from wild rye grown on an uncontaminated medium, which overall demonstrated that plants might alter the extent to which they will promote total rhizosphere biodegradation (Phillips et al., 2012). Thus, it has to be taken into consideration that the overall degree to which plants can exert control over bacterial and fungi communities strictly depends on the concentration of given contaminant and its overall severity of toxic effects (Hao et al., 2018).

It is well established, that soil microorganisms play the crucial role in mineralization of organic matter in soils and also can transform specific contaminants to its different chemical forms including more stable and less toxic ones (Hou et al., 2017). At the same

time, one of the most frequent problems with research on soil microbial communities is that in many cases more than 99% of obtained microbial taxa are not yet cultured. Therefore, using NGS technologies can allow for significantly more thorough analysis of microbial composition and their activity while preserving environmental factors (both abiotic and biotic) that once shaped these microbial communities *in situ* (Song et al., 2016). In addition, to adequately probe the plant-associated microbial communities and examine plant-dependent habitats including plants rhizosphere, phyllosphere and endosphere, shotgun sequencing and amplicon-targeting of genes can be also used as a promising tool (Kumar et al., 2018). In the study by Hao et al. (2018) shotgun high throughput metagenomic sequencing was used in order to capture the PAHs degrading features of *Taxus* rhizosphere microbiome. Study showed numerous functional genes associated with biodegradation and metabolism of xenobiotics as well as multiple genes involved in a range of defence mechanisms against organic pollutants.

Simultaneously, in recent years of phytoremediation research, a lot of interest has also been given to plasmid DNA since contaminated soils, and plants rhizosphere is known to be a hotspot for rapid exchange of plasmids (Chen et al., 2018). During the abiotic stress, plasmids can be used as a reservoir for genomic innovation which can enable the symbiotic bacteria and fungi to adapt to stress conditions. During one research, metagenomic sequencing of plasmid DNA from municipal wastewater-treatment plants displayed, that genes involved in heavy metal resistance are disproportionately abundant in plasmid DNA in comparison to the entire genome. The same study also showed, that genes involved in the transformation and degradation of organic pollutants are also mostly present in plasmids (Balcom et al., 2018). Such knowledge has already been applied, and plasmids with genes responsible for toluene degradation were successfully transferred to aerial plant endophytes and increased the total degradation of contaminant that would have been volatilized (Hao et al., 2018). Besides, metagenomic research performed in changing environmental conditions showed that the microbial communities in terms of its genetic and taxonomic composition could be linked to its activity to metabolize carbon. Also, the analysis of metagenomic DNA showed specific taxa which are involved in the suppression of plants diseases in soils (Benisrael et al., 2019). Experiments by Balcom et al. (2016) displayed the potential of microorganisms to metabolise micropollutants such as xenobiotics during the process of wastewater treatment. Metagenomic analysis reviled microorganisms and specific genes involved in degradation of benzoate. Collectively,

study showed the abundance of xenobiotic metabolism genes present in biofilm of created wastewater treatment plant. In another study, metagenomic approach was applied to investigate the response of rhizosphere microorganisms of rice after exposure to PAHs. Results reviled that the total distance from the surface of roots and PAHs concentration affected the microbial communities in rice rhizosphere. Moreover, the abundance of genes related to PAHs degradation including dioxygenase genes mirrored the potential to PAHs degradation in rice rhizosphere (Ma et al., 2015). A 2019 study applied molecular metagenomic approaches as well as compound-specific isotope analysis in order to check the efficiency and mechanisms of toluene biodegradation in the unsaturated zone where poplars were used to remediate the soil. Experiments reviled spatially-variable numbers of toluene degraders were in roots of hybrid poplars as well as other evidence for toluene biodegradation in the unsaturated zone (Benisrael et al., 2019). Table 1 demonstrates the variability of environmental applications of metagenomic studies from the past five years. To summarize, metagenomic analyses can be used in environmental studies in various ways including: (i) to indicate some functional attributes to selected microbial assemblages; (ii) to identify and determinate the function of microbial genes associated to vast range of functions including the promotion of plants growth or biodegradation of hydrocarbons; (iii) to determinate the distinct environmental conditions that will favour our target symbiotic microorganisms (Kumar et al., 2018, Benisrael et al., 2019). Moreover, it is now possible to determinate the potential of not yet cultured microorganisms in the promotion of plants growth or contaminant biodegradation by the use of single-cell isolation and sequencing technologies. Such methods can not only precisely determine the full genome of selected uncultured strains but also identify new targets to be used in the field tests (Ma et al., 2015).

Table 1. Major metagenomic studies applied for remediation of environmental contamination since 2014

Goal of metagenomic research	**Contaminant**	**Year**	**Reference**
Analysis of an wastewater treatment plants microbial communities and their potential to metabolize pharmaceuticals	Pharmaceuticals	2016	Balcom et al., 2018
Understanding the Mechanism and Function of Plant growth-promoting rhizobacteria (PGPR)	none	2017	Bramhachari et al., 2017
Analysis of the response of microbial metagenome to polycyclic aromatic hydrocarbons (PAHs) degradation in the rice rhizosphere	PAHs	2015	Ma et al., 2015
Perspectives of lindane (γ-hexachlorocyclohexane) biodegradation	lindane	2018	Kumar et al., 2018
Identification of the potential for toluene biodegradation in the vadose zone of a poplar	toluene	2019	Benisrael et al., 2019
Analysis of the influence of cadmium contamination on bioenergy cropping and microbial composition of cadmium contaminated soil	cadmium	2017	Chen et al., 2018
Analysis of the potential of *Taxus* rhizosphere microbiome for bioremediation and phytoremediation	none	2018	Hao et al., 2018
Analysis of rhizosphere microorganisms of PAHs contaminated soil planted with barley and alfalfa	PAHs	2018	Kumar et al., 2018
Identification of the influence of microbial community on the efficiency of metal phytoremediation by *Sedum plumbizincicola*	Cadmium and zinc	2017	Hou et al., 2017

4. PLANTS GENOMIC AND TRANSCRIPTOMIC STUDIES IN THE ASPECTS OF ENVIRONMENTAL CONTAMINATION

4.1 Genomics

The incorporation of genomic approaches into environmental sciences has led to the identification of multiple genes involved in phytoremediation and plant tolerance to abiotic stress, including several contaminants (Das et al., 2016). It is well established, that the high-throughput techniques are especially useful in order to identify plants traits that depend on the specific combination of several genes. As an example, the mapping of quantitative trait loci between hybrids of zinc-tolerant *Arabidopsis helleri* and *Arabidopsis lyrate* which is zinc-intolerant had identified a couple of genomic regions that when combined explained more than 42% of plants tolerance to zinc (Nahar et al., 2017). Similarly, quantitative trait loci for the accumulation of arsenic was successfully identified in leaves and stems of maize. Moreover, this study showed that only one of the quantitative trait loci was present in both tissues which suggested that for arsenic accumulation different genes are responsible in different tissues of the plant (Ceyher-Keskin et al., 2018). It also has to be noticed, that the complexity of plants genomes, which often include extreme polyploidy required extensive genomic characterization across species suitable for phytoremediation in order to identify the specific mechanisms involved in the selected type of remediation of a given contaminant. Therefore, the activity-focused approaches including such technologies as transcriptomics and proteomics might permit the precise examination of the functionality of specific candidate plant species after exposure to distinct environmental conditions including the type and concentration of the given contaminant (Baycu et al., 2016).

4.2 Transcriptomics

Currently, most research in plants omics analyses in the area of environmental contamination has been performed on plants exposed to various heavy metals (Table 2). There are numerous examples of using transcriptomics to identify genes involved in stress tolerance in plants (Table 2). For instance, the stress response of *Brassica chinensis* exposed to chromium had been described using these technologies (Zhou et al., 2016). Work of Yıldırım et al. (2016) applied genome-wide transcriptome profiling in *Populus nigra* grown under exposure to boron and found several candidate genes responsible in

boron uptake, transport and detoxification. During that study, highest induction was recorded for genes encoding: tyrosine aminotransferase, ATP binding cassette transporters, glutathione S transferases and metallochaperones. Moreover, many other genes were showed to be highly upregulated after exposure to boron including genes involved in antioxidative systems, and signalling. Another study, from 2018 reviled in total 72 metabolism pathways, including photosynthesis, phenylalanine metabolism, ribosome, phenylpropanoid biosynthesis, flavonoid biosynthesis and carbon fixation in *Phytolacca americana* exposed to cadmium. In addition, numerus genes related to cadmium tolerance, absorption, transport and accumulation were also determinated, including the total of 11 expansins, 8 nicotianamine synthases, 6 aquaporins, 4 ZRT/IRT-like proteins, 3 ABC transporters and 3 metallothioneins (Chen et al., 2018). In study by Song et al. (2016) analysis of transcriptome led to the determination of functional genes upregulated by stress caused by herbicide – atrazine. Among these were genes encoding the zinc finger proteins, intracellular/extracellular enzymes, structural proteins, anti-stress/anti-disease proteins, and electron transport-related proteins. The results of another researchers showed regulatory mechanisms induced after the exposure to petroleum hydrocarbon stress in *Z. mays* (Ceyher-Keskin et al., 2018). Transcriptomic approach was also applied to investigate the effects of thorium on plants metabolism. Thorium is actinide metal with high potential in nuclear energetics. Contamination by thorium, is mostly caused by mining or spills, and poses a significant threat since its radioactive. Study from 2018 investigated the transcriptomic response of tobacco roots exposed to thorium. Such exposure resulted in up-regulation of the total of 152 genes and down-regulation of 100 genes. The induced genes were mostly related to the production of jasmonic and salicylic acid signalling pathways and various abiotic and biotic stress responsive genes. In addition, up-regulation was noticed in phosphate starvation genes and down-regulation in genes encoding the synthesis of phytic acid which indicated that thorium disturbed phosphate uptake or its signalling. Moreover, the expression of iron responsive genes was also highly influenced by exposure. The down-regulation of some aquaporins showed a severe disturbance of water homeostasis (Mazari et al., 2017).

Overall, transcriptomic analyses can also be performed in a comparative matter, and such a connection between different species suitable for phytoremediation can allow identifying genes responsible for differences in plants response. In one study, researchers compared two species exposed to cadmium – *Solanum torvum* which does not accumulate

significant amounts of cadmium in its tissues, and *Solanum nigrum* which is identified as hyperaccumulator of Cd. Transcriptomic analyses identified a higher expression of multiple genes in *S. nigrum* which were involved in the transportation of metals and were not expressed in *S. torvum* (Wang et al., 2015).

Table 2. Major transcriptomic studies since 2014 of plants exposed to environmental contamination

Species	Contaminant	Year	Reference
Sedum alfredii	Cadmium	2016	Han et al., 2016
Populus nigra	Boron	2016	Yildirum et al., 2016
Phytolacca americana	Cadmium	2018	Chen et al., 2018
Medicago sativa	Atrazine	2016	Song et al., 2016
Landoltia punctata	Cadmium	2018	Zhang et al., 2018
Zea mays	PAHs	2018	Ceyher-Keskin et al., 2018
Festuca arundinacea	Cadmium	2018	Zhu et al., 2018
Nicotiana tabacum	Thorium	2018	Mazari et al., 2017
Sorghum bicolor	Cadmium	2017	Feng et al., 2017

4.3 The use of omics for designing new transgenic species

Currently, there is a growing interest in research focused on improving plants capacity to stabilize, store or remove specific contaminants from water and soil environments (Bernard et al., 2018). Successfully identified genes from microbes and plants are being used to enhance plants ability to survive, tolerate, store and degrade toxic substances (Ren et al., 2018). In the past five years, the use of transgenic plants with specific bacterial genes had been successfully used to decrease the phytotoxic effects of nitroaromatic contaminants which were then used for their removal from soil (Table 3). Moreover, the overexpression of cytochrome P450s genes had been shown to increase the metabolism of a vast variety of herbicides and environmental pollutants in plants (Azab et al., 2016). Also, genes involved in detoxification of heavy metals are used to enhance the efficiency of metal phytoremediation in numerous recent studies (Das et al., 2016, Nahar et al., 2017, Grobelak et al., 2018). One of the studies showed that transgenic plants with bacterial genes could convert herbicide simazine to different and nontoxic forms (Azab et al.,

2016). The study by Nahar et al (2017) cloned, and transformed the *AtACR2* gene (encoding arsenic reductase 2) of *Arabidopsis thaliana* into the genome of *Nicotiana tabacum*. Obtained results revealed that the transgenic tobacco was much more tolerant to arsenic exposure than the wild type. Overall, transgenic plants could grow on medium containing 200 μM of arsenate, whereas the wild could not survive such exposure. Study by Zhang et al. 2018 investigated contamination from the explosives, including hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX), and 2, 4, 6-trinitrotoluene (TNT), on real sites contaminated due to fire training, military ranges in the USA. Such pollution creates a significant threat for both the environment and human health. Phytoremediation and phytodegradation of such explosives is a promising idea but high phytotoxicity of TNT makes it impossible to use on most plants species. Hence, the bacterial genes *xplA* and *xplB*, encoding the ability to degrade RDX and a bacterial nitroreductase gene *nfsI* was cloned and transformed into *Pascopyrum smithii* in order to enhance the capacity of plants to survive and detoxify TNT. All previous studies have used model plant species to demonstrate the efficacy of this action, therefore this time Perennial western wheatgrass was used as it is a United States native species, broadly distributed across all North America and useful for phytoremediation. Study showed that transformed plants removed significantly more RDX when compared to wild-type plants. Furthermore, these plants were also more resistant to TNT toxicity, and detoxified more TNT than wild-type plants.

Table 3. Successful application of transgenic plants during phytoremediation of selected contaminant from past five years

Transgenic species	**Donor species**	**Used gene**	**Contaminant**	**Year**	**Reference**
Nicotiana tabacum	*Arabidopsis thaliana*	*AtACR2* (arsenic reductase 2)	Arsenic	2017	Nahar et al., 2017
Nicotiana tabacum	*Oryza sativa*	*OsMTP1* protein belonging to the cation diffusion facilitator (CDF) and metal tolerance/transport protein (MTP) family	Cadmium	2016	Das et al., 2016
Arabidopsis thaliana	*Homo sapiens*	P450 isozymes *CYP1A2*	Simazine	2016	Azab et al., 2016
Medicago sativa	*Acinetobacter radioresistens*	bacteria 2, 3- dihydroxybiphenyl-1, 2-dioxygenase gene (*bphC. B*)	Polychlorinated biphenyls (PCBs)	2018	Ren et al., 2018
Pascopyrum smithii	Range of bacteria species	*xplA* and *xplB*, confer the ability to degrade RDX in plants, and a bacterial nitroreductase gene *nfsI*	RDX and TNT	2018	Zhang et al., 2018

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5. CONCLUSIONS

Phytoremediation can be a reliable, cost-efficient and vastly socially acceptable technology for dealing with contaminated lands. To increase its efficiency new omics approaches has gained a lot of interest in recent years (Zhang et al., 2018). Our review shows, that such large omics data sets have already identified specific environmental conditions that shape the quantity and quality of microbial communities in soil. The possibility to alter the microbial composition through the introduction of plants or to alter plants growth via manipulation of microbial communities has application not only directly for phytoremediation purposes but also for the management of invasive species the production of crops and biofuel. One of the most prominent advantages of using omics for such research is its continuously-growing sets of data and continuously-decreasing costs. Therefore, the translocation of obtained data and overall, knowledge into useful technologies is depending on research that uses omics analyses with precise applicable goals.

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Bioaccumulation, antioxidative response and metallothionein expression in *Lupinus luteus* L. exposed to heavy metals and silver nanoparticles*

Marta Jaskulak[1,2*], Agnieszka Rorat[2], Anna Grobelak[1], Zayneb Chaabene[2,3], Małgorzata Kacprzak[1], Franck Vandenbulcke[2]

[1]*Institute of Environmental Engineering, Faculty of Infrastructure and Environment, Czestochowa University of Technology, Czestochowa, Poland;*

[2]*University of Lille, Laboratory of Civil Engineering and Environment (LGCgE), Envrionmental Axis, F-59650 Villeneuve d'Ascq, France;*

[3] *Laboratoire des Biotechnologies Végétales Appliquées à l'Amélioration des Cultures, Université de Sfax, Tunisie;*

Abstract:

Yellow-lupin (*Lupinus luteus* L.) was grown on soils contaminated with heavy metals by industrial activities and soils contaminated artificially with single heavy metal including Cd, Pb, Zn, Ni (in nitrate form) and Ag (in nitrate and nanoparticles form). The study was performed to assess plants response to contamination including its antioxidative response and molecular mechanisms involved in metal detoxification through the expression of metallothioneins (*MTs*). Overall, the study provided insights into identification and validation of housekeeping genes (HKG) in *L. luteus* under exposure to metal stress, and showed the effects of selected heavy metals and silver nanoparticles on the expression of metallothioneins, the activity of guaiacol peroxidase (GPX) and bioaccumulation of metals in leaves of *L. luteus*. As such, HKG validation using BestKeeper, NormFinder and geNorm software allowed for selection of four most stable reference genes in a context metal contamination for selected plant. Moreover, a significant increase in the expression levels of *MT* gene was observed in plants grown under heavy metal stress and none on plants grown on 25 mg kg^{-1} of silver nanoparticles. Also, the GPX activity and *MT* expression showed statistically significant changes between different conditions and doses which means that they can be used as highly sensitive stress markers for planning the phytoremediation process on a large scale.

KEYWORDS: phytotoxicity, bioremediation, heavy metals, oxidative stress, biomarkers, phytoremediation

** Jaskulak, M., Grobelak, A., Grosser, A., & Vandenbulcke, F. (2019). Gene expression, DNA damage and other stress markers in Sinapis alba L. exposed to heavy metals with special reference to sewage sludge application on contaminated sites. Ecotoxicology and Environmental Safety, 181, 508–517.*

1. INTRODUCTION

Heavy metal (HM) contamination is one of the major concerns of recent years and primarily a result of industrialization (Cox et al., 2016). Soil and water contamination with HMs creates a direct obstruct in normal growth of plants which in consequence reduces crop yields (Pant et al., 2014). When accumulated into plant tissues, metals may cause a severe threat to animals and humans by entering into the food chain (Balseiro-Romero et al., 2017). Overall, heavy metals cause abiotic stress that results in the improper development of plants, lower yields and finally, at high concentrations, plant death (Placek et al., 2017). It was found that soon after exposure to heavy metals, metabolism of reactive oxidative species (ROS) is altered and oxidative stress is induced (Bolan et al. 2014). The excess of ROS that cannot be adequately neutralized by an antioxidative system causes damage to proteins and DNA (Chibuike et al., 2014). Such toxic effects may lead to the death of plants (Liu et al., 2014). The activity level of antioxidative enzymes can be used to estimate stress levels and to assess environmental toxicity before any visible impact on growth appears. Therefore, such assays may be useful tools for the environmental engineering as indicators of the effects of a soil pollution and/or indicators to follow the impact of remediation processes on organisms (Bernard et al., 2018). Because of this, identification of mechanisms by which plants respond to such exposure is a prime objective in plant research (Grobelak et al., 2017). Studies on plants species like *Trifolium repens* have demonstrated the involvement of specific proteins, i.e., metallothioneins (MTs) in the hyperaccumulation of metals (Bernard et al., 2016). However, the comparison between species is difficult since analysis of heavy metal accumulation in plants other than model species is largely unavailable and MTs have not been characterized in most plant species. Moreover, the physiological roles of MTs are not entirely understood (Chaâbene et al., 2018).

Among heavy metals, cadmium (Cd) and lead (Pb) are one of the most toxic pollutants for organisms (Bolan et al., 2014). Both were/are mostly released to the environment by transportation, mining, ore smelting or as a by-product of mineral fertilizers. They are well known to cause a vast range of phytotoxic effects in plants (Galal et al., 2015). Similarly, nickel (Ni) and zinc (Zn) were shown to cause toxic effects in plants grown under their elevated concentrations caused mostly by irresponsible fertilizing or contamination by metallurgical operations (Rehman et al., 2016, Grobelak et al., 2019).

In recent years, the influence of metal nanoparticles such as silver nanoparticles (AgNPs) gained more attention due to their presence in numerous manufactured products, causing the abundance of silver (Ag) in sewage sludge (Sharma et al., 2016). Since the application of sewage sludge on both, post-industrial and agricultural soils is seen in many countries as a way to improve soil quality and/or a sewage sludge disposal strategy, the eventual effects of metal nanoparticles and of metals contained in metallic nanoparticles on plants need to be determined (Cox et al., 2016).

Currently, many biomarkers may be investigated to assess the biological impact of contamination on organisms. Most of them use plants protective mechanisms against adverse conditions to evaluate the level of toxicity (Ent et al., 2012). Nevertheless, the specificity of plants stress markers and their optimal application in natural, large-scale conditions still requires more research. Standard toxicity tests that involve only germination index and roots length are useful tools to assess the toxicity of selected substance on model plants but were shown to be strongly inaccurate in other than model plant species (Galal et al., 2014). Other markers of oxidative stress are often divided into two main groups (Pal et al., 2010). In the first group, markers focused in biochemical changes including the activity of antioxidative enzymes including superoxide dismutase (SOD), catalases (CAT) and peroxidases (POD) are used alongside the measurement of metabolites that are highly sensitive to adverse conditions like the total concentration of chlorophyll, carotenoids, phenolics, other substances like proline and overall proteins. The second group is consisted of a broad variety of genetic markers used to evaluate the effect of such exposure on the growth and overall, development of plants (Sinha et al., 2015).

During the last decade, qRT-PCR has become a vital tool for gene expression analysis due to its superior sensitivity and overall, accuracy. The utility of this method is also determined by high throughput (Oneto et al., 2016). This method is commonly applied to explore and elucidate many biological processes in response to different conditions including environmental stress. Nevertheless, to rely on data achieved from qRT-PCR, it is necessary to identify reliable house-keeping genes, (HKG) called also as reference genes, which are a critical point to quantify candidate gene expression and data normalization (Sinha et al., 2015, Niu et al., 2017, Chaâbene et al., 2018).

In our study *Lupinus luteus* was used to assess the influence of heavy metals and silver nanoparticles on the selected defence mechanisms. The chosen plant species is a highly resistant species which also had been shown to tolerate and hyperaccumulate elevated concentrations of HMs and other abiotic stressors. Therefore, it has a tremendous potential for application in a phytoremediation of even highly degraded sites. (Gutiérrez-Ginés et al., 2014).

Presented study shows the influence of single metal contamination, industrial contamination and AgNPs on plants development including the germination index, guaiacol peroxidase activity (GPX), protein concentration, metal accumulation, and *MTs* expression. For this purpose, *L. luteus* was exposed to soils contaminated by industrial activities, and soils contaminated artificially by cadmium, lead, nickel or zinc, silver nitrate, silver nanoparticles, and dispersant used in AgNP but without nanoparticles. Contamination-free, the organic soil was used as a reference. Overall, the study was designed to evaluate the effects of heavy metals and silver nanoparticles on bioaccumulation and genotoxic effects of metals and nanoparticles in the *L. luteus.* Selected plant species belongs to *Fabaceae* plants which possess mechanisms allowing for the creation of symbiosis between nitrogen binding bacteria and plant roots, which can significantly improve the nitrogen soil content over time due to the increase in nitrogen fixation and content of organic matter (Balseiro-Romero et al., 2017). Moreover, *L. luteus* has been shown before as able to tolerate metals in soil which allows its use on highly contaminated post-industrial areas (Gutiérrez-Ginés et al., 2014).

Performed experiments provided insights into: (1) identification and expression level of metallothionein in *Lupinus luteus* L.; (2) identification and validation of house-keeping genes (HKGs) in *L. luteus* under metal stress; (3) the ability of the tested gene to serve as toxicity biomarkers on both field-collected and artificially contaminated soil; (4) the influence of heavy metals and silver nanoparticles on the expression of *MTs*.

2. MATERIALS AND METHODS

2.1 Substrates characterization

Two different soils were used in the experiment (Table 1). The first one was highly contaminated with heavy metals and degraded soil collected near to a zinc smelter in Poland (GPS: 50º30'N 18º56'E), collected from surface layer from 0 to 30 cm in depth. The second one was organic peat soil used as control or contaminated artificially with selected heavy metals or silver nanoparticles. All experimental soils have been checked and measured for the chemical and physical soil characteristics both before starting the experiment and after the incubation period. The pH values in all experimental soil mixed were measured in distilled H_2O as well as in 1M KCl in accordance to ISO 10390:2005. For this purpose, standard pH-meter was used (Cole Parmer Model No. 5900200). The cation exchange capacity (CEC) was measured by the standard Kappen method (Kappen et al., 1995). The concentration of total P and K was established by the Egner method (Egner et al., 1960) and the content of total organic nitrogen (TON) in soil samples was recorded by Kjeldahl method (PN-ISO 11261:2002). Concentrations of HMs were measured by ICP-OES using ICP-OES from Thermo apparatus, USA. Reference materials were used for measurement control. Samples were digested before the measurements in a microwave digestion system following EPA 3051 method (US EPA 3051, 1994).

Table 1. Characterization of tested soils and concentrations of heavy metals after 28 days of plants growth.

Growth	pH		CEC	N	TOC	Cd	Pb	Zn	Ni	Ag*
medium	H_2O	KCl	[cmol/kg]	[%]	[g kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]
PS	5.61 ± 0.05	7.12 ± 0.11	33.1 ± 0.21	0.81 ± 0.07	44.0 ± 1.9	1.05 ± 0.2	7.39 ± 1.11	27.4 ± 2.2	1.39 ± 0.5	Nd
CS	5.45 ± 0.04	3.60 ± 0.06	25.2 ± 0.09	0.25 ± 0.12	9.8 ± 1.2	19.5 ± 1.2	1133.8 ± 3.5	733.2 ± 1.4	22.1 ± 1.2	Nd
Zn360	5.57 ± 0.13	7.06 ± 0.02	32.5 ± 0.17	0.76 ± 0.08	46.2 ± 2.4	0.81 ± 0.2	8.21 ± 0.86	315 ± 24.5	0.91 ± 0.8	Nd
Zn720	5.60 ± 0.06	7.10 ± 0.13	32.8 ± 0.54	0.74 ± 0.11	43.5 ± 0.9	0.96 ± 0.3	6.98 ± 1.35	694 ± 31.5	1.27 ± 0.2	Nd
Ni360	5.56 ± 0.02	7.13 ± 0.15	33.3 ± 0.33	0.80 ± 0.10	43.1 ± 1.2	0.79 ± 0.1	7.15 ± 1.71	30.4 ± 4.5	329.13 ± 8.7	Nd
Ni720	5.65 ± 0.07	7.08 ± 0.02	33.0 ± 0.26	0.79 ± 0.04	44.2 ± 1.1	1.08 ± 0.2	6.87 ± 0.98	26.8 ± 3.7	700.62 ± 14.2	Nd
Pb360	5.55 ± 0.11	7.07 ± 0.06	33.4 ± 0.18	0.85 ± 0.08	45.8 ± 2.3	1.02 ± 0.4	321.08 ± 9.75	28.1 ± 1.6	1.38 ± 0.4	Nd
Pb720	5.60 ± 0.04	7.14 ± 0.03	32.9 ± 0.31	0.76 ± 0.05	43.6 ± 1.7	0.92 ± 0.2	701.41 ± 11.54	29.4 ± 1.3	1.22 ± 0.2	Nd
Cd360	5.67 ± 0.09	7.11 ± 0.08	33.1 ± 0.25	0.81 ± 0.14	45.1 ± 1.3	338.76 ± 15.1	8.07 ± 1.32	27.5 ± 2.7	1.29 ± 0.7	Nd
Cd720	5.58 ± 0.12	7.05 ± 0.11	32.7 ± 0.14	0.88 ± 0.09	44.4 ± 0.8	708.44 ± 9.8	7.32 ± 0.51	30.8 ± 2.1	1.08 ± 0.2	Nd
$AgNO_3$	5.62 ± 0.06	7.20 ± 0.06	33.2 ± 0.36	0.75 ± 0.11	46.1 ± 0.6	0.88 ± 0.1	6.75 ± 0.74	29.5 ± 1.9	0.89 ± 0.6	18.76 ± 5.98
AgNP	5.63 ± 0.03	7.18 ± 0.04	32.7 ± 0.09	0.82 ± 0.07	45.9 ± 2.1	0.91 ± 0.1	7.92 ± 0.66	27.1 ± 1.2	1.15 ± 0.5	20.15 ± 4.32
AgDis	5.59 ± 0.07	7.11 ± 0.06	32.8 ± 0.28	0.80 ± 0.03	44.8 ± 1.3	1.01 ± 0.2	8.05 ± 1.08	28.3 ± 2.2	1.28 ± 0.3	Nd

*only values above detection limit are presented;

Nd – metal not detected above the detection limit;

PS – organic, control soil;

CS – soil contaminated by HM from metallurgical industry;

CEC – cation exchange capacity;

TOC – total organic carbon;

Zn360, Zn720, Ni360, Ni720, Pb360, Pb720, Cd360, Cd720 – organic peat soil (PS) contaminated artificially with zinc, nickel, lead or cadmium in two doses - 360 mg/kg of dry weight and 720 mg of dry weight;

$AgNO_3$ – organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nitrate;

AgNP - organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nanoparticles;

AgDis - organic peat soil contaminated artificially with dispersant without nanoparticles;

All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$)

2.2 Soil treatment

In total, study consisted of 13 different experimental treatments prepared in three biological replicates (Figure 1.). Soil was contaminated separately with two different concentrations (360 mg/kg of dry weight or 720 mg of dry weight) of: zinc, cadmium, lead, nickel, and one dose (25 mg/kg dry weight) of silver in nanoparticle or nitrate form. The silver nanoparticles used in this study were Ag NM 300K from European Commission Joint Research Centre (JRC), considered as the standard reference materials and fully characterized (Klein et al., 2011) as spherical. Moreover, AgNPs was also shown to consist of colloidal dispersion with a total, nominal content of silver at about 10.2 w/w %. Nanoparticles were dispersed in 4% w/w of glyceryl trioleate (Tagat® TO, Netherlands) and Tween 20 (polyoxyethylene (20) sorbitan monolauratet), holding > 99% number of particles with a measurement of about 15 nanometres, without any coating. The Transmisson Electron Microscopy (TEM) registered measurements of AgNPs at 17 ± 7 nm. Nanoparticles of smaller (approximately 5 nm) size were also been present. (Mendes et al., 2015). Pure dispersant was then used separately as an additional control condition. Selected metals were treated as separate conditions. Nitrates of all heavy metals were used in the process of artificial contamination of soil. Experimental soil was spiked with selected metal in its nitrate form, watered with 60 ml of distilled water and mixed for 10 minutes in high strength automatic industrial blender (Linkrich, China). After spiking, soil was left in growth chamber for 10 days before sowing seeds. The selected doses of Ni, Zn, Pb, and Cd were close to concentrations seen in soil contaminated by metallurgical industry in Poland (GPS: 50°30’N 18°56’E). In the cases of cadmium and nickel, the doses were chosen also as a most probable positive control for identification and then validation the *mt* expression in all experimental treatments. Moreover, it allowed for an identification of HKGs that remained stable throughout three different sources of metal stress: lab-made contamination with selected HM, lab-made contamination with two forms of silver and the complex contamination of post-industrial soil (Table 1.). The dose of silver nitrate and AgNPs was corresponding to a probable dose of silver that could be found in sewage sludge (Yang et al., 2012). For the control group, peat soil without any heavy metal contamination was selected as a medium which provided an optimum growth conditions. Thus, the influence of all selected metals were assessed in comparison to natural capabilities of *L. luteus* grown without any environmental stressors.

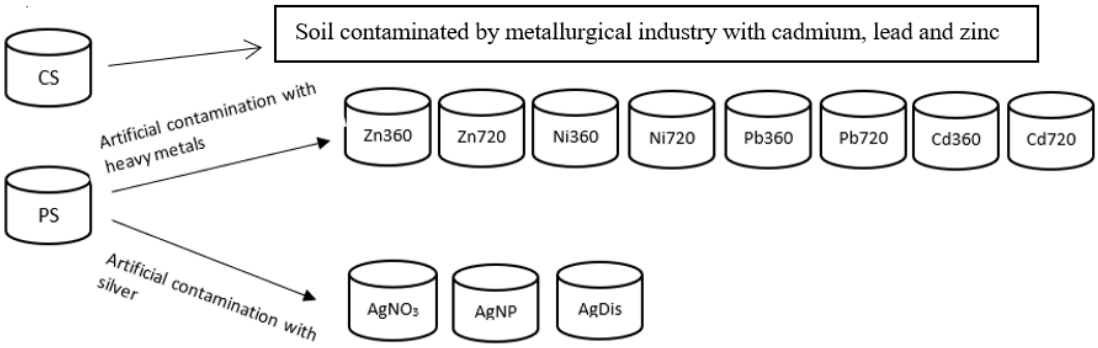


Figure 1. Experimental treatments. PS – organic peat soil, CS – contaminated soil from metallurgical industry, Zn360, Zn720, Ni360, Ni720, Pb360, Pb720, Cd360, Cd720 – organic peat soil contaminated artificially with zinc, nickel, lead or cadmium in two doses - 360 mg/kg of dry weight and 720 mg/kg of dry weight; $AgNO_3$ – organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nitrate, AgNP - organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nanoparticles, AgDis - organic peat soil contaminated artificially with dispersant without nanoparticles.

2.3 Experimental procedure

Before starting the experiment and planting seeds, the soil was transported to the Institute of Environmental Engineering in Poland and then, dried, sieved and thoughtfully mixed. *L. luteus* was then grown independently in 13 different experimental treatments, each in three individual, biological replicates. Certified and commercially available seeds of *L. luteus* were used (FRORMIX, Poland). Seeds quality and vitality was established at approximately 96% following standard germination test in petri dishes based on the norm ISO 11269-2. Each growth pot (square base, H – 15 cm, a – 10 cm) consisted of 200 g of selected soil. For all treatments, 20 seeds were placed in each pot. During the course of the experiment plants were watered using spray bottle with 20 ml of distilled water every other day. Each pot had a separate holder for any leakage. If there was any leakage in the holder then during the next course of watering it was recirculated to prevent the metal escape from the soil. Plants were grown for 4 weeks in a controlled conditions of a phytotron chamber (Biogenet FS360, Poland). For light, photosynthetic LED light was used at intensity of 4000 lx. Temperature during the day 21^{o}C, and during the night 18^{o}C. Photoperiod: 16h/8h (light/dark). After 4 weeks of exposure, plants were harvest from the experimental treatments and their shoots biomass was than stored in -80^{o}C.

2.4 Germination index and metal accumulation

Germination was measured every 24 h during first 14 days of incubation and was acknowledged to properly occur when plants radicles reached 2 mm long. Plantlets with spiral, short or in anyways deformed hypocotyls were recognized as abnormally developed and not included in the calculation of germination index which was then established as a total percentage of correctly germinated plants. Basic growth parameters including plants foliar surface, signs of chlorosis or fungi infection have also been recorded daily by visual observation. No significant ($p < 0.05$) variation between the experimental conditions was observed in that matter (data not shown). Contents of heavy metals were quantified in three independent replicates for each experimental treatment. Samples were harvested after 28 days of plants growth and dehydrated at 40^{o}C. Metal concentrations were determined using ICP-OES same way as for the samples of soil (ICP-OES; Thermo apparatus, USA). Bioconcentration factor (BCF) was calculated using the ration of metal concentration in shoots and in soils respectively [BCF = Cshoot/Csoil].

2.5 Guaiacol peroxidase activity and protein concentration

Guaiacol peroxidase (GPX) activity was recorded by Asada method with minor modification of plants biomass used for the measurement (Asada, 2006). 100 mg of plants shoot tissue was used for the assay. Overall, the activity of GPX was measured by the quantity of oxidation o guaiacol within 2-minute period in the presence of H_2O_2. The activity of GPX then estimated as an amount of enzyme that generated a change in the absorbance at 430 nm of 0.021/min using HACH spectrometer (HACH DR/4000V, USA). Obtained results were recalculated to U/mg of the whole protein concentration in shoots of *L. luteus*. Total protein concentration was measured by standard micro Lowry assay (Lowry et al., 1951). Bovine Serum Albumin was used as a standard (Sigma-Aldrich, USA).

2.6 Gene expression analysis

RNA isolation and reverse transcription. After the experiment, shoot RNA was extracted from tissues with the EURx Universal DNA, RNA, Protein kit following the manufacturer's instructions (EUR_x, Poland). Thus, 100 mg of biomass was used for each RNA extraction. First, frozen tissues were homogenized by the mechanical

homogenizator (IKA Labortechnik, USA). RNA integrity was assessed on 1.5% agarose gel dyed with ethidium bromide (EtBr) (Thermo Scientific, Germany). Reverse transcription of 1.5 μg of extracted RNA was completed using OmniscriptR Reverse Transcription Kit (QIAGEN, Germany) according to manufacturer's instructions. Obtained cDNA was then stored at −20°C and used for further experiments.

Primers design. In our study, expression stability of housekeeping gens (HKGs) for *L. luteus* was examined under 13 treatment conditions. To select adequate HKG for data normalization ΔCt approach and NormFinder software were used following (Brulle et al., 2014) procedure.

The following potential housekeeping genes (HKGs) were tested: tubulin6, β-tubulin, actin and elongation factor 1-α (ef1α) and 18S. As the complete genome as well as HKGs of *L. luteus* were not published yet, sequences of taxonomically related species like *Arabidopsis thaliana* and *Trifolium repens*, were found. The selected genes were checked in BLAST analysis tool, to identify the most conserved sequences, which were used in for primer design using the Primer3Plus software. The length of the amplified fragments including target gene and HKG ranged from approximately 70 to 200 bp. Finally, the control samples with cDNA were used as a template to evaluate the primers by classic PCR in order to make sure the primers were suitable. The obtained PCR products were extracted from gel, purified and sequenced. The sequences allowed the design of specific qPCR primers (Table 2).

Table 2. Primers sequence.

Name	Primer sequence 5′-3′	Primer sequence 5′-3′	Product size (bp)	Tm (^{O}C)
18S	AGTTGAGGGATGATTTGG	TGCAAACCCTCGTCAATT	104	60
EF	GGCTGAGCGTGAAAGAG	CCAGCTTCAAAACCACCA	186	60
β-tubulin	CCAGGCTCCAGATCCAT	GCGAGTCAATGTTTACTA	78	59
Act	CCACCACTAAGAACAATG	TGCTGGATTCATGAGACC	92	60
Tub6	TGCAGAACAAGAACTCCT	ACCCTCCTAAACATCTCT	145	59
MT	GCGAGAGAGTACACACGGA	GCTACAGTTTGATCCGCAG	108	59

EF – elongation factor, Tubb - β-tubulin, Act – actin, Tub6 – tubulin6, MT – metallothionein

PCR and sequencing. PCR reactions were performed as follows: initial denaturation at a temperature of 95°C for total of 5 min followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 60 s. In the end, after cycles, the last step of the program consisted of 72°C for 5 min (MJ Research PTC-200, BIORAD, USA). Promega GoTaqR G2 Flex DNA Polymerase mix was used (Promega, USA) in order to amplify the desired DNA fragments. Magnesium chloride ($MgCl_2$) was added to a concentration of 2 mM. and primers were added in final concentration of: 0.4 μM for each reaction. Each reaction was performed in three biological replicates and two technical ones. The size of each PCR product was examined using a 3% agarose gel stained with EtBr (Thermo Scientific, Germany). Parameters of the electrophoresis were as follows: 150 V, 45 minutes.

Amplified products were purified using the GE Healthcare ilstra GFXTM PCR DNA and GEL Band Purification (GE Healthcare, UK) following the manufacturer's instructions. Then, samples were send for SAGNER sequencing (Genoscreen, France). Using CodonCode Aligner and Sequencher software, a contig was assembled and used to design qPCR primers which were as follows:

- *18S* – 5′ AGTTGAGGGATGATTTGG 3′; 5′ TGCAAACCCTCGTCAATT 3′; product size 104 bp, Tm 60^{o}C;
- *Elongation factor* - 5′ GGCTGAGCGTGAAAGAG 3′; 5′ CCAGCTTCAAAACCACCA 3′; product size 186 bp, Tm 60^{o}C;
- *β-tubulin* - 5′ CCAGGCTCCAGATCCAT 3′; 5′ GCGAGTCAATGTTTACTA 3′; product size 78 bp, Tm 59^{o}C;
- *Actin* - 5′ CCACCACTAAGAACAATG 3′; 5′ TGCTGGATTCATGAGACC 3′; product size 92 bp, Tm 60^{o}C;
- *Tubulinα6* - 5′ TGCAGAACAAGAACTCCT 3′; 5′ ACCCTCCTAAACATCTCT 3′; product size 145 bp, Tm 59^{o}C;
- *Metallothionein* - 5′ GCGAGAGAGTACACACGGAG 3′; 5′ GCTACAGTTTGATCCGCAGC 3′; product size 108 bp, Tm 59^{o}C;

Real-time qPCR amplification. For qPCR, Stratagene Mx3005P thermocycler was used (Agilent Technologies, USA) with MESA Blue qPCR Master Mix Plus SYBR (Eurogene, Belgium), and reactions were performed on plates (twin.tec PCR plate, Eppendorf, Germany). All qPCR reaction were performed as follows: initial denaturation at 95°C for 5 min followed by 40 cycles starting at 95°C 30 s, then 58°C 30 s, and 72°C 60 s. After 40 cycles, the last step of the program consisted of 72°C for 5 min.

The relative expression levels of target gene was established as a total number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of each PCR reaction (C_T). After that, the relative expression level was calculated according to the following formula: $R= (ETg)^{CPTg} /(Eref)^{CPref}$

2.7 Data management and statistical analysis

The validation of House-keeping genes (HKGs) was performed by BestKeeper and NormFinder software. All results are shown as means ± standard deviation, n = 3. All analyses were completed in three biological replicates (n) and at least two technical replicates. Overall, the descriptive statistics and all statistical analyses were prepared using the OriginPro 2015 software. Different letters on bars imply a significant ($p < 0.05$) variation according to ANOVA with post-hoc Tukey's test $p < 0.05$. P values below 0.05 were set as statistically significant.

3 RESULTS

3.1 Germination index and metal accumulation

The initial germination was not influenced in any experimental treatments. From 20 seeds in each pot at least 17 germinated properly (85%) and there were no statistical differences between applied metals or doses (data not shown). Bioconcentration (BCF) was assessed in shoots of incubated plants. BCF values > 1 had been used before to assess the probable potential of selected plant species for phytoextraction and/or phytostabilization of metals in soil. *L. luteus* is a known metal hyperaccumulator. In our experiment high BCF values were obtained for all tested metals (Zn, Pb, Ni, Cd) with an exception of Ag which was not found in plants tissues below the detection limit of ICP-OES (Tab. 3).

Table 3. Bioconcentraction factor (BCF).

Growth medium	**BCF**				
	Zn	**Ni**	**Pb**	**Cd**	**Ag**
PS	1.7 ± 1.3 a	Nd	Nd	Nd	Nd
CS	**7.40 ± 0.55 b**	**3.15 ± 0.5 a**	**11.7 ± 0.9 a**	**5.21 ± 1.60 a**	Nd
Zn360	**27.1 ± 2.1 c**	Nd	Nd	Nd	Nd
Zn720	**31.9 ± 3.2 d**	Nd	Nd	Nd	Nd
Ni360	1.2 ± 0.8 a	**17.2 ± 3.1 b**	Nd	Nd	Nd
Ni720	2.0 ± 1.5 a	**26.1 ± 4.3 c**	Nd	Nd	Nd
Pb360	1.8 ± 0.6 a	Nd	**10.7 ± 1.3 a**	Nd	Nd
Pb720	1.4 ± 0.5 a	Nd	**53.2 ± 8.1 b**	Nd	Nd
Cd360	1.7 ± 1.3 a	Nd	Nd	**12.2 ± 3.81 b**	Nd
Cd720	1.9 ± 1.1 a	Nd	Nd	**11.1 ± 0.25 b**	Nd
$AgNO_3$	2.1 ± 1.0 a	Nd	Nd	Nd	Nd
AgNP	1.5 ± 0.8 a	Nd	Nd	Nd	Nd
AgDis	1.8 ± 0.5 a	Nd	Nd	Nd	Nd

Nd – metal not detected above detection limit, only values above detection limit are presented;

PS – organic, control soil;

CS – soil contaminated by HM from metallurgical industry;

Zn360, Zn720, Ni360, Ni720, Pb360, Pb720, Cd360, Cd720 – organic peat soil contaminated artificially with zinc, nickel, lead or cadmium in two doses - 360 mg/kg of dry weight and 720 mg of dry weight;

$AgNO_3$ – organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nitrate;

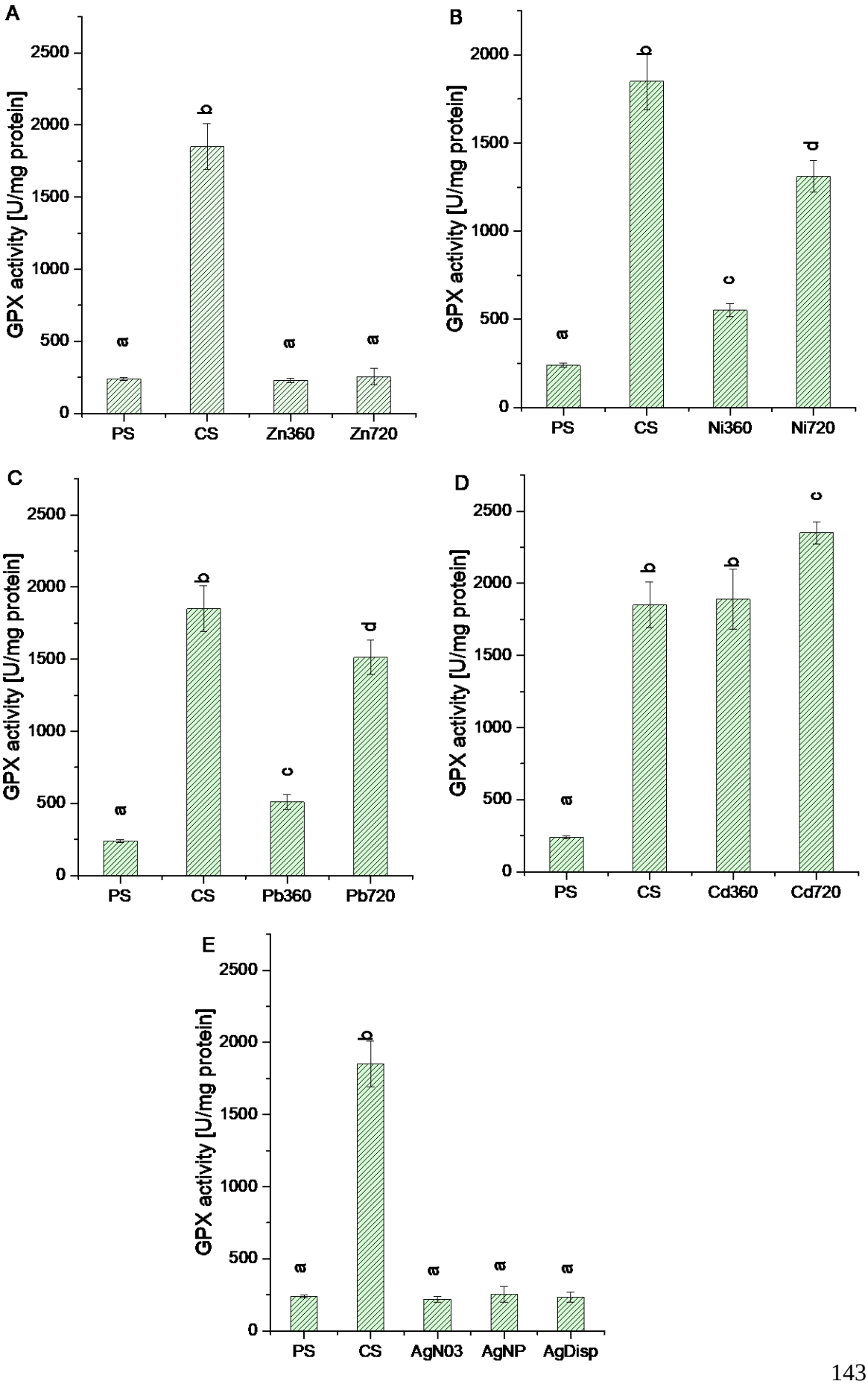
AgNP - organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nanoparticles;

AgDis - organic peat soil contaminated artificially with dispersant without nanoparticles;

All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$).

3.2 GPX activity and protein concentration

Exposure to heavy metals, either added artificially or present in metallurgical industry-contaminated soils significantly ($p < 0.05$) increased GPX activity in comparison to uncontaminated soil (Fig. 2). The highest GPX activity was noticed for plants exposed to soil contaminated by various metals from metallurgical industry and for plants grown on soil artificially contaminated with Cd (Fig. 2D). In such cases, the increase in GPX activity is even 8-10 times compared to the uncontaminated control. The contamination with Zn in lower dose did not cause an increase in GPX activity (Fig. 2A). Moreover, no statistically significant ($p < 0.05$) changes were noticed in GPX activity after treatments with silver nanoparticles and silver nitrate (Fig. 2E). Similarly, the concentration of those proteins in plants shoots was severely impacted by HMs (Fig. 3). The lowest protein concentration was observed again in plants grown on Cd contaminated soil and on soil contaminated by a range of metals from metallurgical industry (Fig. 3D). Again, no statistically significant difference was noticed after exposure to silver nitrate or silver nanoparticles ($p < 0.05$) (Fig. 3E).


A
GPX activity [U/mg protein]
2500
2000
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PS
CS
Zn360
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GPX activity [U/mg protein]
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GPX activity [U/mg protein]
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CS
AgN03
AgNP
AgDisp

Figure 2. GPX activity in plants leaves. PS – organic, control soil, CS – soil contaminated by HM from metallurgical industry, Zn360, Zn720, Ni360, Ni720, Pb360, Pb720, Cd360, Cd720 – organic peat soil contaminated artificially with zinc, nickel, lead or cadmium in two doses - 360 mg/kg of dry weight and 720 mg/kg of dry weight; $AgNO_3$ – organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nitrate, AgNP - organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nanoparticles, AgDis - organic peat soil contaminated artificially with dispersant without nanoparticles. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$)

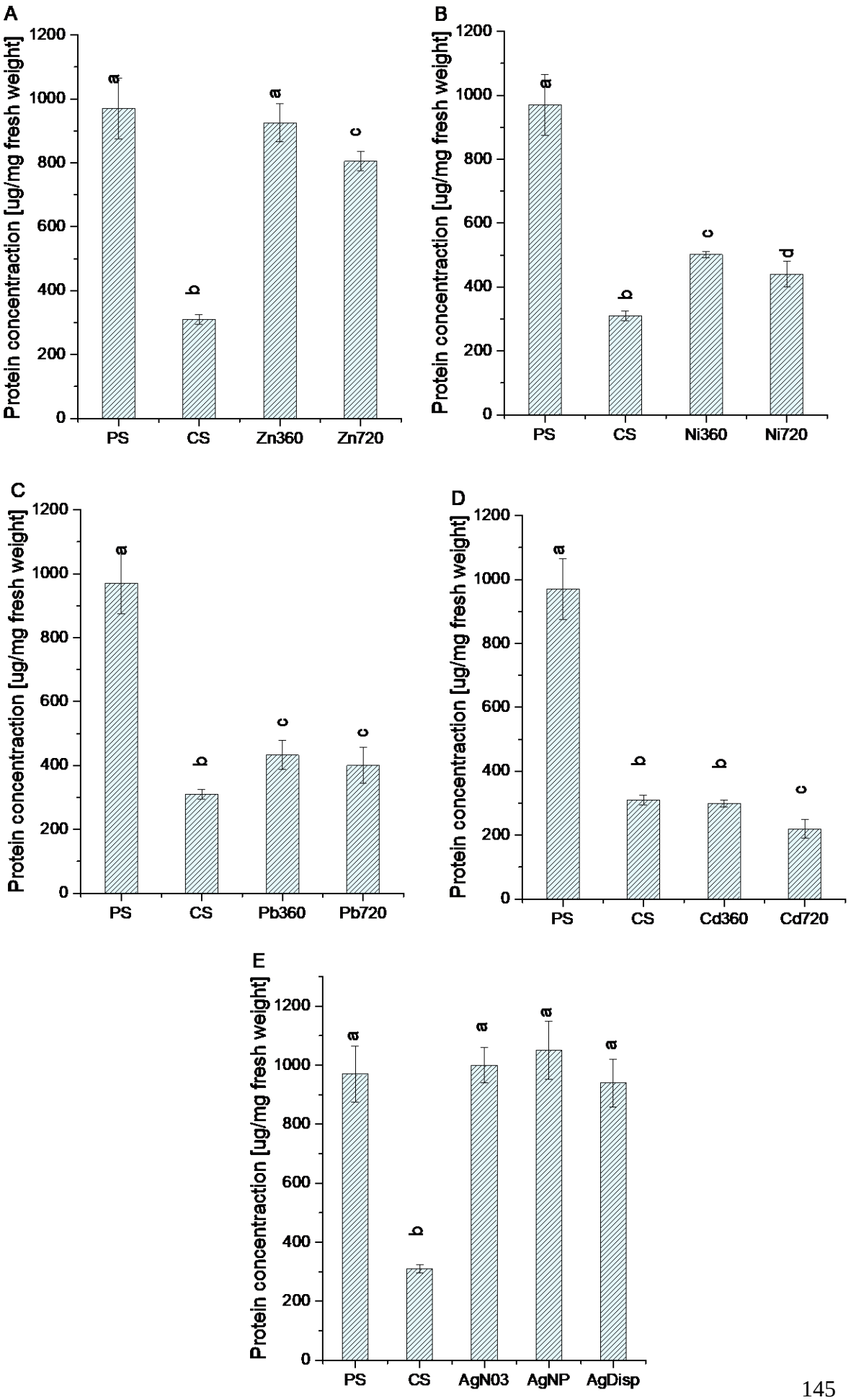
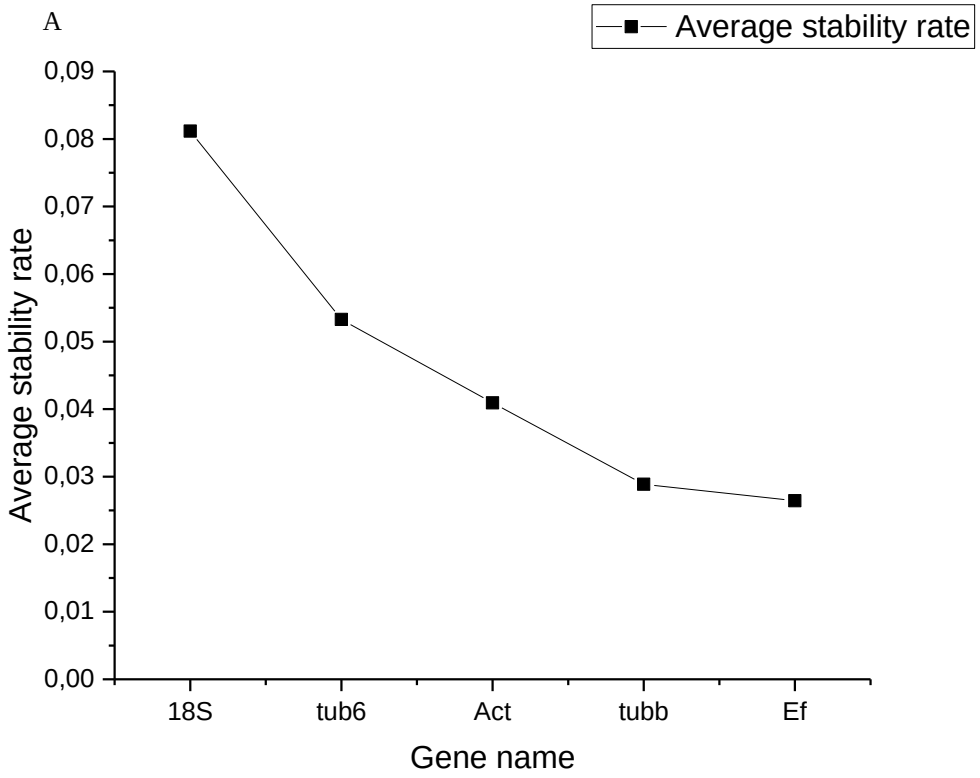

A
Protein concentration [ug/mg fresh weight]
0
200
400
600
800
1000
1200
a
b
a
c
PS
CS
Zn360
Zn720
B
Protein concentration [ug/mg fresh weight]
a
b
c
d
PS
CS
Ni360
Ni720
C
Protein concentration [ug/mg fresh weight]
a
b
c
c
PS
CS
Pb360
Pb720
D
Protein concentration [ug/mg fresh weight]
a
b
b
c
PS
CS
Cd360
Cd720
E
Protein concentration [ug/mg fresh weight]
a
b
a
a
a
PS
CS
AgN03
AgNP
AgDisp

Figure 3. Protein concentration. PS – organic, control soil, CS – soil contaminated by HM from metallurgical industry, Zn360, Zn720, Ni360, Ni720, Pb360, Pb720, Cd360, Cd720 – organic peat soil contaminated artificially with zinc, nickel, lead or cadmium in two doses - 360 mg/kg of dry weight and 720 mg/kg of dry weight; $AgNO_3$ – organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nitrate, AgNP - organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nanoparticles, AgDis - organic peat soil contaminated artificially with dispersant without nanoparticles. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$)

3.3 Validation of house-keeping genes

To determine the best house-keeping genes, the NormFinder and BestKeeper software were used. The Normfinder consists in an algorithm for evaluating the optimal normalization gene among various candidates (Bernard et al., 2018). Vandesompele at al. (2002) evaluated two individual parameters of total HKGs stability (the M value - average expression stability and the V value - pairwise expression). The results are presented as average expression stability M (Fig. 4). A low M value (so the lowest variation coefficient) indicates high stability of tested gene, hence, considerable suitability as a control gene. For *L. luteus* the least stable but still below M – 0.5 genes during abiotic stress were *18S* and *Tub6*, while the most stable HKGs genes were *Ef* and *Tubb* (Fig. 4). The expression stability range was as follows: *Ef* > *Tubb* > *Act* > *Tub6* > *18S*. The average stability $M < 0.5$ indicates good stability and HKGs with lowest values are the most stably expressed. Overall, all selected HKGs had M value lower than 0.5 which means that they can be used to validate results after qPCR (Fig. 4).

A


Average stability rate
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
Average stability rate
18S
tub6
Act
tubb
Ef
Gene name

B

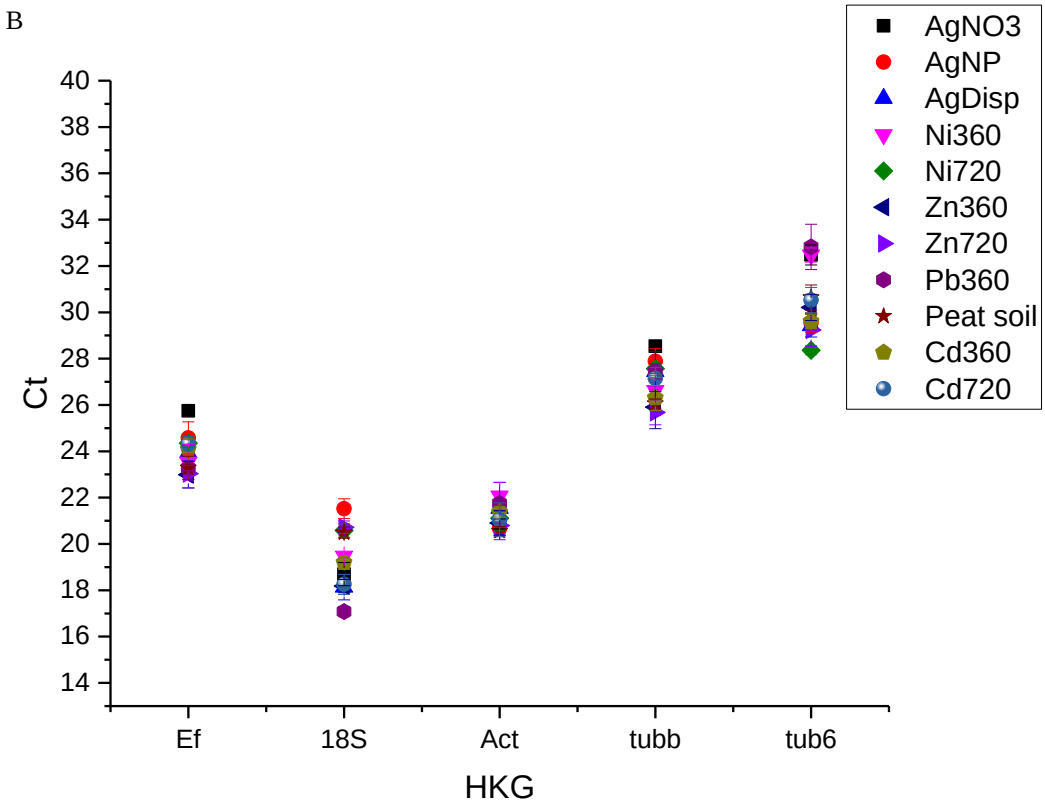

AgNO3
AgNP
AgDisp
Ni360
Ni720
Zn360
Zn720
Pb360
Peat soil
Cd360
Cd720
40
38
36
34
32
30
28
26
24
22
20
18
16
14
Ct
Ef
18S
Act
tubb
tub6
HKG

Figure 4. A - Average expression stability *(M),* of HKG by NormFinder analysis. *EF – elongation factor, Tubb - β-tubulin, Act – actin, Tub6 – tubulin6; B* - the transcriptional profiles of individual HKGs in absolute Ct values over all RNA samples in *Lupinus luteus. EF – elongation factor, Tubb - β-tubulin, Act – actin, Tub6 – tubulin6*. Peat soil – organic, control soil, CS – soil contaminated by HM from metallurgical industry, Zn360, Zn720, Ni360, Ni720, Pb360, Pb720, Cd360, Cd720 – organic peat soil contaminated artificially with zinc, nickel, lead or cadmium in two doses - 360 mg/kg of dry weight and 720 mg/kg of dry weight; $AgNO_3$ – organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nitrate, AgNP - organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nanoparticles, AgDis - organic peat soil contaminated artificially with dispersant without nanoparticles. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$).

3.4 Expression of metallothioneins

The expression profiles of selected target gene has been normalized by the geometric mean of the four most stable HKGs (Fig. 5). Overall, all treatments with Pb, Cd, and Ni had a significant and up-regulated effects on the level of *MT* expression ($p < 0.05$) in comparison to uncontaminated control (Fig. 5B, 5C, 5D). In those treatments, a significant increase was also noticed with a higher dose of selected metal ($p < 0.05$). Nevertheless, major differences had been observed between different metal treatments. The highest increase in *MT* expression was seen after artificial contamination with Cd where we noticed almost 10-fold higher expression rate than in control conditions (Fig. 5D). The induction of target gene showed remarkable sensitivity to different treatments.

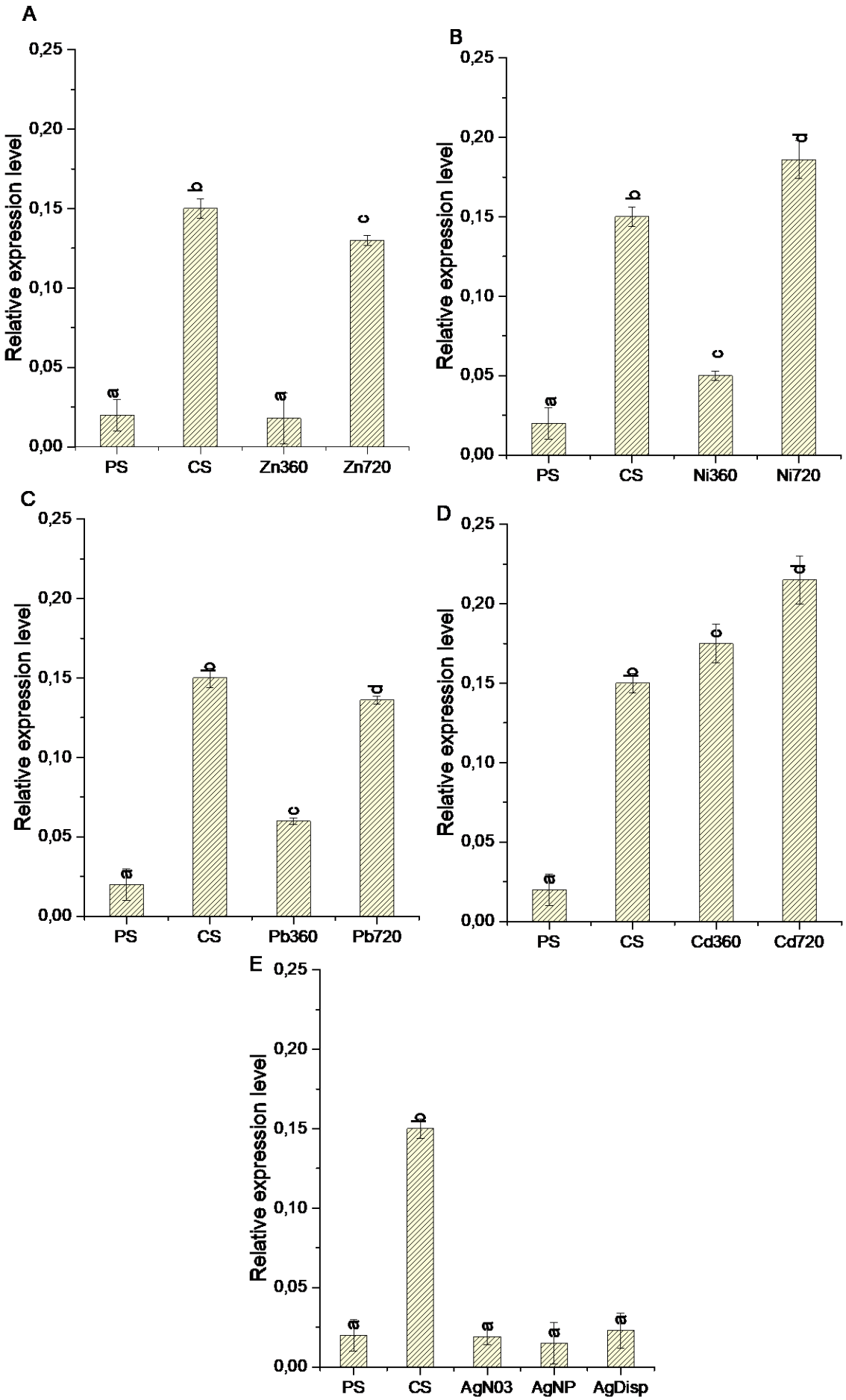

A
Relative expression level
0,25
0,20
0,15
0,10
0,05
0,00
a
b
a
c
PS
CS
Zn360
Zn720
B
Relative expression level
a
b
c
d
PS
CS
Ni360
Ni720
C
Relative expression level
a
b
c
d
PS
CS
Pb360
Pb720
D
Relative expression level
a
b
c
d
PS
CS
Cd360
Cd720
E
Relative expression level
a
b
a
a
a
PS
CS
AgN03
AgNP
AgDisp

Figure 5. The relative expression level of *mt*. PS – organic, control soil, CS – soil contaminated by HM from metallurgical industry, Zn360, Zn720, Ni360, Ni720, Pb360, Pb720, Cd360, Cd720 – organic peat soil contaminated artificially with zinc, nickel, lead or cadmium in two doses - 360 mg/kg of dry weight and 720 mg/kg of dry weight; $AgNO_3$ – organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nitrate, AgNP - organic peat soil contaminated artificially with 25 mg/kg dry weight of silver nanoparticles, AgDis - organic peat soil contaminated artificially with dispersant without nanoparticles. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$).

4 DISCUSSION

In our study, *L. luteus* showed excellent potential for Pb, Cd and Ni uptake which means that it can be also used in the removal of HMs from the soil. It is worth mentioning, that *L. luteus* also has economical value as crop production since it is easily adaptable to abiotic stress and can survive well in a moderate climate allowing for cheaper remediation (Balseiro-Romero et al., 2017). On the other hand, unlike some model species, it was shown to not produce true and accurate results in toxicity tests based only on seed germination index (Jaskulak et al., 2018). Seeds of *Fabaceae* possess a large stock of nutrients for plant germination, even under severe abiotic stress such as contamination with HMs (Balseiro-Romero et al., 2017). Thus, to assess the probability of use *L. luteus* on contaminated site and to assess its success and productivity, more sensitive biomarkers are needed. In this experiment, germination index did not produce any statistically significant results between application of heavy metals and its dose ($p < 0.05$).

Toxic and adverse effects of HMs including Pb, Cd, Ni or Zn on plants' growth have been shown before in a number of studies (Galal et al., 2015, Mitton et al., 2016, Chaâbene et al., 2018). Heavy metal (HM) contamination of soils in Europe is mostly caused by industrialization, mainly past mining and smelting activities. Nowadays, a tremendous increase in land contamination is observed yearly and continuously more areas suffer chronically from contamination and decrease in organic matter caused mostly by overexploitation and droughts. Moreover, the presence of contamination in soils is most often assessed only by chemical analyses of samples. The assessment of toxicity of such sites on living organisms can be a useful addition to monitor and control the contamination better (Caverzan et al., 2014). Such biomonitoring can involve both: the presence of given contaminant in the living organisms and its influence on physiological, metabolical and molecular changes caused by its toxicity (Soudani et al., 2017). Physiological and molecular changes in plants after the exposure to heavy metals can be a critical element for planning the phytoremediation or any other restoration since it can provide necessary information concerning the detoxification capabilities involved and chance of plant survival (Mitton et al., 2016, Soudani et al., 2017).

In this experiment, GPX activity and protein concentration were used as physiological biomarkers of plant stress after the influence of heavy metals and silver nanoparticles. Overall, the highest activity of GPX was observed on soil contaminated artificially by Cd

and on soil contaminated by HMs from metallurgical industry in Poland. Moreover, both of those markers showed a significant response to selected treatments ($p < 0.05$). GPX activity was elevated vastly after the exposure to cadmium, nickel, and lead and in its highest point, it equated almost ten times the activity on the uncontaminated soil. Similar results were reported in study by Anjum et al. 2014, where after exposure of another *Fabaceae* species - *V. radiata* to cadmium a significant increase in GPX activity was observed. Also, GPX activity was highest after exposure to high levels of cadmium in comparison to other metals like lead which was also noticed in Anjum et al., 2014 study. Moreover, In 2007, Semane et al. studied seedlings of *A. thaliana* exposed to Cd solutions and APX, GR, CAT, SOD and GPX activities were measured in their shoots and leaves. Overall the activity of APX, SOD and GPX were elevated in all samples with cadmium which can indicate that GPX activity can be a sensitive biomarker not only to different metals but also different plants species since *A. thaliana* belongs to *brassicaceae* and our plant *L. luteus* to *fabaceae* species. In our experiment for all treatments with the exception of silver, the protein concentration declined severely in comparison to the control. Moreover, no influence of silver in both nitrate form or its nanoparticles was noticed on the GPX activity and protein concentration.

The necessity for accurate and quick toxicity assessment is gaining more interest as a mean to assess the severity of soil contamination and to assess its impact on organisms growing in contamination areas. In combination with other biomarkers, gene expression study is a powerful tool to follow the crop production and phytoremediation operations in contaminated sites. Accurate, selected choice of reference genes is a prerequisite to quantify the expression of target genes. Moreover, HKGs used as a reference gene in qRT-PCR should show high stability regardless of each biological conditions (Lambret-Frotté et al., 2015, Martins et al., 2017). During our experiment, the identification and validation of stable house-keeping genes and the isolation of target metallothionein gene in stress resistance and hyperaccumulating plant – *L. luteus* allowed to explore the probability of its involvement in metal detoxification and to use this gene as a potential biomarker. To the best of our knowledge, this is the first work investigating stable house-keeping genes and expression of metallothioneins in *L. luteus*. In a case of HKG it is important to find and properly validate their stability for the chosen organism since it was shown before that such stability is different not only in regards to different conditions but also for each species. Niu et al (2017) noticed that in *Poa pratensis* L., under various

stress conditions, different genes were stable. For heat stress high stability showed *eIF4A*, *TBP-1* and *E2* but for ABA treatment, genes such as *eIF4A*, *TEF1*, and *E2* had the high expression stability (Huang et al., 2014). Those genes are considered as suitable and reliable reference genes for gene expression normalization for this plant exposed to abiotic stress, however each of them is more suitable for specific treatment conditions. Similar tendency was noticed by Chen et al. (2015) who showed that the difference in stability of *ACT* in *Agrostis stolonifera* leaves depends on environmental conditions. This gene showed high stability under salt treatment and significantly lower stability under cold stress ($p < 0.05$). Nevertheless there are some genes that are stable under many treatments. Martins et al. (2017) in their work presented, that the most commonly used genes for normalization like *GAPDH* in their experiment showed high expression variability against to less known *KIN*, *BIND* and *SDH*. Moreover, they also noticed that factor as tissue type, cell age and even abiotic stress type significantly influence on gene expression stability, thus it is required to enhance selection of the most suitable reference gene to each particular conditions ($p < 0.05$) and for the studied species.

Presented work is the first in-depth study aimed to find and evaluate the optimal control genes (HKG) for the quantification of transcript levels during metal stress in *L. luteus* development using RT-PCR technology. As a result of this assessment, we recommend stable HKG for *L. luteus* as references for normalization of any target gene expression. All the HKGs listed in Table 2 showed stability (M) lower than 0.5 which means that they can be used to validate the qPCR results.

In order to deal with exposure to HMs, minimize the oxidative stress and restore internal homeostasis, plants possess a vast range of mechanisms regulating the uptake and detoxification of HMs. Among them, there are HM chelators like metallothioneins (Bolan et al., 2014). Overall, MTs constitute to a family of low-molecular-weight proteins (approximately 4-15 kDA) with broad distribution in plants and are characterized by high content of cysteine (Cys) and the capability to binding with other metals including Pb, Ni, Zn, Cu and Cd in a stable manner (Fosso-Kankeu et al., 2014). Genes that code MTs have been found in many plant species including both, *monocotyledons* and *dicotyledons*. Their expression had been also reported in several different plant tissues including roots, stems and leaves (Sharma et al., 2016). Although MTs existence had been known for several years, their functions are still not completely clarified, but they have been shown

to play a crucial role in metal tolerance, protection against oxidative damage, also during initial germination and roots development (Fosso-Kankeu et al., 2014, Sharma et al., 2016, Chaâbene et al., 2018). Thus, the identification of such markers in non-model plant species, which can be directly applied in remediation technics, can be used to evaluate the impact of contamination on organisms and to plan large-scale remediation by, for example spreading of specific fertilizers or waste products. Measuring the level of expression of specific genes as a toxicity biomarkers gained more interest in recent years, but the necessary data like sequences for stable HKG or target genes in non-model plant species are still mostly unavailable (Chen et al., 2015). In the described experiment, *MT* gene expression level was highly affected by different metal treatment and showed statistically significant results between different kinds of soils and various metals ($p < 0.05$). Interestingly, in samples contaminated by the smallest dose of Zn and in all samples containing Ag, there were no statistically important changes in comparison to the uncontaminated soil which suggest the lack of toxicity in those samples which correlates to other measurement endpoints like GPX activity and protein concentration. Moreover, provided results are evidence for metallothionein involvement in metal tolerance and its detoxification in *L. luteus.* In 2016 work of Bernard et al., significant increase in *Mt1* expression was noticed in another *fabaceae* species - *Trifolium repens* after exposure to cadmium and lead. Similarly to our study, the highest expression of *Mt* was noticed after exposure to cadmium by itself and lower after treatments with mixtures of cadmium and lead.

5 CONCLUSIONS

The study gave an in-depth insight into the antioxidant and molecular basis of HM resistance in *L. luteus*. Overall, the physiological and molecular changes in *L. luteus* were assessed after its exposure to different metal treatments to better understand the mechanisms of its metal tolerance which can be successfully exploited in remediation techniques including the removal of metals from contaminated sites. The study showed the effects of HMs and silver nanoparticles on the level of *MT* expression, GPX enzyme activity and the bioaccumulation of HMs in leaves of *L. luteus*. As such, a significant increase in the expression levels of *MT* was observed in plants grown under heavy metal stress and none on plants grown on 25 mg kg^{-1} of silver nanoparticles. The differences in GPX activity, and *MT* expression showed statistically significant changes between different medium and applied doses which can mean that presented assay can be used as a sensitive stress marker for planning the phytoremediation process.

Acknowledgements

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Gene expression, DNA damage and other stress markers in *Sinapis alba L.* exposed to heavy metals with special reference to sewage sludge application on contaminated sites*

Marta Jaskulak[1,2], Anna Grobelak[1], Anna Grosser[1], Małgorzata Kacprzak[1], Franck Vandenbulcke[2]

[1]Institute of Environmental Engineering, Faculty of Infrastructure and Environment, Czestochowa University of Technology, Czestochowa, Poland;

[2]University of Lille, Laboratory of civil engineering and environment (LGCgE), Envrionmental Axis, F-59650 Villeneuve d'Ascq, France;

Bioindicators are promising tools used to detect the long-term effects of selected biosolids on plants development and should be implemented before large-scale supplementation of sewage sludge into the soil. The presented study shows the impact of sewage sludge application on metal-sensitive toxicity biological parameters (biomarkers) in S*inapis alba* including: germination, root length, the activity of guaiacol peroxidase, the chlorophyll content, the level of DNA damage and the expression level of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) and metallothionein (*mt*). We evaluated data from selected biomarkers in order to broaden our understanding of plants defence mechanisms against heavy metal contamination and the application of sewage sludge into soils. Overall, in contaminated soil after supplementation with both municipal sewage sludges, an increase in toxicity was noticed in DNA damage, *mt* and *rbcl* expression and total chlorophyll content. The supplementation of both soils with municipal sewage sludge caused a two-time induction in the *mt* expression. Moreover, clean soil supplemented with sewage sludge caused an increase in DNA damage shown as the tail moment from approximately 12μm on control to 40μm after supplementation. Even if those biosolids increased the initial germination, roots length, and biomass in comparison to the unamended soil, the toxicity was evidenced with other stress markers. Results showed, that in order to accurately assess the influence of sewage sludge application on plants the use of several specific biomarkers is required for safe land restoration. The conducted study also confirmed, both under biochemical and genotoxic tests, that iron enrichment for biosolids or contaminated soil can significantly reduce the bioavailability and toxicity of other metals.

**Jaskulak, M., Grobelak, A., Grosser, A., & Vandenbulcke, F. (2019). Gene expression, DNA damage and other stress markers in Sinapis alba L. exposed to heavy metals with special reference to sewage sludge application on contaminated sites. Ecotoxicology and Environmental Safety, 181, 508–517. doi:10.1016/j.ecoenv.2019.06.025*

Highlights

- Gene expression and DNA damage in *Sinapis alba* exposed to metals and biosolids
- Increase in toxicity was noticed after supplementation with municipal sewage sludge
- The use of several specific biomarkers is required for safe land restoration
- Selected endpoints can be use as sensitive biomarkers for phytoremediation

KEYWORDS: biomarkers, phytoremediation, metallothioneins, heavy metals, sewage sludge

1 INTRODUCTION

The contamination of soil with toxic elements such as heavy metals (HMs) is becoming a global concern across the world (Ali et al., 2013). The major contribution to this contamination comes from anthropogenic activities such as rapid industrialization, transport, and the improper use of fertilizers and pesticides (Fellet et al., 2014). The constant increase in the human population is also causing a rise in the quantities wastewater produced across the globe leading to the production of increasing volumes of sewage sludge (SS) which have to be treated and disposed (Grobelak et al., 2017). In spite of possibility of the presence of various toxic compounds, especially HMs, sewage sludge are often used as a fertilizer, and land spreading has become a more common practice in recent years. It was shown before that soil supplementation with sewage sludge (SS) can improve soil properties and increase crop productivity and can also be used to maintain and restore the quality of highly degraded post-industrial soils (Homa et al., 2015). Such practice can significantly decrease the costs of soil restoration since it is cheaper than using synthetic fertilizers or other synthetic compounds and provides a lot of organic matter which is often missing in highly degraded soils (Kumar et al., 2017). It has been also shown in recent decades that more and more areas had been chronically suffering from a decrease in the content of organic matter which is mostly caused by droughts, industrial contamination or overexploitation (Peng et al., 2016). Therefore, the reuse of sewage sludge is often proposed as an alternative solution for the improvement of soil quality at low costs (Fellet et al., 2014). However, it needs to be remembered that sewage sludge can contain various contaminants, including heavy metals which can further contaminate the soil. Thus, the land application of SS needs to be done with caution (Goss et al., 2013).

Toxic effects of HMs on organisms are well documented (Emamverdian et al., 2015). For instance, an excess of HMs in soil may cause adverse effects in plants including an increase in oxidative stress which can cause severe damage to photosynthetic apparatus and other unfavourable physiological effects (Ali et al., 2013). The efficiency of carbon dioxide fixation in plants is known to be remarkably sensitive to changes in environmental conditions. In the land species of plants, it is controlled by the RuBisCO protein (ribulose-1,5-bisphosphate carboxylase/oxygenase) which consists of eight small and eight large subunits encoded by a single chloroplast gene (Bourioug et al., 2015).

Overall, the toxicity of HMs can also be magnified by their ability to bind with different molecules, including enzymes, which leads to enzyme inactivation and causes even more toxic alterations (Busch et al., 2013). Moreover, metal ions also trigger a vast cascade of protective, antioxidant responses in plants to minimize their toxic effects or to prevent further damage and excessive production of reactive oxygen species (ROS) (Elloumi et al., 2014). Such mechanisms allow for an increase in plants tolerance to adverse environmental conditions and can reduce the bioaccumulation of metals in plant tissues, for example by restricting its translocation to plant shoots (Emamverdian et al., 2015). Furthermore, some protective mechanisms allow for the creation of specific complexes between metals and thiol-rich molecules in order to be compartmentalized in vacuoles. This improves plant tolerance to excessive concentration of HMs and helps to maintain the cellular homeostasis (Gagetti et al., 2016). The well-known Metallothioneins (MTs) are thiol-rich, small peptides that can bind with several metals. Stable complexes between HM ions and MTs formed in cells cytosol may be transported and compartmented in vacuoles, thus reducing the chance of the metal interacting with essential molecules (Chaâbene et al., 2018). Recent molecular studies indicate that these proteins are crucial for metal tolerance in plants exposed to HMs such as lead (Pb), cadmium (Cd), copper (Cu), nickel (Ni) and zinc (Zn). In most cases, metal exposure strongly induces MT expression in plants (Sharma et al., 2016).

MTs constitute a family of low-molecular-weight proteins (approximately 4 - 15 kDa) with a broad distribution in plants and are characterized by a high content of cysteine (Cys). They have the capability to bind with other metals, including Pb, Ni, Zn, Cu, and Cd in a stable manner. Genes that encode MTs have been found in many plant species, both *monocotyledons*, and *dicotyledons*. Their expression had been reported in various plant tissues (Gagetti et al., 2016, Chaâbene et al., 2018). Although the existence of MTs had been known for several years, their functions still have not been completely clarified. They have been shown to play a significant role in metal tolerance, protection against oxidative damage, signalling against pathogens, and also during initial germination and root development (Elloumi et al., 2014).

In an attempt to assess the impacts and measure the effects of contaminants on organisms and potentially on ecosystems, selected and characterized biomarkers are needed. The

underlying principle of biomarkers is to analyse the physiological or biochemical response of plants to (a) particular toxic element(s). The concept of biomarkers derives from an idea that adverse effects will manifest themselves first at a subcellular level – before any visible changes could be noticed (Hattab et al., 2016, Javed et al., 2016). The measurement of such responses may serve as a useful tool to improve the assessment of exposure to a toxic compound and enhance the ability to assess the risks of toxic effects and the overall survival of exposed populations (Hsu et al., 2016). Besides, molecular markers linked to heavy metal tolerance can also be exploited to elucidate the molecular mechanisms of tolerance (i.e. detoxification) and to advance phytoremediation technologies further (Jaskulak et al., 2018). Understanding the molecular and genetic mechanisms of heavy metal detoxification is also a crucial aspect for identifying and development of new plants as agents for large-scale phytoremediation of contaminated sites (Javed et al., 2018). Therefore, the study was designed to examine the toxicity consequences in *Sinapis alba* grown in soil supplemented with sewage sludge. Two municipal sewage sludges containing different trace elements amounts including Cd, Pb, Ni and Zn, and two industrial sewage sludges containing much less HMs were applied to degraded and contaminated soils, with a low quantity of organic carbon.

The main aim of the presented work was to determine the influence of sewage sludge application on the phytotoxicity of degraded soils. The specific objectives of the study were as follows: (1) to assess the phytotoxic effects of soils supplemented with sewage sludge on *S. alba* (2) to compare the phytotoxic effects between soils previously contaminated by HMs and non-contaminated ones (3) to evaluate the usability of standard toxicity markers for prediction of phytotoxic consequences after sewage sludge application (5) to assess the influence of sewage sludge on DNA damage, and the expression of genes encoding metallothioneins and RuBisCO in the shoots of *S. alba*.

2 MATERIALS AND METHODS

2.1 Substrates characterization

Two soils were used in the experiment. Both were collected from the surface layer (0 – 30 cm) of landless sites and were characterized by low fertility, microbial activity, sorption capacity and low content of organic matter (Figure 1). According to the World Reference Base for Soil Resources (WRB) classification, both soils belong to podzols and can be described as a sandy loam with approximately 65% sand, 20% silt and 15% clay. The first soil (A) was also highly contaminated with heavy metals from past emissions from the metallurgical industry in Poland (GPS: 50º30'N 18º56'E). The second one (B) was uncontaminated by heavy metals (GPS: 51.33°N / 19.11°E) (Table 1). Both soils were also characterised by similar cation exchange capacity (CEC). For S1: 14.1 ± 0.32 cmolc/kg; for S2: 15.1 ± 0.17 cmolc/kg.

Dewatered sewage sludge was obtained from 4 different wastewater treatment plants from the Silesia region in Poland. Overall, two sewage sludges (SS1 and SS2) were municipal sludge, the third sewage sludge (SS3) derived from a wastewater treatment facility from the production of mineral waters and beverages, and the fourth sewage sludge (SS4) derived from a water treatment station and is characterized by high levels of iron due to the process of water de-ironing. Sludges were transported to Faculty of Infrastructure and Environment (Czestochowa University of Technology, Czestochowa, Poland), where the experiment started immediately. Before mixing with soils, dewatered sewage sludges were tested for their physical and chemical properties (Figure 1).

Both soils were dried, sieved and mixed with sewage sludges. The doses of sewage sludges were selected to meet the EU nitrogen standards (170 kg of nitrogen/year/ha) (EUR-Lex - 31991L0676 e EN). In summary, after adjusting to each SS dry mass and content of nitrogen, the total weight of added SS per pot was as follows: SS1: 95.7 g, SS2: 96.8 g, SS3: 105.4 g, SS4: 462.5 g. Prior to planting seeds and after 28 days of the experiment, all experimental treatments were tested for their physical and chemical properties (Table 1).

A standard pH meter was used for pH determination (Cole Parmer Model No. 59002e00). The pH values of mixtures were measured in distilled water and 1 M solution of KCl

following ISO 10390:2005. The content of total organic nitrogen was established using the Kjeldahl method (PN-ISO11261:2002) (Bradstreet, 1940), while the concentration of heavy metals were determined by ICS-OES (ICP-OES; Thermo apparatus, USA) after digestion in a microwave digestion system according to EPA method 3051. The content of total phosphorus and potassium was measured following the Egner method (Egner et al., 1960).

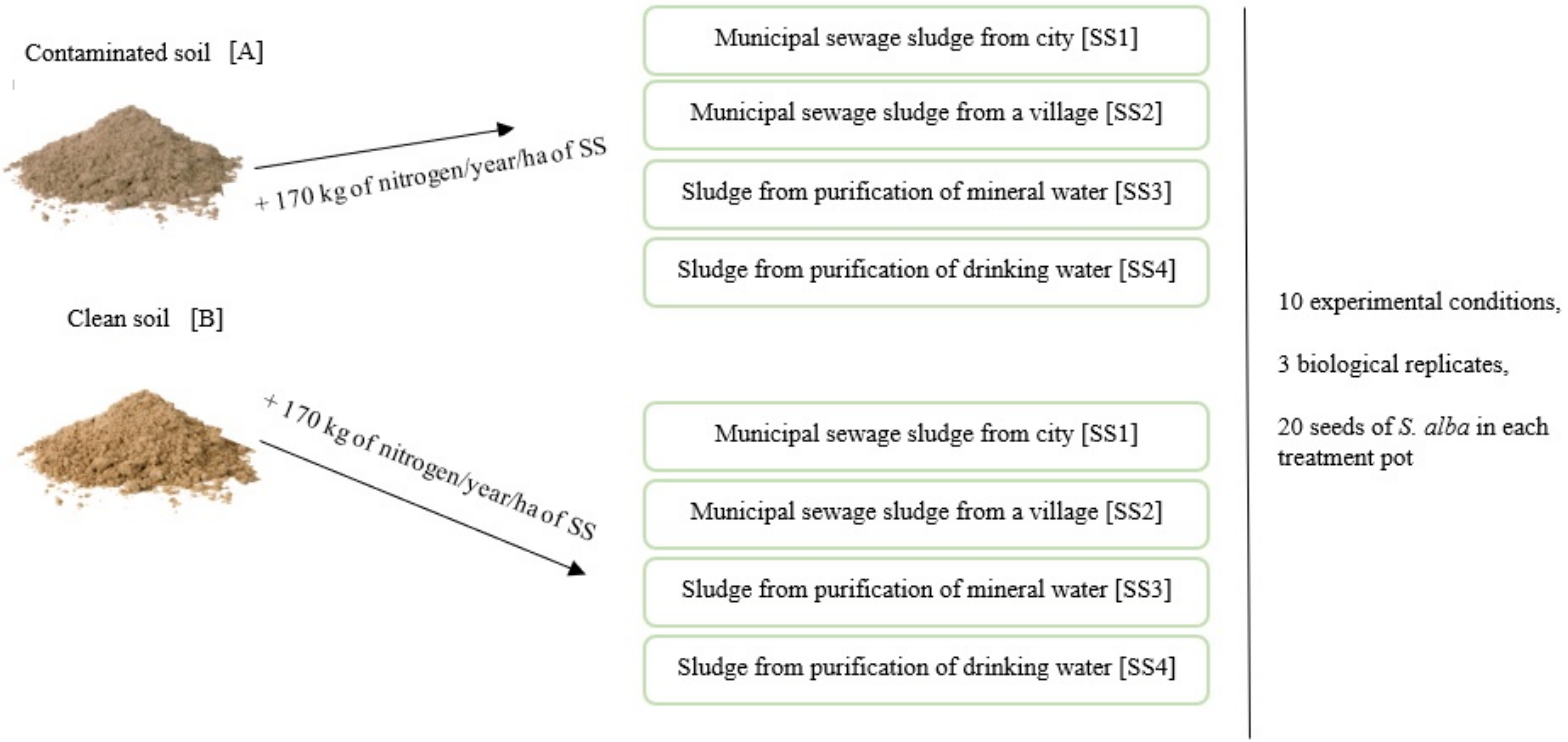


Figure 1. Experiment design and substrates characterisation. A – degraded soil contaminated with HM by industrial activities; B – degraded, uncontaminated soil; SS1 and SS2 – municipal sewage sludge selected for the experiment; SS3 – industrial sewage sludge after purification of mineral water, SS4 - industrial sewage sludge after purification of drinking water, TOC – total organic carbon. Results shown as means ± standard deviation, n = 3

Table 1. Substrates characterization. A – degraded soil contaminated with HMs by industrial activities, B – degraded, uncontaminated soil, SS1 and SS2 – municipal sewage sludge selected for the experiment SS3 – industrial sewage sludge after purification of mineral water, SS4 - industrial sewage sludge after purification of drinking water, TOC – total organic carbon. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$).

Substrate	pH		N Kjeldhal	P total	TOC	Cd	Pb	Zn	Fe
	H_2O	KCl	[mg kg^{-1}]	[mg kg^{-1}]	[g kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]	[mg kg^{-1}]
A	5.47 ± 0.05a	5.15 ± 0.07a	658.04 ± 15.11a	76.57 ± 8.2a	9.6 ± 1.5a	19.8 ± 0.8a	1135.8 ± 4.2a	773.5 ± 2.5a	34.5 ± 6.2a
B	5.53 ± 0.07a	5.12 ± 0.08a	831.41 ± 10.05b	88.65 ± 10.1a	12.2 ± 2.1a	1.12 ± 0.3b	13.9 ± 1.24b	56.7 ± 3.1b	48.1 ± 8.1a
SS1	7.11 ± 0.13b	5.87 ± 0.04b	14364.07 ± 233.10c	3359.13 ± 38.2b	684.4 ± 18.7b	7.62 ± 0.6c	242.84 ± 3.7c	2471.15 ± 7.1c	291.7 ± 33.8c
SS2	6.86 ± 0.05c	5.61 ± 0.16c	12624.54 ± 120.42d	3124 ± 59.4c	647.5 ± 47.3b	4.27 ± 0.4d	64.55 ± 2.2d	733.36 ± 2.6d	258.3 ± 32.4c
SS3	7.45 ± 0.12d	6.23 ± 0.11d	17825.71 ± 57.12e	6 015 ± 66.1d	812.3 ± 69.1c	0.87 ± 0.2e	15.7 ± 1.5b	91.7 ±. 4.2e	352.5 ± 14.6d
SS4	7.51 ± 0.14d	6.41 ± 0.09e	17911.45 ± 61.21e	10 723 ± 87.5e	854.6 ± 75.2c	1.05 ± 0.1e	17.4 ± 1.9b	74.2 ± 3.6f	2 520.4 ± 20.4e

2.2 Experimental procedure

Commercially available, certified and high-quality seeds of *S. alba* were used (Dary-Podlasia, Poland). Each pot (H - 25 cm, a - 12 cm, b – 25 cm) contained 4 kg of soil. Prepared growth media were left in phytotron chamber for 7 days prior to the planting of seeds. 20 seeds of *S. alba* were sown approximately two cm deep. Subsequently, plants were incubated for 28 days in a growth chamber (Biogenet FS360, Poland) under controlled conditions: photoperiod: 16 h light 8 h dark, temperature during the day 21^{o}C, night 18^{o}C, light intensity 4000 lx (photosynthetic LED light) watering every 3 days according to plants requirements. After 28 days, plants were extracted from soil, washed thoroughly in distilled water and the biomass was stored in -80^{o}C for further analyses. Overall, the experiment consisted of 10 different treatments (Figure 1) including contaminated soil (A), contaminated soil with 4 different sewage sludges (SS1, SS2, SS3, SS4), uncontaminated soil (S2), uncontaminated soil with 4 sewage sludges (SS1, SS2, SS3, SS4).

2.3 Basic toxicity assessment – germination index, roots length, plants biomass

Germination was recorded every 24 hours for the first 14 days of incubation and was considered to occur when the seedlings were at least 4 mm long. Seedlings with short or deformed hypocotyls were considered as abnormally germinated. After 14 days the germination index was calculated as a percentage of properly germinated plantlets.
Root length and biomass were measured after extraction of plants from the soil at day 28 of the experiment. 10 plants were measured from each pot for their biomass and roots length.

2.4 Metal accumulation

The content of heavy metals in plants shoots was also quantified in three independent replicates each experimental treatment. Bioconcentration factor (BCF) was calculated using the ratio of total metal accumulation in plants shoots and in soil respectively [BCF = C_{shoot}/C_{soil}].

2.5 Activity of guaiacol peroxidase (GPX)

The activity of guaiacol peroxidase (GPX) was recorded following the Asada et al., 2006

method. 100 mg of plants shoot tissues were used to perform the assay. Overall, GPX activity was evaluated by a level of guaiacol oxidation in the presence of hydrogen peroxide (H_2O_2) in 2 minutes of guaiacol oxidation. Enzyme activity was then estimated as an amount of GPX that produced a change in absorbance at 430 nm of 0.021/min (HACH DR/4000V, USA). Final results were normalized to U/mg of total protein concentration in plant biomass which was measured by standard Lowry assay (Lowry et al., 1951) with bovine serum albumin (BSA) (Sigma-Aldrich, USA) as a standard, using HATCH spectrometer (HACH DR/4000V, USA).

2.6 Content of chlorophyll

Total content of chlorophyll (*Chl*) in plants leaves was estimated by Aarnon method (Aarnon et al., 1949) with minor modifications. Overall chlorophyll was extracted by homogenization of 100 mg of fresh leaf with 10 mL of 80% acetone in a mortar. After that, samples were centrifuged for 15 minutes at 6000 g, and chlorophyll concentration in the supernatant was established spectrophotometrically (HACH DR/4000V, USA) at wavelengths 663 nm and 645 nm.

2.7 DNA damage

DNA damage in plant leaves was established by the alkaline version of the Comet Assay (single-cell gel electrophoresis SCGE) according to Lanier et al., 2016 with minor modifications to *S. alba*. In summary, after cultivation of *S. alba* plants on contaminated and uncontaminated soil with sewage sludge, plants leaves were placed in a cold 60 mm Petri dish, kept tilted on the ice and spread with 250 mL of cold Tris buffer (pH 7.5). Using a razor blade, leaves were sliced, and nuclei were collected in a Tris buffer. The whole procedure was conducted under dim light in three biological replicates and three technical ones.

Microscope slides were dipped into a 1% solution of NMA (normal melting agarose), prepared with distilled water at boiling temperature. Slides were cleaned from one side, and the other side was left to dry overnight at room temperature. Nuclear suspension (50 μL) with 1% LMA (low-melting-point agarose) prepared in Tris buffer at 40^{o}C was added to previous slides. The nuclei suspension was gently mixed with 1% LMA, and after application to slides, coverslips were placed. Slides were then cooled on ice for at least 5

minutes before the coverslips were removed and the final layer of 0.5% LMA was placed onto each slide and covered with a coverslip.

Prepared slides were placed in a tank for horizontal electrophoresis with fresh and cold electrophoresis buffer (pH > 13, 1 mM Na_2EDTA and 300 mM NaOH). Plants nuclei were incubated in the electrophoresis buffer for 15 minutes prior to electrophoresis to allow the DNA to unwind. Then, electrophoresis was performed at 25 V, 300 mA for 30 minutes at 4^{o}C.

After electrophoresis, the slides with plant nuclei were neutralized by washing in cold Tris buffer and stained with 20 mL of SimplySafe® dye (Eur_x, Poland), for 10 minutes. The excess dye was removed by dipping slides in ice-cold distilled water and slides were then covered with coverslips. For each prepared slide, 25 randomly chosen nuclei were analysed under a fluorescence microscope with barrier filter of 590 nm and an excitation filter of BP 546/10 nm. For data analysis, Komet version 3.0 software was used (Komet version 3.0, Kinetic Imaging Ltd., Liverpool, UK). DNA damage was then presented as the tail moment (TM) – the quantity of DNA in the comet tail times the tail length, divided by 100 and shown in micrometres.

2.8 Expression of *rbcL* and *mt*

2.8.1 Extraction of RNA and reverse transcription

Expression analyses of two target and 4 house-keeping genes (HKG) were performed on total RNA extracted from *S. alba* shoots by Eur_x Universal DNA/RNA/Protein kit (Eur_x, Poland), according to manufacturer's instructions. Overall, 100 mg of plant shoots were used for each extraction. The quantity and quality of isolated RNA were established spectrophotometrically using spectrometer with a nanodrop (Eppendorf BioPhotometer D30, USA). RNA integrity was confirmed on 1,5% agarose gel stained with SimplySafe® (Eur_x, Poland). The amount of isolated RNA allowed to perform the reverse transcription of 1.5µg cDNA using NG dART RT-PCR kit (Eur_x, Poland) according to manufacturer instructions. cDNA samples were then stored in -20^{o}C until further analyses.

2.8.1 House-keeping genes and target genes primers design and qPCR

To select stable HKG for data normalisation $_{\Delta}$Ct approach was used following previously described procedures (Brulle et al., 2014, Jaskulak et al., 2019). Stability of 4 HKG was tested in all experimental conditions including *actin*, *β-tubulin*, *elongation factor 1-α (ef1α)* and *18S*. Sequences of taxonomically related species including *Brassica napus*, *Brassica oleracea* and *Arabidopsis thaliana* were used to select both: HKG and two target genes: *rbcL* and *Mt*, due to the lack of information in *S. alba* plants. Selected HKG were analysed by BLAST and highly conserved sequences were identified. Based on selected sequences, primers were design using Primer3plus software. Amplificated fragments ranged in size between 100 and 250 bp.

PCR reactions were performed in the following temperature and time conditions: initial denaturation at 95°C for 5 min and then 40 cycles starting at 95°C for 30 s, followed by 58°C for 30 s, and 72°C 60 s. After 40 cycles, the last step of the qPCR was the final extension in 72°C for 5 min (Mastercycler® ep realplex, Eppendorf, Germany). For all samples, Taq PCR MasterMix (Eur$_x$, Poland) was used to amplify DNA fragments. The concentration of primers chosen for reaction was 0.4µM. After PCR, products were checked on 2% agarose gel with SimplySafe® (Eur$_x$, Poland) as a stain. Electrophoresis was performed in the following conditions: 150V for 45 minutes. After electrophoresis, products were extracted from the gel and sequenced via SAGNER sequencing (Genoscreen, France). Obtained sequences were then deposited in GenBank under the following accession numbers: *18S:* MK212131, *Elongation factor*: MK228841, *β-tubulin:* MK228842, *Actin:* MK226405, *RuBisCO:* MK217151, *Metallothionein:* MK226406. Also, the obtained sequences were used to design specific qPCR primers for qPCR which are presented below:

18S	5′ AGTTGAGGGATGATTTGG 3′; 5′ TGCAAACCCTCGTCAATT 3′; product size 104 bp, Tm 60^{o}C;
Elongation factor	5′ GGTCCCTTGTACCAGTCA 3′; 5′ GGAGGTGTCTTCCTACTT 3′ ; product size 126 bp, Tm 60^{o}C;
β-tubulin	5′ CCAGGCTCCAGATCCAT 3′; 5′ GCGAGTCAATGTTTACTA 3′; product size 78 bp, Tm 59^{o}C;
Actin	5′ AGCAATACCAGGGAACAT 3′; 5′ GACATCAGAAAGGACTTG 3′; product size 102 bp, Tm 60^{o}C;
RuBisCO	5′ GTTGATTTACTGCGCGATGA 3′; 5′ GAGCTACTCGGTTGGCTACG 3′; product size 350 bp, Tm 58^{o}C;
Metallothionein	5′ GCGAGAGAGTACACACGGAG 3′; 5′ GCTACAGTTTGATCCGCAGC 3′; product size 108 bp, Tm 59^{o}C;

Real-time qPCR amplification. For qPCR, Mastercycler® ep realplex, Eppendorf thermocycler was used (Eppendorf, Germany) with SYBR™ Select Master Mix (Thermo Fisher Scientific, USA) and reactions were performed in strip tubes (MicroAmp Optical 8-Tube Strip, 0.2 mL - Thermo Fisher Scientific). All qPCR reactions were performed as follows: 95°C 5 min and 40 cycles at 95°C 30 s, 58°C 30 s, and 72°C 60 s. After 40 cycles, the last step of the program consisted of 72°C for 5 min. The average stability of HKG was calculated by NormFinder software. Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential

phase of PCR reaction (Ct). The relative expression level was calculated according to the following formula:

$$R = 2^{\delta Ct(target\ gene) - \delta Ct(reference\ gene)}$$

2.9 Data analysis

All primers were designed using Primer3Software. HKG stability was quantified using software's BestKeeper and NormFinder. For DNA damage, the tail moment was calculated by Komet 3.0 software. All results are expressed as means ± standard deviation, n = 3. Tukey's test ($p < 0.05$), after ANOVA, same letters indicate the non-significant difference (ANOVA $p > 0.05$). Descriptive statistics and statistical analyses (one-way and multi-variance ANOVA) were produced using the OriginPro 2015 software and Statistica. Different letters "a", "b" "c", "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey's test $p < 0.05$. For all tests, p values below 0.05 were considered statistically significant. Correlations were evaluated using the Pearson correlation coefficient and the associated statistical test used to evaluate whether the correlation was significant. The strength of the association between variables was based on absolute value of the coefficient and in accordance to the Evans scale, classifying following r values as follows: .00-.19 – 'very weak'; .20-.39 – 'weak'; .40-.59 – 'moderate'; .60-.79 – 'strong'; .80-1.0 – 'very strong' (Evans, 1996).

3 RESULTS

3.1 Soil characterization

Changes in the physical and chemical parameters of the soil were recorded after treatments with sewage sludge (Table 2). The selected dose of sludge did not cause statistically significant ($p < 0.05$) changes to its pH values which stayed at approximately 5.55 on average (in H_2O) for both soils and regardless of the sewage sludge. The content of Kieldahl nitrogen was established on a stable level since all sewage sludges were added to soil in a dose adjusted to the European Norm, focused on the nitrogen dose applied to the soil. Overall, in our study comparing different sewage sludge, the influence of pH and nitrogen on the phytotoxicity levels was eliminated by keeping them at a similar level for both tested soils. Without SS, soil A was characterized by approximately 658 mg kg^{-1} of N, whereas soil B was characterized by 831 mg kg^{-1} of N. The supplementation with SS caused an increase of roughly 200 mg kg^{-1} of N in all experimental treatments. Total organic carbon (TOC) significantly ($p < 0.05$) increased after application of all sewage sludge in both soils. Furthermore, soil A was characterized by a high content of heavy metals, including Cd, Pb, and Zn. For Cd, the soil exceeded the safe threshold for agricultural use by more than 6 times and for Pb by more than 3 times. Soil B was not contaminated with these metals. Supplementation of sewage sludge in soil A did not cause statistically significant ($p < 0.05$) changes in the content of Cd for SS1 and SS2 municipal sewage sludge but lowered the Cd concentration after supplementation with SS3 and SS4. For Pb and Zn, supplementation with SS1 did not produce any significant changes, whereas the SS2 caused an increase in the presence of Zn in soil. Similarly to Cd, the Pb values were lower after application of SS3 and SS4. The results for soil B were comparable to soil A as the supplementation with two municipal sludges caused an increase in heavy metal concentration.

Table 2. Sewage sludge and soil mixtures characterization. A – degraded soil contaminated with HM by industrial activities, B – degraded, uncontaminated soil, SS1 and SS2 – municipal sewage sludge selected for the experiment SS3 – industrial sewage sludge after purification of mineral water, SS4 - industrial sewage sludge after purification of drinking water, TOC – total organic carbon. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$).

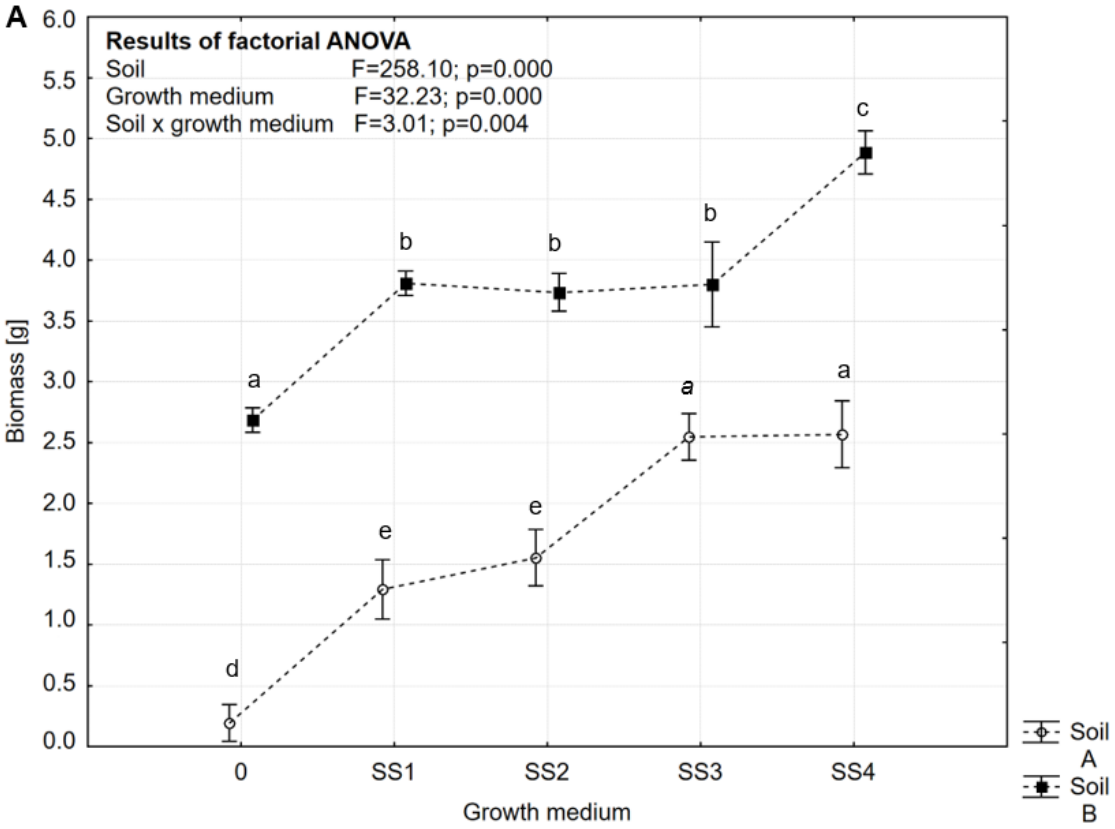
Growth medium	**pH**		**N Kjeldhal [mg kg^{-1}]**	**TOC [g kg^{-1}]**	**Cd [mg kg^{-1}]**	**Pb [mg kg^{-1}]**	**Zn [mg kg^{-1}]**
	H_2O	KCl					
A	5.47 ± 0.05a	5.15 ± 0.07a	658.04 ± 15.11a	9.6 ± 1.5a	19.8 ± 0.8a	1135.8 ± 4.2a	773.5 ± 2.5a
A+SS1	5.62 ± 0.23a	5.25 ± 0.06a	841.77 ± 23.14b	17.3 ± 1.2c	18.83 ± 0.4a	1142.63 ± 4.6a	781.17 ± 6.2a
A+SS2	5.56 ± 0.07a	5.21 ± 0.18a	894.54 ± 42.62b	13.5 ± 1.3b	19.97 ± 0.5a	1137.51 ± 2.8a	793.48 ± 5.5b
A+SS3	5.48 ± 0.16a	5.13 ± 0.12a	825.71 ± 57.15b	19.8 ± 1.2d	15.21 ± 0.7b	1031.5 ± 3.4c	791.74 ±. 4.1b
A+SS4	5.59 ± 0.11a	5.21 ± 0.08a	911.45 ± 61.27b	20.2 ± 1.7d	14.05 ± 0.2c	1015.4 ± 3.9d	794.2 ± 4.5b
B	5.53 ± 0.07a	5.12 ± 0.08a	831.41 ± 10.05a	12.2 ± 2.1a	1.12 ± 0.3a	13.9 ± 1.24a	56.7 ± 3.1a
B+SS1	5.63 ± 0.09a	5.18 ± 0.12a	1012.25 ± 33.1b	22.1 ± 3.05b	3.54 ± 0.7c	64.1 ± 2.4c	120.5 ± 5.6d
B+SS2	5.55 ± 0.13a	5.22 ± 0.17a	988.41 ± 14.54b	20.7 ± 1.8b	1.91 ± 0.2b	27.5 ± 3.1b	89.8 ± 3.5c
B+SS3	5.67 ± 0.05a	5.28 ± 0.8a	1054.61 ± 42.1b	26.4 ± 1.6c	1.07 ± 0.5a	14.3 ± 1.3a	70.6 ± 3.8b
B+SS4	5. 59 ± 0.17a	5.23 ± 0.7a	1036.55 ± 21.7b	29.1 ± 1.7d	0.91 ± 0.3a	14.21 ± 1.2a	61.5 ± 5.1a

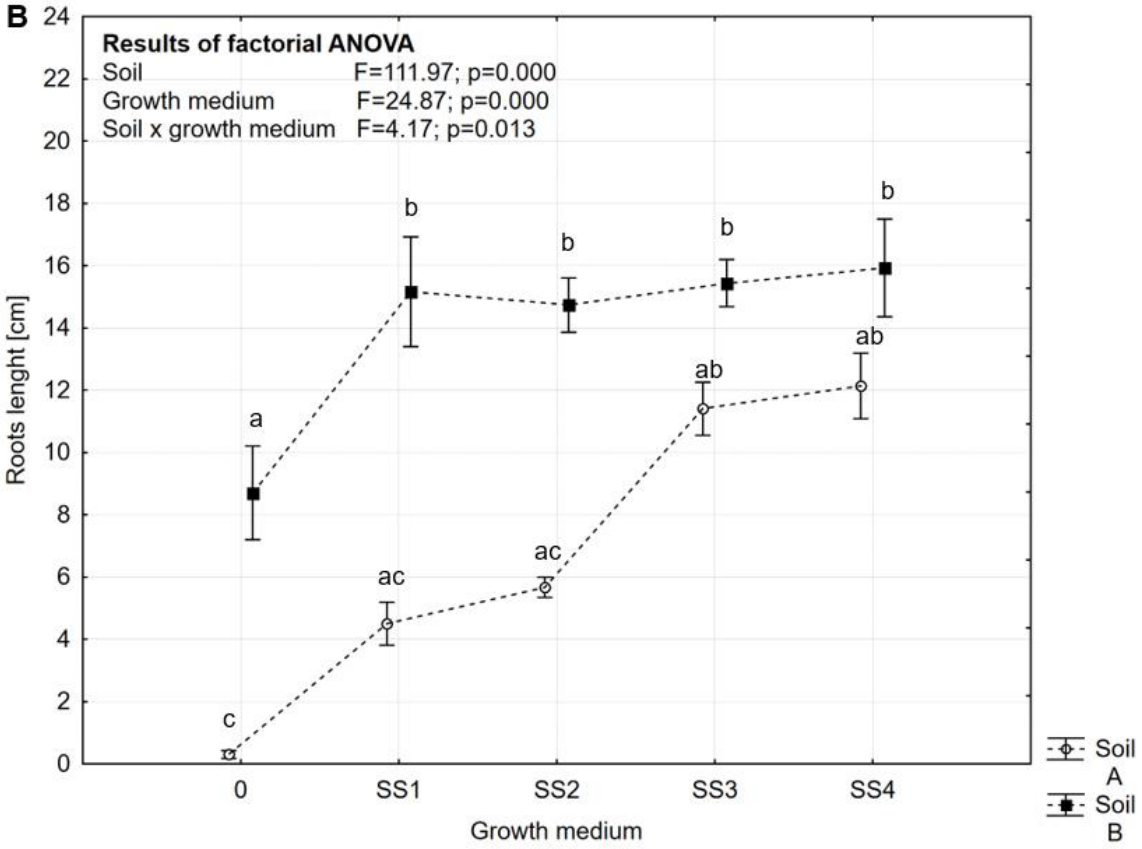
3.2 Germination index, root length, biomass and metal content

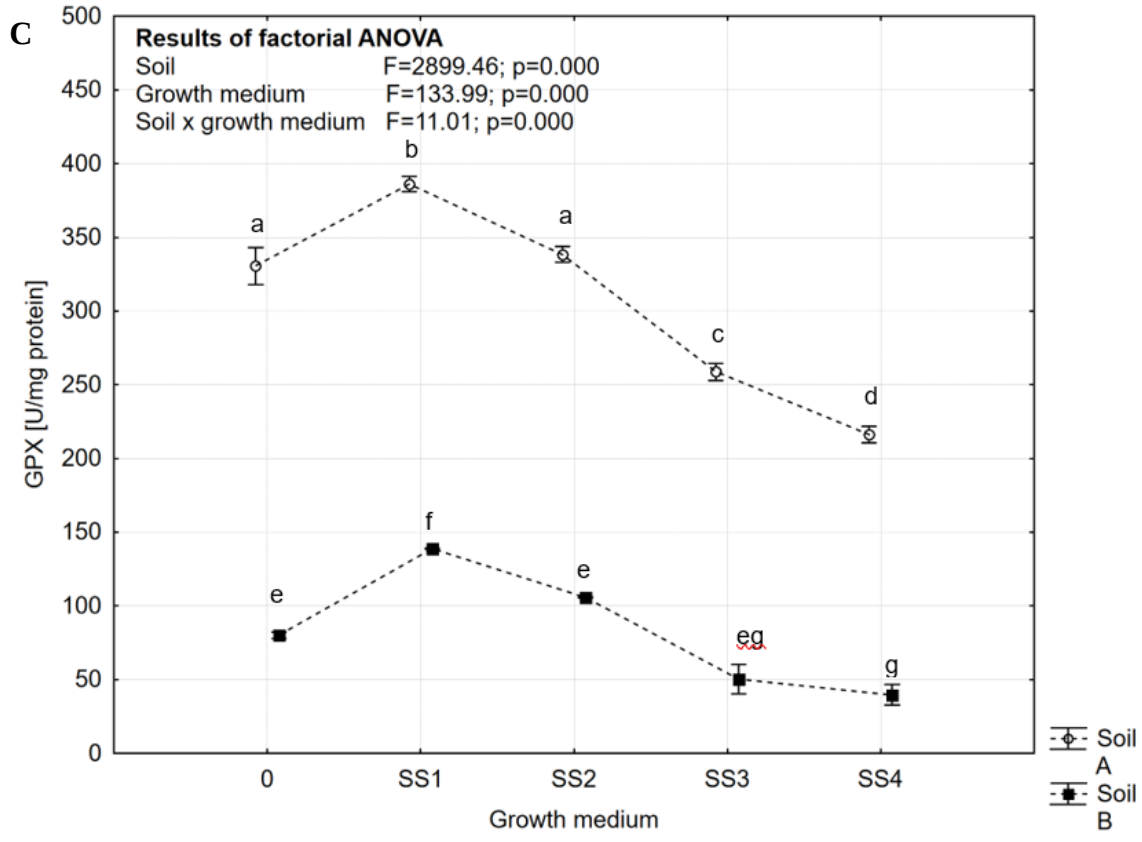
Basic growth parameters like germination index, roots length, and shoot biomass were profoundly affected by heavy metal contamination in soil A in comparison to the control conditions of soil B (Table 3, Figure 2). The germination index on the non-contaminated soil without any supplementation was reported at approximately 96% whereas on contaminated soil without supplementation it decreased to about 13%. Overall, in contaminated soil (A) all sewage sludges caused a significant ($p < 0.05$) increase in all basic growth parameters – germination index was the highest after supplementation with SS4, root length and shoot biomass also showed an increase after supplementation with 1 - 4 SS. The bioconcentration factor (BCF) was also highly influenced by the supplementation of contaminated soil with sewage sludge and was the lowest after supplementation with sludges SS3 and SS4. The highest decrease of BCF was noted for SS4 treated soil, while the plant biomass was similar to SS3 treatment, eliminating the BCF decrease as a "dilution" effect of origin. In control, uncontaminated soil (B) sewage sludge application increased the length of the roots and shoot biomass of plants. The germination index stayed at a stable level for all treatments with soil B, and the bioconcentration factor increased after supplementation with SS1 and SS2. For other treatments in soil B, the concentrations of selected HMs in plant shoots were too low to be detected by ICP-OES. Moreover, in soil A, for Pb BCF and germination index, similar statistical groups were noted, indicating the strong correlation between these two parameters.

Table 3. Germination index, metal concentrations and bioconcentration factor (BCF) in *S. alba* after 28 days of incubation. Nd – values below the detection limits of ICP-OES. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$).

Growth medium	Average germination index [%]	Cd [mg kg^{-1}]	Cd BCF	Pb [mg kg^{-1}]	Pb BCF
A	13a	103.7 ± 9.2a	5.24 ± 1.5 a	1419.7 ± 94.1a	1.25 ± 0.8a
A+SS1	43b	99.2 ± 5.3a	5.27 ± 1.6 a	731.3 ± 52.4b	0.64 ± 0.3b
A+SS2	38b	116.0 ± 12.8a	5.81 ± 1.1a	693.8 ± 55.2b	0.61 ± 0.5b
A+SS3	49c	41.1 ± 3.4b	2.7 ± 0.8b	556.7 ± 41.7c	0.54 ± 0.4c
A+SS4	73d	19.7 ± 2.1c	1.4 ± 0.5c	162.4 ± 11.6d	0.16 ± 0.1d
B	96 a	Nd	Nd	Nd	Nd
B+SS1	92a	3.9 ± 2.8a	1.1 ± 0.4a	3.3 ± 2.7a	0.12 ± 0.06a
B+SS2	95a	1.6 ± 2.5a	1.5 ± 0.2a	1.1 ± 0.5a	0.07 ± 0.05a
B+SS3	96a	Nd	Nd	Nd	Nd
B+SS4	98a	Nd	Nd	Nd	Nd


A
Results of factorial ANOVA
Soil F=258.10; p=0.000
Growth medium F=32.23; p=0.000
Soil x growth medium F=3.01; p=0.004
Biomass [g]
Growth medium
0
SS1
SS2
SS3
SS4
Soil A
Soil B


B
Results of factorial ANOVA
Soil F=111.97; p=0.000
Growth medium F=24.87; p=0.000
Soil x growth medium F=4.17; p=0.013
Roots lenght [cm]
Growth medium
0
SS1
SS2
SS3
SS4
Soil A
Soil B


C
Results of factorial ANOVA
Soil F=2899.46; p=0.000
Growth medium F=133.99; p=0.000
Soil x growth medium F=11.01; p=0.000
GPX [U/mg protein]
Growth medium
0
SS1
SS2
SS3
SS4
Soil A
Soil B

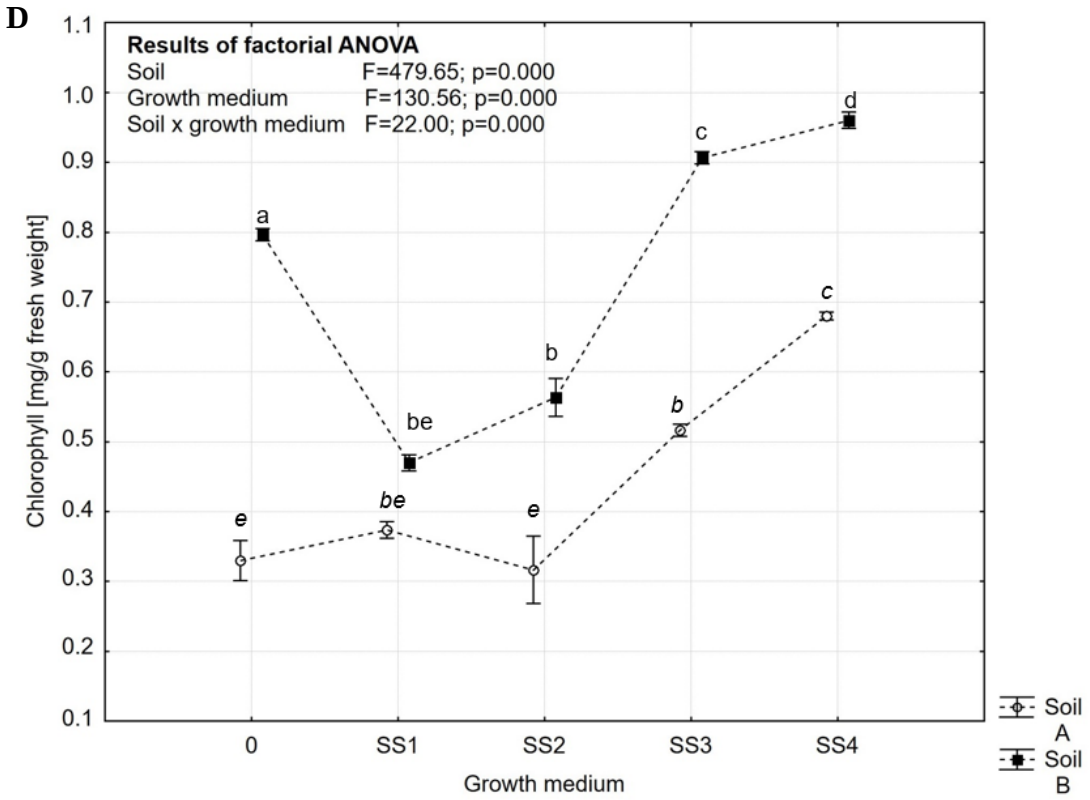


Figure 2. Biomass [**A**], roots length [**B**], GPX activity [**C**] and chlorophyll content [**D**] in *S. alba* after 28 days of incubation. Soil A – degraded soil contaminated with HM by industrial activities, Soil B – degraded, uncontaminated soil, 0 – soil A or B without supplementation, SS1 and SS2 – municipal sewage sludges selected for the experiment SS3 – industrial sewage sludge after purification of mineral water, SS4 - industrial sewage sludge after purification of drinking water, All results are expressed as means ± standard deviation, n = 3. Different letters "a", "b" "c", "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey's test $p < 0.05$.

3.3 GPX activity and content of chlorophyll

The activity of guaiacol peroxidase in plants grown on contaminated soil without sludge application was more than 4 times higher than in plants grown on the uncontaminated soil (Figure 2C). In both soils, supplementation with municipal sludge SS1 caused an increase in GPX activity, whereas SS3 and SS4 showed positive effects by lowering the activity of GPX. The lowest enzyme activity of about 42 U/mg of protein was observed in uncontaminated soil supplemented with SS4. Similar results were found for the content

of chlorophyll (*Chl*), which increased after supplementation with uncontaminated sludge SS3 and SS4 but decreased significantly ($p < 0.05$) after supplementation with municipal sludge SS1 and SS2 (Figure 2D). In the uncontaminated soil B, supplementation with SS1 and SS2 did not show any significant ($p < 0.05$) effects in the concentration of chlorophyll while supplementation with SS3 and SS4 caused an increase in *Chl* content from 0.32 mg/g^{-1} in the control soil to 0.51 mg/g^{-1} and 0.67 mg/g^{-1} after supplementation with SS3 and SS4 respectively.

3.4 DNA damage

In leaves of *S.alba*, the contaminated soil caused an approximately 6-fold increase in the level of DNA damage in comparison to the uncontaminated soil (Figure 3). In soil A, the application of municipal sludge SS1 caused a statistically significant ($p < 0.05$) rise in DNA damage. The other municipal sludge did not influence DNA damage, whereas the two uncontaminated sludges caused a substantial decrease in reported DNA damage. For the uncontaminated soil, the municipal sludge caused even 9-fold increase in TM moments in comparison to the control soil. Similarly to soil A, in soil B, SS3, and SS4 also caused a substantial decrease in DNA damage (Figure 3).

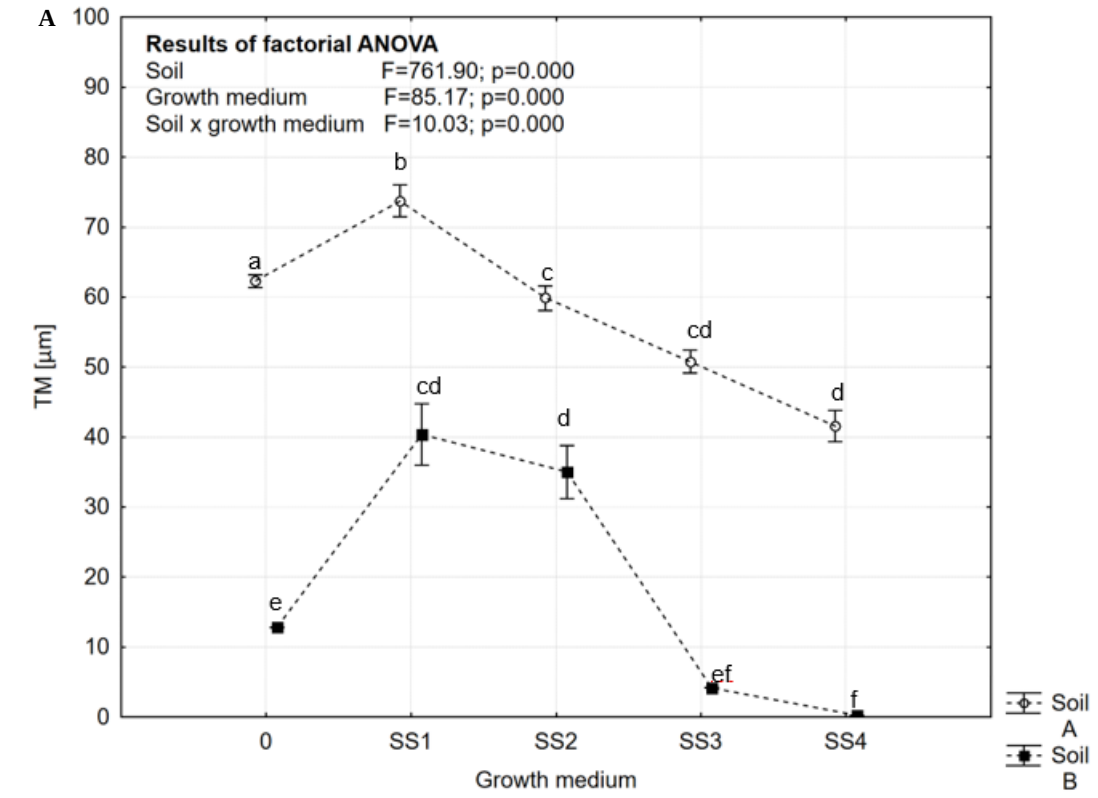
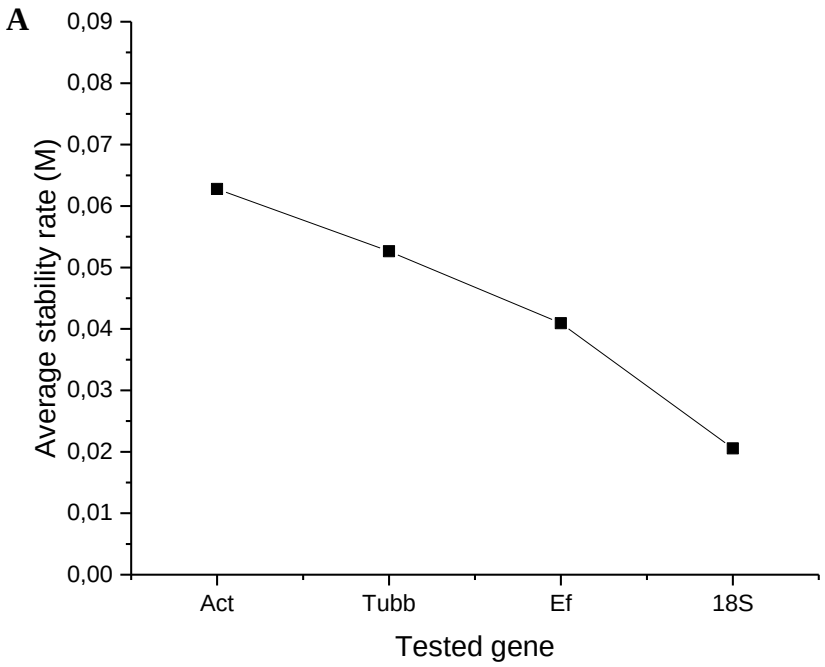


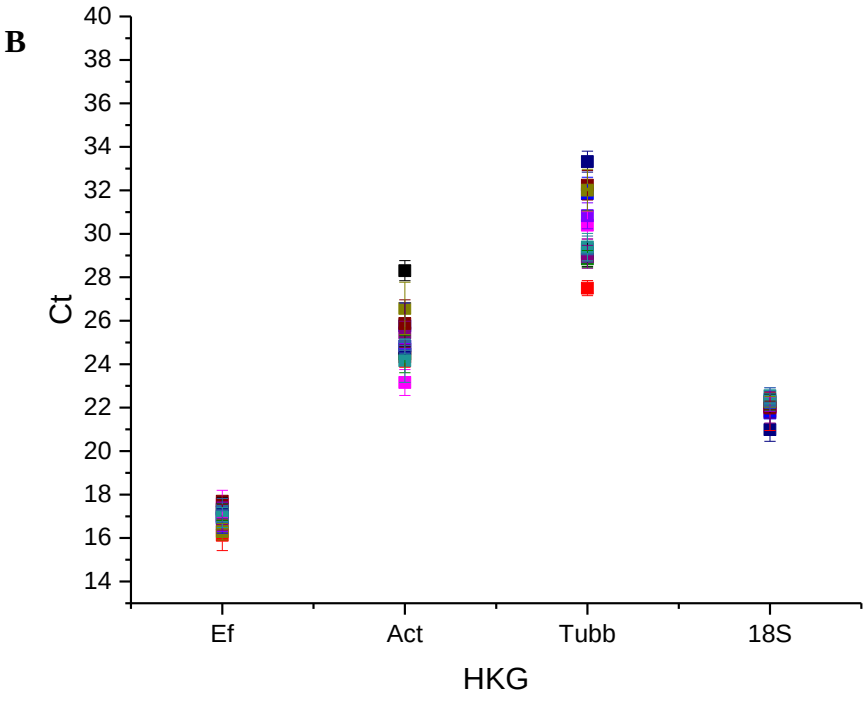
Figure 3. DNA damage in leaves of *S. alba* after 28 days of incubation [A]. Soil A – degraded soil contaminated with HM by industrial activities, Soil B – degraded, uncontaminated soil, [B] – Nuclei seen after comet assay in plants grown on control soil B, [C] – Nuclei seen after comet assay in plants grown on A soil. 0 – soil A or B without supplementation, SS1 and SS2 – municipal sewage sludge selected for the experiment SS3 – industrial sewage sludge after purification of mineral water, SS4 - industrial sewage sludge after purification of drinking water. All results are expressed as means ± standard deviation, n = 3. Different letters "a", "b" "c", "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey's test $p < 0.05$.

3.5 HKG validation and level of expression of rbLc and mt

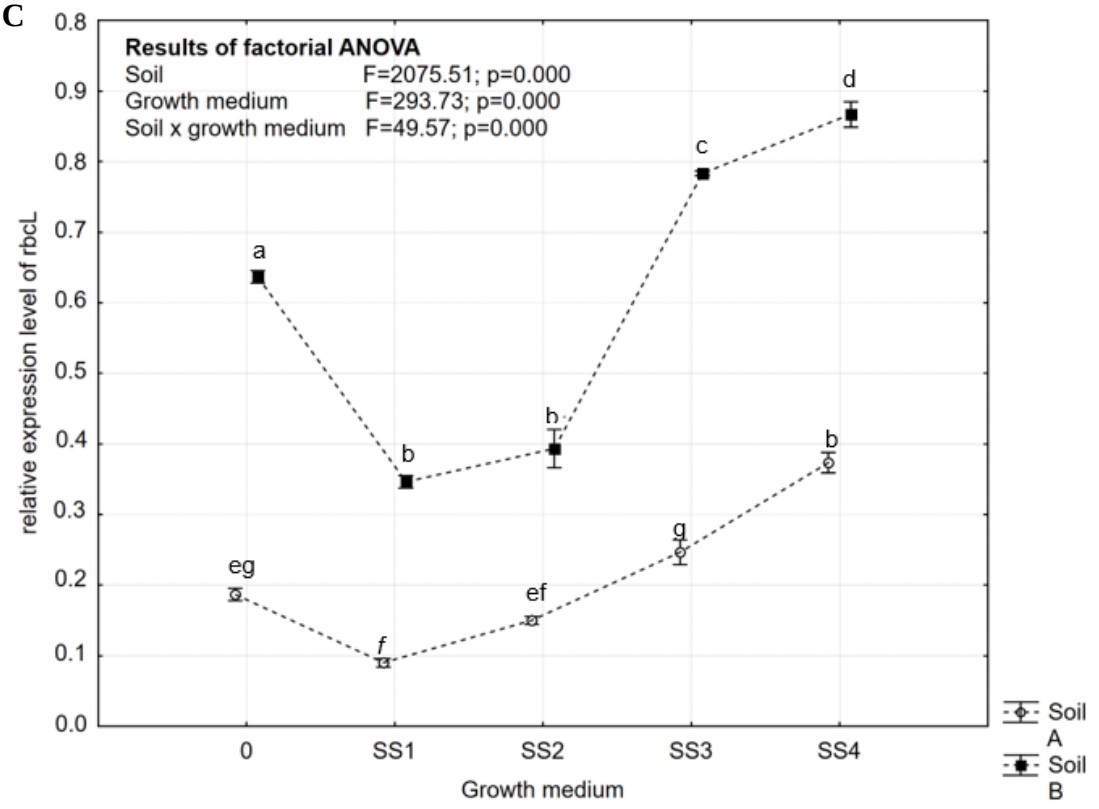
To evaluate the best HKG, the NormFinder and BestKeeper software were used according to the methodology of Brulle et al., 2014. The results are presented as average expression stability M. A lower M value indicates higher stability. The most stable HKG in *S. alba* was *18S*, then *β-tubulin*, *elongation factor* and the least was *actin* (Figure 4).

The contamination of soil A with heavy metals severely decreased the expression of the *rbcL* gene in comparison to uncontaminated soil B (Figure 4C). Moreover, supplementation of such contaminated soil with SS3 and SS4 caused a significant ($p < 0.05$) increase in *rbcL* expression in plants and thus, in photosynthesis efficiency. Municipal sewage sludge SS1 (the most contaminated) caused a decrease in *rbcL* expression in both soils, while SS2 caused a reduction in soil B, but did not influence *rbcL* expression in soil A. Overall, a significant ($p < 0.05$) increase was also noticed after supplementation of soil B with SS3 and SS4. Similarly, the expression of *mt* was also significantly ($p < 0.05$) influenced after SS application in both soils (Fig. 4D). In the uncontaminated soil B, and after its supplementation with uncontaminated SS3 and SS4, the relative expression level of *mt* was minimal, almost negligible. In the contaminated soil A, and also after supplementation with contaminated sludge SS1 and SS2 a significant increase in *mt* expression was observed. Furthermore, the supplementation of contaminated soil A with SS4 caused a substantial decrease in *mt* expression.


A
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
Average stability rate (M)
Act
Tubb
Ef
18S
Tested gene


B
40
38
36
34
32
30
28
26
24
22
20
18
16
14
Ct
Ef
Act
Tubb
18S
HKG

C


Results of factorial ANOVA
Soil F=2075.51; p=0.000
Growth medium F=293.73; p=0.000
Soil x growth medium F=49.57; p=0.000
relative expression level of rbcL
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
a
b
b
c
d
eg
f
ef
g
b
0
SS1
SS2
SS3
SS4
Growth medium
Soil A
Soil B

D

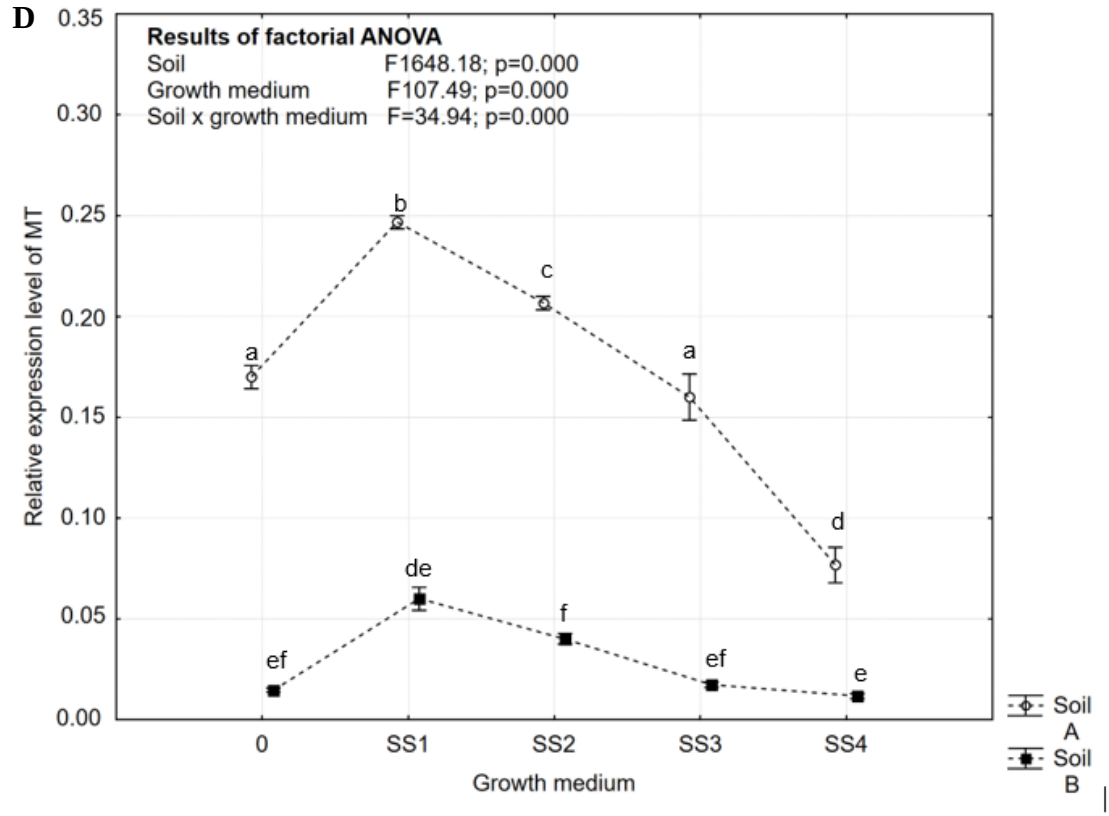

Results of factorial ANOVA
Soil F1648.18; p=0.000
Growth medium F107.49; p=0.000
Soil x growth medium F=34.94; p=0.000
Relative expression level of MT
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
a
b
c
a
d
ef
de
f
ef
e
0
SS1
SS2
SS3
SS4
Growth medium
Soil A
Soil B

Figure 4. [**A**] - Average expression stability *(M),* of HKG by NormFinder analysis. *EF – elongation factor, Tubb - β-tubulin, Act – actin, 18S;* [**B**] - The transcriptional profiles of individual HKGs in absolute Ct values over all RNA samples. *EF – elongation factor, Tubb - β-tubulin, Act – actin,* [***C***] - expression of *rbcL* gene and *mt* gene [**D**] in *S. alba* shoots. A – relative expression of *mt* in plants grown on degraded soil contaminated with HM by industrial activities, B – relative expression of *rbcL* in plants grown on degraded, uncontaminated soil, C – *rbcL* expression in arbitrary units [AU]. SS1 and SS2 – municipal sewage sludge selected for the experiment SS3 – industrial sewage sludge after purification of mineral water, SS4 - industrial sewage sludge after purification of drinking water. All results are expressed as means ± standard deviation, n = 3. Different letters "a", "b" "c", "d" on top of bars indicate a significant difference according to ANOVA with post-hoc Tukey's test $p < 0.05$.

3.6 Correlations between tested markers

We evaluated correlations between obtained data from selected biomarkers in order to broaden our understanding of plant defence mechanisms against heavy metal contamination and the application of sewage sludge in soils (Table 4). Overall, a number of 'very strong', 'strong' and 'moderate' correlations had been observed including the strongest positive correlation between GPX activity and the bioaccumulation of Cd ($r = .94$, $p < 0.05$); the level of DNA damage and activity of GPX ($r = .88$, $p < 0.05$) the expression of large rubisco subunit to total chlorophyll content ($r = .90$, $p < 0.05$); the expression of metallothionein to the activity of GPX ($r = .94$, $p < 0.05$) and the metallothionein expression to the level of DNA damage ($r = .94$, $p < 0.05$). In addition, the strongest negative correlations had been observed between the bioaccumulation of Pb and root length and plants biomass, the chlorophyll content to bioaccumulation of Cd and the activity of GPX, the expression of *rbcL* to bioaccumulation of Cd, GPX activity and level of DNA damage and expression of *mt* to the expression of *rbcL*. Moreover, there is a very strong correlation between TM, *mt* and, GPX and as one set of data is strongly correlated with Cd BCF, indicating that the four parameters are a sensitive biomarkers of Cd toxicity and consequences in shoot biomass presence. For Pb such effects were not so clear, confirming low bioconcentration in shoots, thus a less sensitive response in genotoxicity markers (TM, *mt*, GPX), but strong response in biomass (root, shoot) parameters.

Table 4. Pearson's correlation coefficient among different tested biomarkers in *S. alba*. Correlations are not shown if they are below an absolute value of r = 0.4.

	Roots lenght	**Biomass**	**Cd BCF**	**Pb BCF**	**GPX**	**Chlorophyll**	**TM**	***rbcL***
Biomass	0.9907							
Cd BCF	-0.8594	-0.7836						
Pb BCF	-0.9084	-0.9293	0.6992					
GPX	-0.8010	-0.7181	0.9405	0.5919				
Chlorophyll	0.8628	0.7962	-0.9840	-0.7748	-0.8899			
TM	-0.7752	-0.6998	0.8796	0.5925	0.9844	-0.8322		
rbcL	0.7172	0.6224	-0.9289	-0.5608	-0.9755	0.9011	-0.9627	
Mt	-0.6067	-0.5014	0.8733	0.4498	0.9436	-0.8410	0.9376	-0.9889
p value	$p < 0.001$	$p < 0.01$	$p < 0.05$					

4 DISCUSSION

The pollution of soil and water with heavy metals is considered a critical issue due to their persistence and the potential risk of bio-accumulation through the food chain (Dong et al., 2010). Most HMs do not possess any recognized biological role in living organisms and are severely toxic even at very low concentrations. Moreover, HMs are currently the most common contaminants in soils across the world (Placek et al., 2017). At the same time, many countries face the challenge of disposing of millions of tons of sewage sludge each year, and due to its high content of organic carbon, nitrogen and other nutrients, it is often proposed to use it as a fertilizer (Kupper et al., 2014). Such a solution would also be a practical option for the management of produced biosolids. However, sewage sludge contains several potentially toxic substances including organic pollutants and HMs that can be highly persistent in topsoil (Fellet et al., 2014). For instance, conducted studies confirmed that municipal sewage sludge can contain very high levels of toxic metals (SS1). Even though the phytotoxic effects of heavy metals have been extensively studied for many years, those studies are mostly focused on crops and/or artificial contamination most often (Soudek et al., 2014). Studies on the effects of HM exposure on plants used for phytoremediation usually take into consideration only basic toxicity tests like the germination index and roots development (Peng et al., 2016). However, these markers had been shown to give vastly inconsistent results across different species and soils. Thus, the investigation into the genotoxic properties of heavy metals is therefore much needed since it can provide more accurate toxicity results for planning/following a remediation process on a larger scale (Chang et al., 2016).

The aim of our study was to evaluate the effects of sewage sludge application on metal-sensitive mechanisms in *S. alba* including the activity of the antioxidative enzyme GPX, the efficiency of photosynthesis by the content of chlorophyll and expression level of the *rbcL* gene, the level of DNA damage through the SCGE technique and the synthesis of metal chelators – metallothioneins as expression rate. We measured all these biomarkers in order to gain insight into the acclimation mechanisms implemented in *S. alba* growing in soil supplemented with sewage sludge. This is fundamental for a better understanding of HMs detoxification mechanisms which can be applied to optimize the selection of a proper kind of sewage sludge and its dose for a large scale phytoremediation program.

The bioconcentration factors (BCF) for Cd and Pb in our study are in agreement with other studies reported in related species including *Brassica rapa* (Khan et al., 2015), Chinese leaf mustard (Chang et al., 2014), and *Brassica juncea* (Chowdhary et al., 2018). Similar results after soil supplementation with sludge have also been observed in *Sorghum* (Alvarenga et al., 2016). Overall, the supplementation of contaminated soil with different SS showed no significant effect on BCF for municipal sludge which were contaminated with HMs, and at the same time, a significant decrease in BCF was noticed after supplementation with uncontaminated sludge. For most metals, statistically significant ($p < 0.05$), differences were observed in plant BCF factors between the application of different biosolids were observed. In most cases, municipal sewage sludge slightly increased or did not cause a considerable change in the BCF factor, while the two uncontaminated sludge caused a significant ($p < 0.05$) decrease in BCF. The "dilution" effect of metals in plants biomass can be dismissed in this study since plants biomass was the same for SS3 and SS4 while BCF for SS4 was much lower. The significant decrease in BCF after SS4 treatment was probably connected to high Fe content, Fe being a competitor of toxic heavy metals. This confirms that Fe enrichment for biosolids or soil can significantly reduce the bioavailability of other metals. In that sense, the results obtained were quite similar to Antoniadis et al. (2008) in which substantial differences in concentrations of Ni, Cd, and Pb were noticed in *Ryegrass* after supplementation with biosolids.

The biomass and root length increased in both soils after supplementation with all kinds of sewage sludge, and such results had been observed before in Khan et al., 2015 and are thought to be caused by an addition of organic matter and nutrients to the degraded soil. However, our study shows that the sludge contaminated with HM still produced other toxic effects in plants tissues that were not seen (or not yet) in biomass and root lengths. Such effects include the accumulation of DNA damage, an increase in *MT* gene expression and the activity of GPX as well as a decrease in photosynthesis activity including the total content of chlorophyll and expression of *rbcL*. However, sewage sludge from industrial water treatments that were almost free from the presence of toxic trace metals did not cause any adverse effects of the same biomarkers and significantly improved the physiological condition and development of plants on contaminated soil. The results of biomass and root length agree with the hydroponic studies on *Dianthus*

carthusianorum (Wójcik et al, 2014) where low concentrations of trace metals did not reduce biomass and roots length, but toxicity effects could be observed at a cellular level. However, there were no previous studies on the effect of sewage sludge application on DNA damage and the expression of RuBisCO and metallothionein.

Since many HMs exert adverse effects to organisms through redox reactions which lead to excessive production of reactive oxygen species (ROS), antioxidative enzymes such as peroxidases (POD), catalases (CAT), glutathione S transferase, and superoxide dismutase (SOD) play an essential role in the neutralization and detoxification of HMs (Bernard et al., 2016). Thus, those enzymes had been extensively studied in both animals and plants. Nevertheless, the majority of such research on plants had been focused on artificial, lab-made contamination or in water solutions (Emamverdian et al., 2015). The potential application of such technology as biomarkers for environmental exposure is of great interest, especially since it has been previously observed that oxidative damage is sensitive even to low levels of contamination and therefore could be routinely used in toxicity studies (Hattab et al., 2016).

In the works of Chowdhary et al., (2018), in shoots of *Triticum aestivum* and *Brassica juncea*, SOD was shown to not be affected by low concentrations of Cd, Hg, and Cu while the activity of peroxidases was strongly elevated even after short exposure time. Interestingly, peroxidase activity was inhibited by Hg but activated by most of the other heavy metals. Furthermore, HMs including Pb and Cd has been shown before to induce oxidative stress in several plant species, including *Brassicae* plants (Khan et al., 2015). Such effects have also been visible in our study where GPX activity was highly increased by heavy metal contamination in comparison to uncontaminated soil and was also increased in uncontaminated soil supplemented with contaminated sewage sludge which shows that it is a sensitive marker even at low metal concentrations. Moreover, the addition of sewage sludge SS4 containing a high concentration of iron significantly decreased the activity of this enzyme, even on contaminated soil. The depletion of the photosynthesis rate in plants after HMs exposure has been reported before in the work of Willam et al., 2012. In our study, the contamination of heavy metals resulted in the severe loss of chlorophyll content and decreased the expression of the *rbcL* gene in comparison to plants grown on uncontaminated soil. Moreover, the supplementation with municipal

sewage sludge contaminated with trace metals in clean soil also caused a significant decrease in those markers. Similar adverse effects have been observed on artificially spiked soils in different plant species such as in poplar trees (Chandra et al., 2016) and several species of vegetables (Chang et al., 2014), and it has been shown to occur due to the disruption of chloroplasts and a decrease in chloroplast volume induced by HMs.

A comet assay is a viable tool for DNA damage assessment in eukaryotic cells (Javed et al., 2016). The alkaline version of this method can quantitatively measure DNA damage including single and double strand brakes, incomplete excision repair sites, DNA cross-links, and alkali-labile sites. Although this method is mostly applied to animal cells, the incorporation of SCGE techniques in plants can be a useful tool in applied studies on environmental mutagenesis (Mišík et al., 2016, Cortés-Eslava et al., 2018). In previous years, the effects of HM contamination on DNA damage have been reported in many crops and vegetables. For example, Gishner et al. 2007 reported that Cd^{2+} severely induced DNA damage in *Nicotiana tabacum*. Moreover, a dose-dependent response against HM contamination was reported in roots od *Allium cepa*, *Vicia faba*, (Cortés-Eslava et al., 2018) and *Tradescantia* (Mišík et al., 2016) after artificial contamination with Cd^{2+} and Pb^{2+}. Moreover, in *Tradescantia* plants cultivated on soils from metal smelters, the contamination resulted in a 10 to even 15 fold increase in DNA damage in comparison to plants grown on uncontaminated soil (Mišík et al., 2016). In our work, *S. alba* plants which showed increased DNA damage were also severely injured in other toxicity markers such as the content of chlorophyll, activity of GPX and expression levels of *rbcL* and *mt*. All together, these biomarkers show that the application of contaminated sewage sludge inhibits the whole photosynthetic apparatus and also flames up the protective mechanisms.

Another mechanism that can be triggered by toxic metals to minimize the induced cellular damages is the synthesis of metal chelators. Metallothioneins are small proteins with abundant cysteine residues able to chelate metals (Bulgarelli et al., 2016). It was previously shown that the family of *mt* genes is regulated in different ways, yet at the same time is essential for metal detoxification at a cellular level. Overall the amount of metallothioneins can vary between species, certain plant tissues, and environmental factors (Sharma et al., 2016). In the present study, soil contaminated by HMs increased

the *mt* expression in plant shoots. The application of municipal sewage sludge that contained HMs caused a higher increase in *mt* expression even if the sewage sludge application increased the germination and biomass of plants. Moreover, the application of sewage sludge on uncontaminated soils also caused an increase in *mt* expression which shows how sensitive the mechanisms of *mt* expression are. *Mt* expression in plants grown on contaminated soil significantly decreased after sewage sludge treatment with high Fe content. The industrial sewage sludge (SS3) without trace metals did not cause any significant ($p < 0.05$) increase in *mt* expression and therefore did not increase phytotoxicity levels in plants. Similar results, meaning the induction of *mt* expression after exposure to HMs, have been observed before on heavy metal contaminated soils or after artificial contamination of samples in poplar, tomatoes and, *Trifolium repens* (Madejón et al., 2015, Bernard et al., 2016) but this is a first study investigating such influences after sewage sludge application. One of the most interesting correlations identified in this study was the relationship between the expression of *mt* and *rbcL* genes. Obtained results combined with the Pearson correlation coefficient indicated that the induction of *mt* expression was strongly correlated with the activity of an antioxidative enzyme – GPX and the level of DNA damage.

5 CONCLUSIONS

Presented study provided insights into plants stress markers that can be used to assure safe application of sewage sludge into soils. Research showed that ecotoxicological markers such as the level of DNA damage, the content of chlorophyll and the expression of *rbcL* and *mt* could provide a more accurate description of the influence of specific sewage sludge on plants metabolism.

Acknowledgements

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Effects of sewage sludge supplementation on heavy metal accumulation and the expression of ABC transporters in *Sinapis alba* L. during assisted phytoremediation of contaminated sites*

Marta Jaskulak[1,2*], Anna Grobelak[1], Franck Vandenbulcke[2]

[1]Institute of Environmental Engineering, Faculty of Infrastructure and Environment, Czestochowa University of Technology, Czestochowa, Poland;

[2]University of Lille, Laboratory of civil engineering and environment (LGCgE), Environmental Axis, F-59650 Villeneuve d'Ascq, France;

Abstract:

ATP binding cassette (ABC) transporters, types C, G, and B were monitored via qPCR in order to investigate the influence of heavy metal (HM) contamination of post-industrial and post-agricultural soils, and the effects of its supplementation with sewage sludge, on *Sinapis alba* plants. Five house-keeping genes were selected and validated to ensure the best reference points. The relative expression of *ABC* types *C* and *G* genes was profoundly affected by experimental conditions and included their upregulation after plants exposure to heavy metals and downregulation after supplementation with sewage sludge. However, *ABC* type *C* was more responsive then type *G*. The experimental conditions altered the expression of *ABC* type *C* gene faster than *ABC* type *G* and thus, the expression of ABC type C can therefore potentially be used as a bioindicator during assisted phytoremediation of degraded sites. In clean soil, supplementation with sewage sludge with a slight content of heavy metals still caused an upregulation in the expression of ABC types C and G, which showed that proper toxicity assessments are necessary to ensure safe application of sewage sludge into soils. Results showed that the analysed genes take a significant part in plants metal detoxification and that their expression is regulated at transcriptional level after exposure to soil contaminated with heavy metals by both, industrial activities and by sewage sludge supplementation. Thus, their expression can potentially be used as an early-warning biomarker when soil supplementation with sewage sludge is incorporated into the soil-management process.

KEYWORDS: biomarkers, phytoremediation, ABC transporters, heavy metals, sewage sludge

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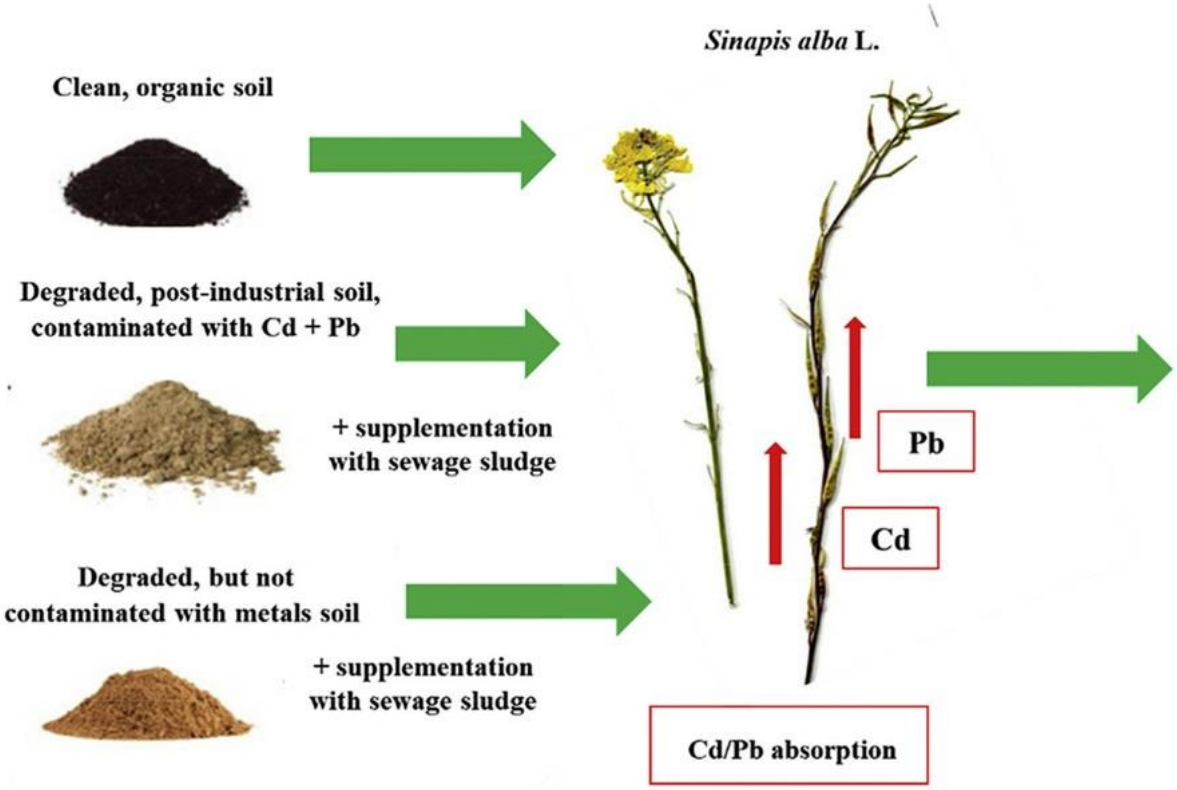
Highlights

- Effects of heavy metals and sewage sludge (SS) on the expression of ABC transporters
- Soil supplementation with SS alters the expression of ABC transporters in plants
- Type C and G of ABC transporters are highly overexpressed by metal contamination
- Expression of ABC transporters as effective biomarkers for phytoremediation

Graphical abstract:



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**Jaskulak, M., Grobelak, A., & Vandenbulcke, F. (2020). Modelling assisted phytoremediation of soils contaminated with heavy metals – main opportunities, limitations, decision making and future prospects. Chemosphere, 126196. doi:10.1016/j.chemosphere.2020.126196*

1 INTRODUCTION

In past years, soil contamination with toxic elements including heavy metals (HMs) has become a global concern across the world (Sarwar et al., 2017). An excess of HMs has a negative impact on biochemical and physiological processes in all living organisms, which poses a major risk not only to all members of the surrounding environment but also through the food chain - to human health (Dubey et al., 2018). Depending on the specific chemical properties of metals, their toxicity is commonly attributed to the following mechanisms: (1) the excessive production of reactive oxidative species, (2) the interference in proteins functional sites, (3) the disruption of enzymatic functions via the displacement of essential elements (Bernard et al., 2018). Plants exposed to metal-contaminated soils commonly suffer from decreased growth and thus, heavy metals alongside droughts are considered the most common abiotic stressors and are currently the leading cause of crop loss (Roy et al., 2015, Thakur et al., 2016).

Chemical and physical methods currently available and employed for heavy metal removal from soils are extremely perplexing and expensive (Gantait et al., 2019). Due to those reasons, in recent years more attention had been given to investigate phytoremediation which can be more of a cost-effective approach to deal with this issue by exploiting plants remarkable capabilities to metabolize, concentrate and store metals in their tissues (Agnihotri et al. 2019). At the same time, in the last two decades, phytoremediation had been also seen as a beneficial way to deal with the problem of constantly increasing volumes of produced sewage sludge (SS) across the world (Thomaidi et al., 2016). However, safe soil application of sewage sludge is a challenging and disputable environmental issue (Alvarenga et al., 2016). Nevertheless, in the case of areas degraded by past and current industrial activities, the use of SS for land reclamation seems to be a beneficial method of recycling the organic matter and crucial nutrients (Almasi et al., 2018). Such actions can allow maintaining and restoring the quality of previously degraded soils, as well as reduce the need for the application of synthetic fertilizers and thus, reduce the costs of soil remediation (Kubátová et al., 2016).

The selection of plants as well as the selection of the kind and the dose of soil amendment for assisted phytoremediation of soils contaminated by heavy metals is an intricate and multi-levelled query (Mahar et al., 2016). The selected plant has to be able to survive

given conditions and provide maximum biomass growth in a short period of time. Moreover, it needs to be able to uptake and store the highest possible concentration of given metal and at the same time, depending on if we want to stabilize or extract the contamination, it must have a wide range of mechanisms enabling transfer (or lack thereof), of metals to the shoots without impeding proper cellular functions (Nissim et al., 2018).

Since avoidance of harmful elements cannot be achieved in plants by their stationary vegetation, to minimalize the detrimental effects of heavy metals, plants developed a vast range of detoxification pathways that allow them to survive the exposure to contamination (Jaskulak & Grobelak, 2019). However, the mechanisms of tolerance and susceptibility to given abiotic stress are incredibly complex, multigenic and thus difficult to examine and control (Nahar et al., 2016). In recent years, ABC transporters had been recognized in many crucial processes associated with substance translocation through biological membranes (Hwang et al., 2016). Their contribution to detoxification against various hazardous substances is undisputed and consistently intriguing for researchers. Thus, understanding the physiological role of ABC proteins can be essential for a better understanding of many other detoxification processes in plants (Wang et al., 2016).

The ATP binding cassette (ABC) transporters are the superfamily of proteins conserved with integral membranes, responsible for allocating and distributing a broad scope of metabolites and other substances including xenobiotics and metals (Shabani et al., 2015). In all plants species, ABCs play a crucial role in basic vital processes including germination, the development of lateral root, stomatal movement and the response to various environmental stress responses (Adebesin et al., 2017). ABC transporters belong to a taxonomic superfamily within each every member shares two basic domains, TMD (transmembrane domain) that is primarily created with six membrane-spanning α-helices and NBD (nucleotide-binding domain), a cytosolic domain involved in ATP (Shibata et al., 2016).

Currently, only in two model plant species including *Arabidopsis thaliana* and *Zea mays*, genes encoding the ABC transporters have already been identified (Demessie et al., 2017). At the same time, only a small fraction of those genes had been functionally

characterized (Do et al., 2018). Since ABC transporters in plants are more diverse then their homologs in all other organisms, some researchers suggest, that it is possible that they are also more specialized (Brunetti et al., 2015., Lefèvre et al., 2015). Moreover, the ABC transporters from other plant species including species suitable for phytoremediation were not previously investigated (Lane et al., 2016). Thus, understanding the role of ABC transporters in plants, after real, complex stress conditions can potentially be also exploited as potential biomarkers to further advance the phytoremediation technologies including safety of sewage sludge or other biosolids application into soils.

For those reasons, our study was designed to investigate the effects of soil heavy metal contamination and sewage sludge (SS) supplementation on *S. alba* grown on degraded soils - post-industrial and post-agricultural. The main aim of the study was to assess the influence of soil supplementation with SS on the expression levels of ABC transporters in shoots of *S. alba* in order to determinate the effects of complex metal contamination and soil supplementation with SS on those transporters. Overall, three types of ABC transporters had been chosen for the study: types C, G, and B that are mostly researched and thought to take a significant part in metal detoxification.

2 MATERIALS AND METHODS

2.1 Substrates characterization

All experiments were conducted in 2018, and the study consisted of two separate experiments with two different soils performed simultaneously. Both soils were collected from the top layer (0 – 30 cm) of landless areas, and both were characterized by low fertility, sorption capacity, the content of organic matter, and almost no microbial activity (Grobelak et al., 2019). Soil A was also severely contaminated with heavy metals, specifically cadmium and lead, as an effect of former emissions from zinc smelter in Silesia, Poland (GPS: 50º30'N 18º56'E). Soil B was degraded by post exploitation for agricultural purposes, uncontaminated by heavy metals but in other physical and chemical parameters similar to soil A (GPS: 51°33'N 19°11'E) (Table 1). According to the classification of World Reference Base for Soil Resources (WRB), both soils belong to podzols and can be described as a sandy loam with a composition of approximately 15% clay, 20% silt and 65% sand. Both soils were also characterized by similar cation exchange capacity (CEC). For S1: 16.2 ± 0.27; for S2: 17.5 ± 0.32. The uncontaminated by metals soil B was therefore used as a control to soil A in order to establish only the influence of heavy metals on plants but in order to see the severity of such influence, high in nutrients and organic matter, peat soil was also used as a clean control, providing optimal conditions for plants growth. Such action allowed assessing if sewage sludge application not only improved plants growth and development but also to study, how far it was from plants proper, optimal development. Prior to the experiments, collected soils were dried, sieved and finally mixed with sewage sludge.

In total, four types of sewage sludges, deriving from four separate facilities in Silesia region (Poland) were used in the experiment (Figure 1). Two of which consisted of municipal sewage sludge (S1 and S2), and two were from industrial water-treatment plants: one from the production of mineral water and sweet beverages (S3) and the second one form treatment of drinking water which was characterized with high concentration of iron due to water treatment process (S4). Three of the selected sewage sludges (S2, S3, S4) were clean from metal contamination and met the standards of EU norm (Directive 86/278/EEC) regulating their use as soil fertilizers. On the other hand, S1 was characterized by slight metal contamination and did not meet the EU standards, which

allowed to also asses the consequences of introducing such sewage sludge to selected endpoints as well as to compare chosen soil additives.

Figure 1. Experiment design. [0 – organic, peat soil, A – soil contaminated with HM by industrial activities; B – degraded but uncontaminated, post-agricultural soil; S1 – municipal sewage sludge contaminated by HMs; S2 – uncontaminated, municipal sewage sludge; S3 – industrial sewage sludge after purification of mineral water and beverages, S4 - industrial sewage sludge after purification of drinking water with high iron content].

Sludges were transported in a dewatered state to the Faculty of Infrastructure and Environment, Czestochowa, Poland, where the experiment started immediately. Both, soil and sewage sludge had been tested in their dewatered forms for their physical and chemical properties before mixing and experimental procedures (Table 1). Prior to the experiment, soil was dried sifted, and selected sewage sludge was added in a dose corresponding to the EU nitrogen standards (170 kg of nitrogen/year/ha) (EUR-Lex - 31991L0676 e EN), to ensure an equal amount of nitrogen to each treatment. After adjusting to each SS dry mass, its content of nitrogen, and pot size, the total weight of added SS per pot was as follows: S1: 94.5 g, S2: 97.2 g, S3: 100.8 g, S4: 161.8 g. Each mix of soil with sewage sludge as well as all control samples were tested for their physical and chemical properties both, before and after the plants growth (Table 1, Table 2).

Table 1. Substrates characterization prior to the experiments [0 – organic, peat soil, A – soil contaminated with HM by industrial activities; B – degraded, uncontaminated soil; S1 – municipal sewage sludge contaminated by HMs; S2 – uncontaminated, municipal sewage sludge; S3 – industrial sewage sludge after purification of mineral water and beverages, S4 - industrial sewage sludge after purification of drinking water with high iron content]. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey test ($p < 0.05$) after a one-way ANOVA.

Substrate	pH		N Kjeldahl [mg kg^{-1}]	P total [mg kg^{-1}]	TOC [g kg^{-1}]	Cd [mg kg^{-1}]	Pb [mg kg^{-1}]	Zn [mg kg^{-1}]	Fe [mg kg^{-1}]
	H_2O	KCl							
0	5.97 ± 0.04b	5.75 ± 0.03b	2632.17 ± 39.54c	180.5 ± 5.21b	44.8 ± 2.14b	0.55 ± 0.1a	8.22 ± 0.2a	31.6 ± 3.3a	87.6 ± 2.6c
A	5.39 ± 0.06a	5.08 ± 0.06a	621.30 ± 22.35a	75.63 ± 27.64a	8.8 ± 1.9a	22.42 ± 1.1d	1252.6 ± 7.1e	795.6 ± 5.7e	32.1 ± 3.8a
B	5.44 ± 0.05a	5.18 ± 0.10a	809.87 ± 9.78b	91.52 ± 16.56a	11.9 ± 3.2a	1.08 ± 0.5a	14.5 ± 2.16b	54.2 ± 2.8b	45.7 ± 2.9b
S1	7.12 ± 0.11d	5.92 ± 0.06c	15243.37 ± 193.81d	3633.72 ± 42.11c	701.44 ± 24.41c	8.15 ± 0.4c	253.66 ± 8.6d	1455.26 ± 8.4f	273.4 ± 32.6d
S2	6.74 ± 0.13c	5.88 ± 0.11d	11576.54 ± 201.70e	3276.18 ± 62.14d	686.31 ± 35.25c	3.88 ± 0.5b	47.82 ± 3.5c	774.89 ± 4.8e	262.5 ± 35.7d
S3	7.51 ± 0.08e	6.61 ± 0.09e	18153.45 ± 198.77f	6154.48 ± 51.36e	798.15 ± 75.61d	0.75 ± 0.2a	13.8 ± 2.2b	133.76 ±. 7.1d	370.8 ± 11.3e
S4	7.44 ± 0.10e	6.75 ± 0.13e	17417.36 ± 55.31f	11162.23 ± 92.71f	825.61 ± 73.22d	1.12 ± 0.3a	18.5 ± 4.3b	94.4 ± 2.5c	2735.5 ± 48.4f

For the determination of pH, standard pH meter was used and the pH values were assessed in distilled water as well as in 1 M KCl solution in accordance to ISO 10390:2005. The content of total organic nitrogen was measured by following the Kjeldahl method (PN-ISO11261:2002) (Bradstreet, 1940). Total organic carbon (TOC), was measured using multi N/C 2100 (Analytik Jena, Germany) with an attachment for measuring solids (HT 1300, Analytik Jena, Germany) according to the manufacturer's instructions. The content of phosphorus and potassium was assessed by Egner method (Egner et al., 1960), and the concentrations of heavy metals in substrates and later in plants, were determined using spectrometer (ICP-OES; multi view, Spectroapparatus, USA) after digestion in a microwave digestion system according to the EPA method 3051.

2.2 Experimental procedure

Sinapis alba L., member of *Brassicaceae* was chosen to the study due its strong ability to accumulate heavy metals and high resistance to other abiotic stressors which makes it a suitable species for phytoremediation (Agnihotri et al., 2019). Certified, high-quality and commercially available seeds were used for all experiments (Dary-Podlasia, Poland). Prior to the soil exposure, seeds were germinated for 7 days on a sprouted trays with filter paper and water in strictly controlled conditions of a growth chamber (Biogenet FS360, Poland). During this time, the seeds germination index was recorded and was considered to properly occur when the seedlings were at least 4 mm long. Germination index of *S. alba* seeds was thus estimated to be around 95% (percentage of properly germinated plantlets out of 500 germinated seeds, data not shown).

Each experimental pot (H - 25 cm, a - 12 cm, b – 25 cm) contained 2 kg of soil and all experimental treatments were prepared in three independent biological replicates. After mixing the soil with sewage sludge and adjusting soil water content to approximately 50%, the prepared growth media were left in the growth chamber for 7 days prior to planting germinated seedlings. After that, 15 plantlets were transported into each experimental pot and planted approximately 3 – 5 cm deep. The exposition time weas chosen as follows: 3 days, 7 days, 14 days, 21 days and 28 days. Similarly to seeds germination, plants were incubated in a phytotron chamber (Biogenet FS360, Poland) under controlled conditions including: photoperiod: 16 h of light, 8 h of dark, temperature during the day 21^{o}C, during the night 18^{o}C, light intensity 4000 lx (photosynthetic LED

light), watering every 3 days according to plants requirements. After each selected time-point, plants were extracted from soil, washed thoughtfully in distilled water, the length of roots and plants fresh biomass were measured, and plants biomass was stored in -80^{o}C for future analyses. In total, study consisted of 11 different experimental treatments in 5 time-points each and included: the degraded, contaminated soil (A), the same contaminated soil with four different sewage sludges (S1, S2, S3, S4), the degraded but uncontaminated soil (B), the same uncontaminated soil with four sewage sludges (SS1, SS2, SS3, SS4), and organic peat soil (0) as a reference point to the optimum conditions of plants growth.

2.3 Basic toxicity assessment – plants biomass and roots length

Roots length and biomass were measured after extraction of plants from the soil after each selected time-point of the experiment. From each experimental treatment in each time-point, the 3 plants were extracted and measured for their biomass and roots length. Basic growth parameters including plants' foliar surface, signs of chlorosis, or fungi infection have been also recorded daily by visual observation.

2.4 Metal accumulation in plants shoots

The content of metals in plants shoots after selected exposure timepoints was also quantified in three independent replicates for all experimental treatments. Prior to the ICP-OES analysis, plants were air dried at 40^{o}C and digested. Overall, the following metals and metalloids were analysed: Ag, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Mg, Mn, Na, Ni, P, Pb, Sr, Ti, Zn, but due to the content below contamination norms or completely below measurement threshold, they were not taken into account. Bioconcentration factor (BCF) was calculated using the ratio of total metal accumulation in plants shoots and in soil respectively [$BCF = C_{shoot}/C_{soil}$]

2.5 Expression of ABC transporters

2.5.1 Extraction of RNA and reverse transcription

Expression analyses of three target and five house-keeping genes (HKG) were performed on the total RNA extracted from shoots of *S. alba* by Eur_x Universal DNA/RNA/Protein kit (Eur_x, Poland), according to manufacturer's instructions. For each extraction, 100 mg of plants frozen biomass was used, and the quantity and quality of isolated RNA were measured spectrophotometrically, by using spectrometer with a nanodrop (Eppendorf BioPhotometer D30, USA).The integrity of the extracted RNA was also validated on 2% agarose gel stained with SimplySafe® (Eur_x, Poland). The total concentration of isolated RNA allowed performing the reverse transcription to a final concentration of 1.0µg/µl cDNA via using NG dART RT-PCR kit (Eur_x, Poland) and by following manufacturer instructions. cDNA samples were stored in -20^{o}C until further analyses.

2.5.2 Primers design, the evaluation of house-keeping genes and qPCR

In order to select the most stable HKG for data normalization and after selected exposure, the $_{\Delta}$Ct approach was chosen following (Brulle et al., 2014) procedure. The stability of five HKG was measured for all experimental conditions and included genes encoding: *actin, GAPDH, β-tubulin, elongation factor 1-α (ef1α)* and *18S*. The selection of five potential HKG was performed based on sequences available in GeneBank from taxonomically related species such as *Brassica napus, Brassica oleracea* and *Arabidopsis thaliana* due to the lack of information on stable HKG for *S. alba* plants exposed to metal stress. The selected reference genes were then analysed by BLAST in order to identify highly conserved fragments, and based on those fragments primers were designed using Primer3Plus software. In a case of target genes, the sequences encoding fragments of ABC transporters types C, G, and B in *S. alba* were already deposited in GeneBank. Thus, conserved fragments were also identified via BLAST and primers were designed using Primers3Software. All of the amplificated fragments ranged in their size between 100 and 300 bp. The specific qPCR primers are presented below:

18S:	5′ AGTTGAGGGATGATTTGG 3′;
	3′ TGCAAACCCTCGTCAATT 5′;
	product size: 104 bp; melting temperature 60^{o}C;
Elongation factor:	5′ GGTCCCTTGTACCAGTCA 3′;
	3′ GGAGGTGTCTTCCTACTT 5′;
	product size 126 bp; melting temperature 60^{o}C;
β-tubulin:	5′ CCAGGCTCCAGATCCAT 3′;
	3′ GCGAGTCAATGTTTACTA 5′;
	product size: 140 bp; melting temperature 59^{o}C;
Actin:	5′ AGCAATACCAGGGAACAT 3′;
	3′ GACATCAGAAAGGACTTG 5′;
	product size: 102 bp; melting temperature 60^{o}C;
GAPDH:	5′ GCTGCTTCATTCAACATC 3′;
	3′ CATCATCCTCGGTGTATC 5′;
	product size: 170 bp; melting temperature 60^{o}C;
ABCC:	5′ TTACACAAGAGGAAGAAGTAGA 3′;
	3′ ATACGGTTGCTGATAGAAGA 5′;
	product size: 185 bp; melting temperature 59^{o}C;
ABCG:	5′ CCGATGAAGATAACGAGAAG 3′;
	3′ TAAGGCAACATTAGGTAACG 5′;
	product size: 160 bp; melting temperature 60^{o}C;
ABCB:	5′ GAAGTCGTTGGAAGAATGTC 3′;
	3′ ACAAGAAGAGGTATTGAAGA 5′;
	product size: 260 bp; melting temperature: 58^{o}C;

The PCR parameters were conducted in the following manner: 95°C for 5 min and 40 cycles at 95°C 30 s, 56°C 30 s, and 72°C 60 s. After 40 cycles, the last step of the program consisted of 72°C for 5 min (MJ Research PTC-200, BIORAD, USA). To amplify the desired DNA fragments, Promega GoTaqR G2 Flex DNA Polymerase mix was used (Promega, USA) was used with an addition of magnesium chloride ($MgCl_2$) to a final concentration of 2 mM. All selected primers were added to the reaction in a concentration of 0.4 μM. All of the PCR reactions were executed in three biological replicates and two technical ones. After the reactions, the size and quality of desired products were checked on a 2% stained with EtBr (Thermo Scientific, Germany).

Parameters of the electrophoresis: 150 V, 45 min. After that process, obtained products were extracted from the gel, purified by GFXTM PCR DNA and Gel Band Purification (GE Healthcare, UK), and sequenced using standard SANGER sequencing (Genomed, Poland). Obtained sequences were then deposited in GenBank and used to design qPCR primers.

2.5.3 qPCR amplification

All qPCRs were performed in strip tubes MicroAmp Optical 8-Tube Strip, 0.2 mL (Thermo Fisher Scientific) with SYBR™ Select Master Mix (Thermo Fisher Scientific, USA), and Mastercycler® ep realplex, Eppendorf thermocycler was used (Eppendorf, Germany). The specific parameters for qPCR regarding the used temperatures were as follows: 95°C for 5 min and 40 cycles at 95°C 30 s, 56°C 30 s, and 72°C 60 s. After cycles, last step of the program consisted of 72°C for 5 min. The stability of reference genes was calculated using NormFinder software and by following (Jaskulak et al., 2019) procedure. After stability evaluation, three of the most stable house-keeping genes were chosen for calculation of the relative expression level of the *ABCC*, *ABCG* and *ABCB* gene. The levels of expression were determined as the number of cycles necessary for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (Ct). After that, the relative expression level was carried out according to standard formula: $R= (E_{Tg})CP_{Tg} /(E_{ref})CP_{ref}$

2.6 Data analysis

Presented results are expressed in tables and graphs as means ± standard deviation, n = 3. All of analyses with plants biomass were performed in three independent biological repetitions as well as two technical ones. For statistical analysis, Tukey's test ($p < 0.05$) was used, after one way (for soil analyses) or two-way ANOVA (for plant analyses with timepoints), same letters indicate the non-significant difference (ANOVA $p > 0.05$). Descriptive statistics and statistical analyses were obtained via OriginPro 2015 software. Different letters "a", "b" "c", "d" on top of graph points and in table columns indicate a significant difference according to ANOVA with post-hoc Tukey's test $p < 0.05$. For all tests, p values below 0.05 were considered statistically significant. Specific primes were designed using Primer3Software and the HKG stability was quantified using softwares BestKeeper and NormFinder by following previously described settings (Jaskulak et al., 2019).

3 RESULTS

3.1 Soil characterization

The effects of soil supplementation with chosen sewage sludge are shown in Table 2. Overall, the addition of all types of sewage sludge did not cause any statistically significant ($p < 0.05$) changes in regards to soil pH. On average in distilled water, the pH of contaminated soil (A) measured at 5.4 and for soil B at 5.6. Since the selected dose of sewage sludge was based on the EU nitrogen norms for soil fertilization, after the supplementation with SS, the content of nitrogen remained stable for all samples. Thus, applying the nitrogen norm allowed as to eliminate another variable in our conditions, which enabled for a more accurate assessment of the influence of heavy metals on phytotoxicity since all of the experimental conditions received the same dose of nitrogen with sewage sludge. Without any supplementation, the content of nitrogen (N) in soil A is measured at around 710 mg kg^{-1} of N and for soil B at about 800 mg kg^{-1} of N. The addition of SS caused a significant ($p < 0.05$) increase in the content of N at on average 1050 mg kg^{-1} for all experimental conditions.

Moreover, the content of organic carbon (TOC) and cation exchange capacity (CEC) also increased significantly ($p < 0.05$) after the supplementation. The contamination of soil A deriving from past smelting activities in Silesia Poland, showed that the total concentration of Cd in collected soil exceeded the safe threshold for agricultural use more than 8 times according to the EU norms. The contamination of soil A with Pb exceeded the safe limit approximately 3 times. The application of municipal sewage sludges (S1 and S2) to soil A did not cause any significant changes to soil concentration of Cd and Pb whereas the supplementation with S3 and S4 caused a slight decrease in those values. However, the differences between soil A and soil B were noticed after the supplementation with sludge with minor contamination of heavy metals, which caused a slight increase in Cd and Pb concentration in soil B.

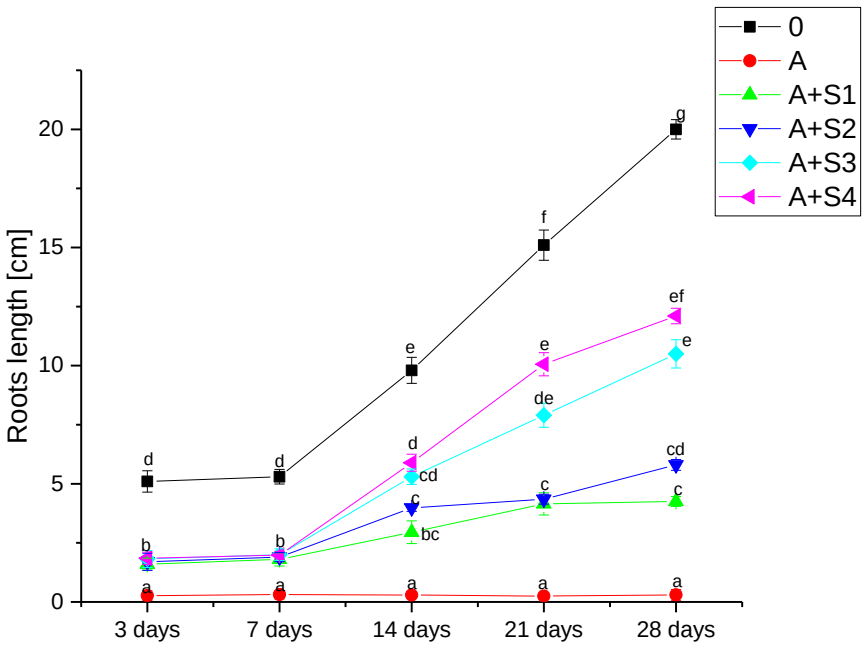
Table 2. Soil characterization after 28 days of the experiment. [0 – organic, peat soil, A – soil contaminated with HM by industrial activities; B – degraded, uncontaminated soil; S1 – municipal sewage sludge contaminated by HMs; S2 – uncontaminated, municipal sewage sludge; S3 – industrial sewage sludge after purification of mineral water and beverages, S4 - industrial sewage sludge after purification of drinking water with high iron content, CEC – caption exchange capacity, TOC – total organic carbon]. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey's test ($p < 0.05$).

Growth medium	**pH**		**N Kjeldahl [mg kg^{-1}]**	**CEC**	**TOC [g kg^{-1}]**	**Cd [mg kg^{-1}]**	**Pb [mg kg^{-1}]**
	H_2O	KCl					
0	5.94 ± 0.05a	5.61 ± 0.04a	2601.32 ± 24.5a	31.2 ± 0.45a	43.9 ± 1.4a	0.44 ± 0.2a	7.63 ± 0.3a
A	5.49 ± 0.04b	5.25 ± 0.12b	710.08 ± 22.34b	16.2 ± 0.27b	9.4 ± 1.2b	19.95 ± 0.6b	1076.8 ± 4.7b
A+S1	5.58 ± 0.18b	5.15 ± 0.07b	1498.41 ± 43.26c	18.5 ± 0.62c	15.4 ± 1.5c	19.50 ± 0.5b	1094.52 ± 5.1b
A+S2	5.61 ± 0.05b	5.18 ± 0.03b	1532.27 ± 51.22bc	17.9 ± 0.71c	13.2 ± 1.0c	18.92 ± 0.3b	1115.42 ± 7.6b
A+S3	5.54 ± 0.12b	5.20 ± 0.10b	1575.59 ± 67.28c	22.5 ± 0.55d	18.9 ± 1.7c	16.31 ± 0.7c	1001.65 ± 12.5c
A+S4	5.48 ± 0.17b	5.23 ± 0.15b	1487.98 ± 53.27c	23.1 ± 0.47d	19.7 ± 1.3c	16.02 ± 0.1c	1024.21 ± 33.4c
B	5.55 ± 0.09b	5.17 ± 0.13b	1472.75 ± 75.02c	17.5 ± 0.35c	12.8 ± 2.0d	0.88 ± 0.2d	12.5 ± 1.18d
B+S1	5.60 ± 0.15b	5.11 ± 0.05b	1518.75 ± 28.90c	19.2 ± 0.18c	22.5 ± 3.2c	4.02 ± 0.5e	43.5 ± 0.9e
B+S2	5.49 ± 0.18b	5.25 ± 0.10b	1587.60 ± 35.64c	19.5 ± 0.41c	21.0 ± 1.9c	1.64 ± 0.3f	22.8 ± 4.6f
B+S3	5.65 ± 0.07b	5.18 ± 0.11b	1477.81 ± 42.17c	23.6 ± 0.38d	25.8 ± 1.4d	0.79 ± 0.5d	12.7 ± 1.7d
B+S4	5. 58 ± 0.21b	5.16 ± 0.5b	1583.52 ± 33.65c	24.3 ± 0.28d	28.5 ± 1.8d	0.90 ± 0.1d	13.2± 2.1d

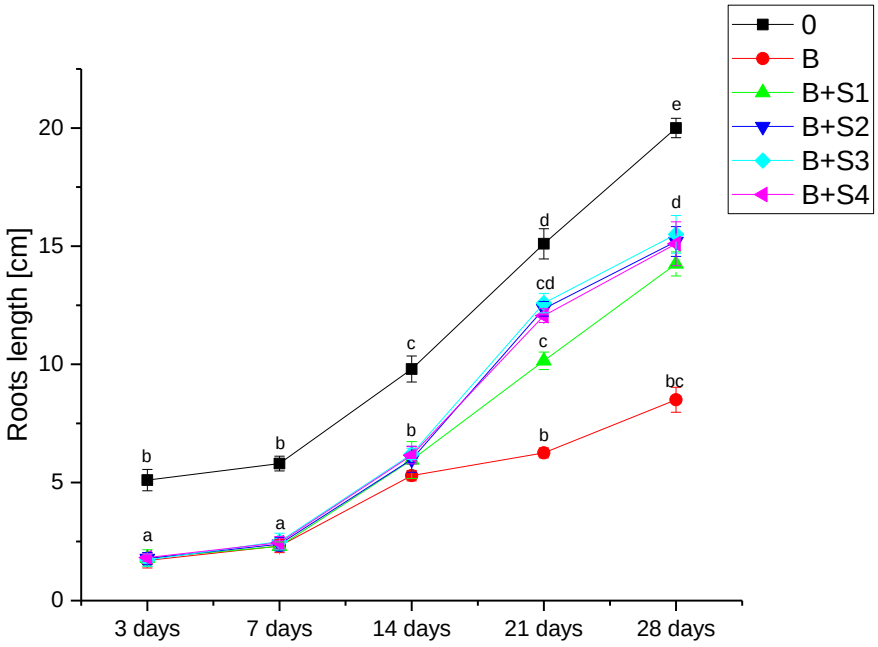
3.2 Basic growth parameters: roots length and plants biomass

The primary growth parameters including roots length and plants biomass were immensely impacted by soil A in comparison to optimal growth conditions of organic peat soil as well as in contrast to also degraded but uncontaminated soil B (Figure 2). Equally on both soils, the different effects of each sewage sludge supplementation started to occur around 14 days of exposure whereas earlier, all four sewage sludges caused approximately the same increase in plants biomass and roots length. Overall, the roots elongation was entirely inhabited on contaminated soil without any supplementation, the lengths of the roots stayed at the same level of approximately 4 mm which is 50 times shorter than in optimal conditions after 28 days of the experiment. Supplementation with all types of sewage sludge caused a beneficial impact on plants biomass and roots length but the differences between specific sludges were prominent and continued to differ across timepoints. Overall the supplementation of soil with S4 caused a 4-fold increase in roots length then supplementation with S1 after 28 days of the experiment. However, such variances were not noticeable before 14 days of the experiment which shows the need to perform long-term tests or find even more sensitive biomarkers before planning of phytoremediation and land application of sewage sludge. Similar tendencies were observed for biomass weight. Moreover, in plants grown on soil A without any sewage sludge and with S1, severe chlorosis and necrosis was observed starting at day 7 and persisted until day 28. No significant ($p < 0.05$) variation between any other experimental conditions was found in that matter (data not shown).

A


0
A
A+S1
A+S2
A+S3
A+S4
Roots length [cm]
0
5
10
15
20
3 days
7 days
14 days
21 days
28 days

A


0
B
B+S1
B+S2
B+S3
B+S4
Roots length [cm]
0
5
10
15
20
3 days
7 days
14 days
21 days
28 days

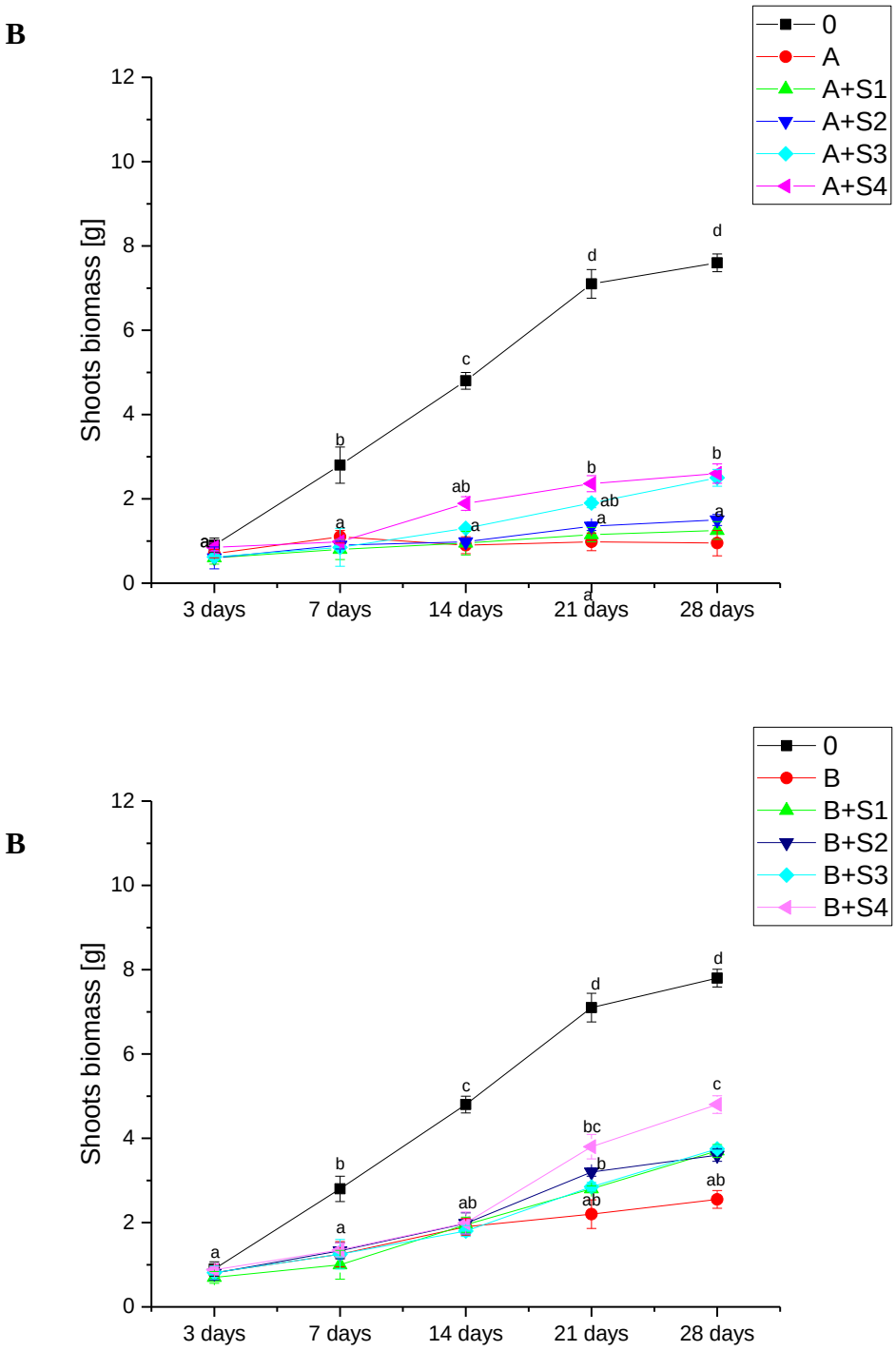


Figure 2. Roots length (A) and plants biomass (B) [0 – organic, peat soil, A – soil contaminated with HM by industrial activities; S1 – municipal sewage sludge contaminated by HMs; S2 – uncontaminated, municipal sewage sludge; S3 – industrial sewage sludge after purification of mineral water and beverages, S4 - industrial sewage sludge after purification of drinking water with high iron content]. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the

different letters are significantly different according to Tukey's test ($p < 0.05$). Different letters "a", "b" "c", "d" indicate a significant difference according to two-way ANOVA with post-hoc Tukey's test $p < 0.05$.

3.3 Metal content in plants

The accumulation of Cd and Pb in plants shoots was also severely affected by both: heavy metal contamination and its supplementation with sewage sludge (Table 3). Similarly to biomass and roots length results, the accumulation of Cd and Pb on soil A escalated after 14 days of the experiment. Overall, supplementation of sewage sludges caused a significant ($p < 0.05$) decrease in metal accumulation after 28 days of the experiment, but the degree of that effect varied severely. On the contaminated soil A, for the accumulation of Cd in plants shoots, municipal sewage sludge (S1) did not cause any statistically significant decrease ($p < 0.05$), S2 decreased the metal accumulation by approximately 40%, S3 by about 60% and S4 by almost 75%. At the same time, sewage sludge application on soil A caused a decrease in the accumulation of Pb by 22% for S1, 55% for S2, 72% by S3 and even 88% for S4. On the not contaminated soil B, supplementation of sewage sludge did not cause any statistically significant variations in metals accumulation ($p < 0.05$) (Table 3)

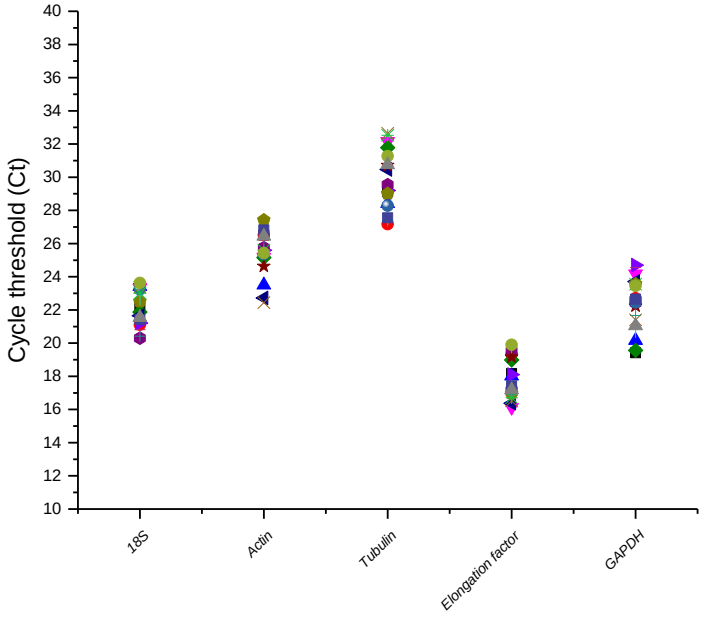
Table 3. Cadmium and lead accumulation in shoots of *S. alba*. [0 – control soil, A – soil contaminated with HM by industrial activities; B – degraded, uncontaminated soil; S1 – municipal sewage sludge contaminated by HMs; S2 – uncontaminated, municipal sewage sludge; S3 – industrial sewage sludge after purification of mineral water and beverages, S4 - industrial sewage sludge after purification of drinking water with high iron content]. All results are expressed as means ± standard deviation, n = 3, means within **rows** marked with "*" and with bold font are statistically different according to Tukey Test ($p < 0.05$) after a two-way ANOVA, means within **columns** marked with different letters are statistically different according to Tukey Test ($p < 0.05$) after a one-way ANOVA.

A	**Time**	**Treatment**									
	[days]	**0**	**A**	**A+S1**	**A+S2**	**A+S3**	**A+S4**	**B**	**B+S1**	**B+S2**	**B+S3**	**B+S4**
Cd [mg kg^{-1}]	3	Nd.	3.8 ± 0.7a	3.4 ± 0.8a	4.2 ± 1.4a	2.8 ± 0.6a	**0.8 ± 0.2a***	Nd.	**0.6 ± 0.4a***	Nd.	Nd.	Nd.
	7	Nd	4.7 ± 2.3a	3.9 ± 1.4a	5.1 ± 1.2a	3.4 ± 0.5a	4.5 ± 0.6b	Nd.	**0.7 ± 0.5a***	Nd.	Nd.	Nd.
	14	Nd.	38.6 ± 3.5b	40.2 ± 2.3b	40.9 ± 2.7b	**10.9 ± 2.2b***	**8.6 ± 1.1c***	Nd.	**1.1 ± 0.4a****	Nd.	Nd.	Nd.
	21	Nd.	72.8 ± 4.1c	68.5 ± 5.2b	**48.7 ± 3.1c***	**27.5 ± 3.6c****	**12.5 ± 2.5d*****	Nd.	**1.3 ± 0.6a******	**0.6 ± 0.3a******	Nd.	Nd.
	28	Nd.	103.2 ± 4.3d	99.1 ± 11.8c	63.5 ± 7.2d*	41.3 ± 1.8d**	**17.4 ± 1.4e*****	Nd.	**2.2 ± 0.8a******	**0.8 ± 0.2a******	Nd.	Nd.
Pb [mg kg^{-1}]	3	Nd.	20.5 ± 3.6a	22.5 ± 2.7a	19.6 ± 2.2a	20.1 ± 1.9a	**5.8 ± 0.3a***	Nd.	Nd.	Nd.	Nd.	Nd.
	7	Nd	57.4 ± 5.3b	53.9 ± 5.4b	55.8 ± 7.5b	**25.3 ± 0.8b***	**9.8 ± 0.7b****	Nd.	Nd.	Nd.	Nd.	Nd.
	14	Nd.	175.2 ± 7.5c	167.7 ± 9.1c	**75.9 ± 5.8c***	**70.8 ± 2.2c***	**18.2 ± 3.1c****	Nd.	**1.7 ± 0.6*****	Nd.	Nd.	Nd.
	21	Nd.	352.1 ± 8.1d	**268.5 ± 8.4d***	**128.3 ± 7.5d****	**86.5 ± 5.1d*****	**32.5 ± 2.7d******	Nd.	**3.2 ± 0.3*******	Nd.	Nd.	Nd.
	28	Nd.	419.2 ± 11.2e	**324.5 ± 9.4e***	**185.2b ± 8.2e****	**114.7 ± 4.9e*****	**47.8 ± 2.8e******	Nd.	**5.1 ± 0.4*******	Nd.	Nd.	Nd.
Cd BCF	3	Nd.	0.19 ± 0.2a	0.17 ± 0.1a	0.22 ± 0.2a	0.17 ± 0.3a	**0.05 ± 0.2a***	Nd.	0.15 ± 0.1a	Nd.	Nd.	Nd.
	7	Nd	0.24 ± 0.2a	0.20 ± 0.3a	0.27 ± 0.3a	0.21 ± 0.1a	0.28 ± 0.2a	Nd	0.17 ± 0.2a	Nd.	Nd	Nd
	14	Nd.	1.94 ± 0.4b	2.06 ± 0.4b	2.16 ± 0.5b	**0.67 ± 0.5a***	**0.54 ± 0.4a***	Nd.	**0.27 ± 0.2a***	Nd.	Nd.	Nd.
	21	Nd.	3.64 ± 0.3c	3.51 ± 0.5c	**2.58 ± 0.6b***	**1.69 ± 0.4b****	**0.78 ± 0.6a*****	Nd.	**0.32 ± 0.3a*****	**0.37 ± 0.2a*****	Nd.	Nd.
	28	Nd.	5.17 ± 0.5d	5.08 ± 0.3d	**3.36 ± 0.3c***	**2.53 ± 0.3c****	**1.09 ± 0.5a*****	Nd.	**0.55 ± 0.4a******	**0.49 ± 0.2a******	Nd.	Nd.
Pb BCF	3	Nd.	0.02 ± 0.3a	0.02 ± 0.1a	0.01 ± 0.2a	0.02 ± 0.2a	0.01 ± 0.2a	Nd.	Nd.	Nd.	Nd.	Nd.
	7	Nd	0.05 ± 0.4a	0.05 ± 0.3a	0.05 ± 0.4a	0.03 ± 0.1a	0.01 ± 0.3a	Nd	Nd.	Nd.	Nd	Nd.
	14	Nd.	0.16 ± 0.2b	0.15 ± 0.4b	0.07 ± 0.2a	0.07 ± 0.4a	0.02 ± 0.6a	Nd.	**0.42 ± 0.1a***	Nd.	Nd.	Nd.
	21	Nd.	0.33 ± 0.3b	0.25 ± 0.2b	**0.11 ± 0.6a***	**0.09 ± 0.5a****	**0.03 ± 0.4a****	Nd.	**0.80 ± 0.2b*****	Nd.	Nd.	Nd.
	28	Nd.	0.41 ± 0.3b	**0.30 ± 0.3b***	**0.17 ± 0.2b****	**0.11 ± 0.6a****	**0.05 ± 0.2a*****	Nd.	**1.26 ± 0.3c******	Nd.	Nd.	Nd.

3.4 HKG validation

In order to select the best HKGs, for data normalisation the NormFinder and BestKeeper software were used according to Brulle et al., 2014 methodology. The results are presented as their cycle threshold between all samples and as an average expression stability M. The lower M value indicates higher stability. The most stable HKG in *S. alba* for given experimental conditions was *18S*, then, *elongation factor*, *actin*, *tubulin* and at least *GAPDH* (Figure 3).

A



B

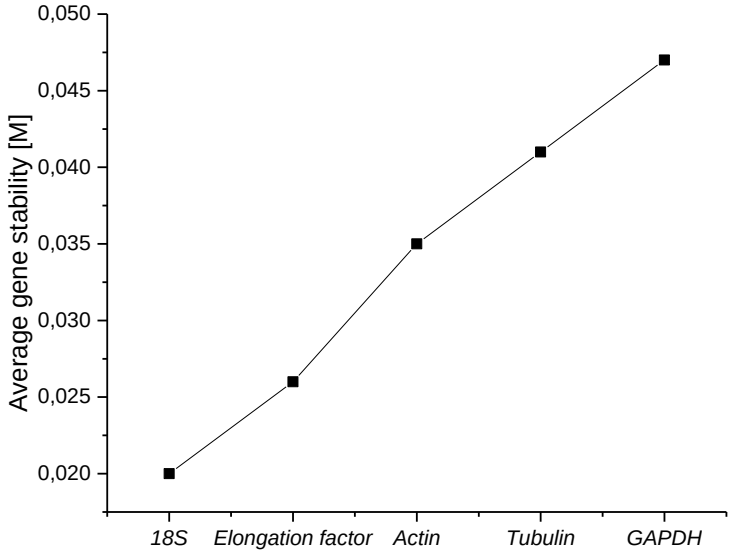


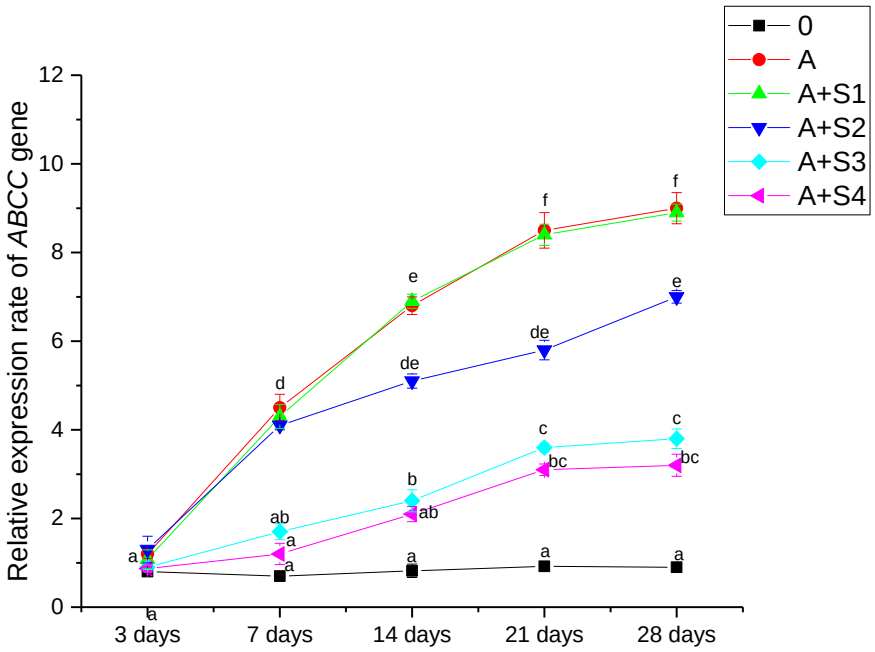
Figure 3. A - Cycle threshold (Ct) of all candidate reference genes for *S. alba* exposed to experimental conditions. Data presented are means ± standard deviation. B – average gene stability of candidates for reference genes in experimental conditions ranked from the most stable to the least stable via NormFinder and BestKeeper software

3.5 Expression of ABC transporters

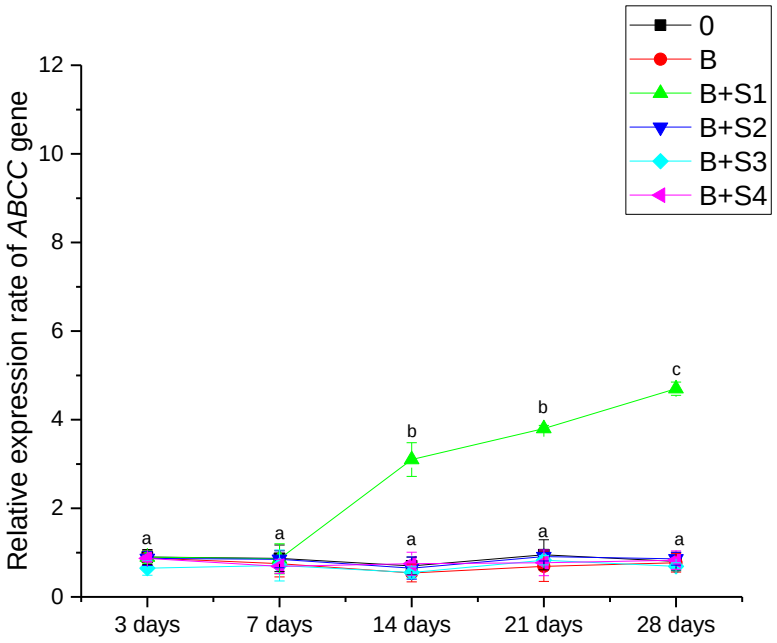
Contamination of soil with heavy metal caused a severe overexpression of *ABCC* and *ABCG* transporters, while *ABCB* remained stable and unaffected. In comparison to plants grown on clean organic soil (soil 0), the contamination (soil A) induced the expression of *ABCC* 8 times and *ABCG* 6.5 times (Figure 4). The supplementation of contaminated soil with sewage sludge led to different reactions of all discussed genes regarding the type of sewage sludge. For both *ABCC* and *ABCG*, the expression rate in plants shoots after 28 days in the experiment was not changed by the supplementation with S1, whereas all other sludges caused the decrease of expression in those genes. However, chosen genes differ between each other in regards to the time of their induction. For the *ABCC* gene, in plants grown on contaminated soil (A), the tremendous increase was observed after 7 days of exposure, whereas *ABCG* was induced after 14 days of the experiment. No significant ($p < 0.05$) change in expression of *ABCB* was observed for all experimental condition, suggesting that it is not influenced by industrial contamination and sewage sludge supplementation (Figure 4).

Interestingly, on degraded but not contaminated soil B, a significant ($p < 0.05$) increase in the expression of *ABCC* gene was observed after supplementation with S1 containing high amounts of trace metals. Since the *ABCG* gene was not found to be induced by S1, *ABCC* seems to be much more sensitive to even small concentrations of heavy metals. At the same time, this result suggests that soil application of municipal sewage sludge, containing even trace amounts of heavy metals, can alter plants gene expression.

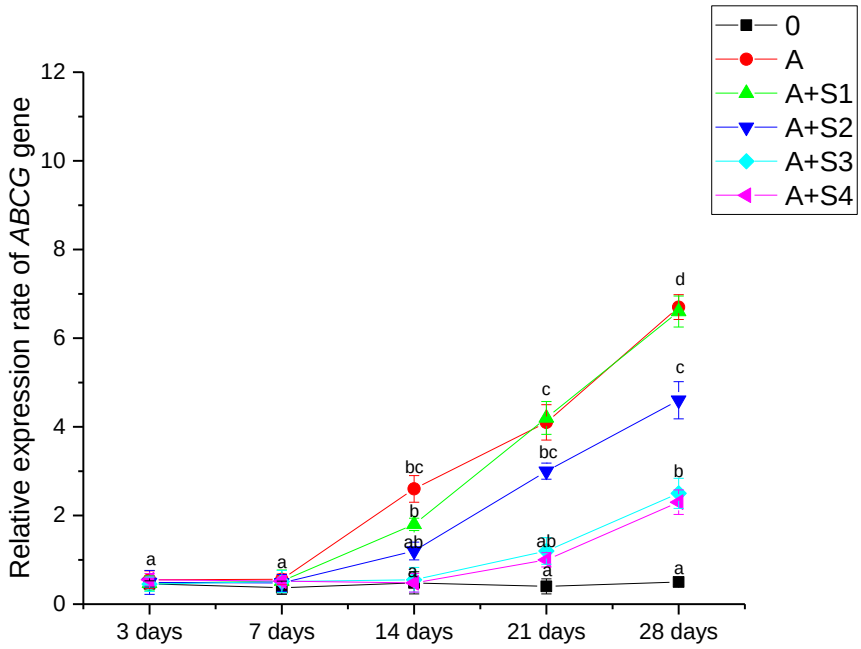
A


0
A
A+S1
A+S2
A+S3
A+S4
Relative expression rate of ABCC gene
3 days
7 days
14 days
21 days
28 days

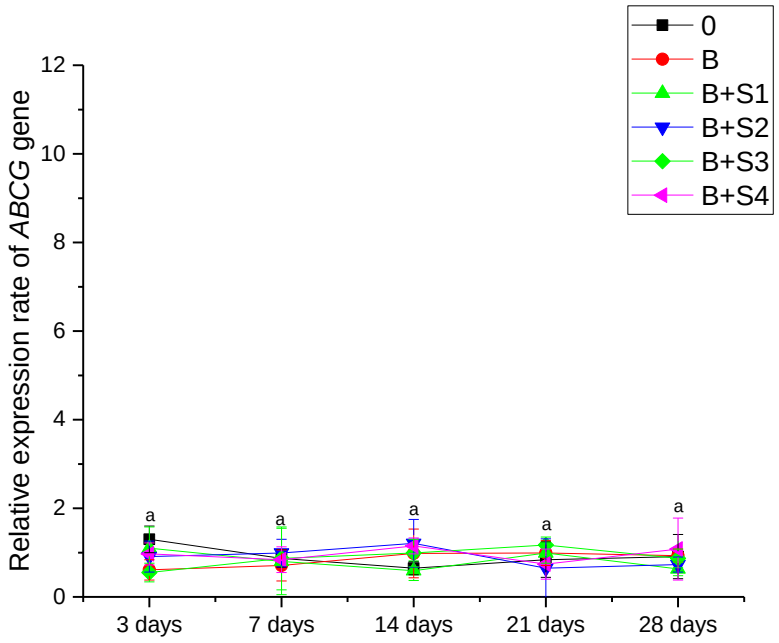
B


0
B
B+S1
B+S2
B+S3
B+S4
Relative expression rate of ABCC gene
3 days
7 days
14 days
21 days
28 days

A


0
A
A+S1
A+S2
A+S3
A+S4
Relative expression rate of ABCG gene
0
2
4
6
8
10
12
3 days
7 days
14 days
21 days
28 days
a
a
bc
b
ab
c
bc
ab
d
c
b
a

B


0
B
B+S1
B+S2
B+S3
B+S4
Relative expression rate of ABCG gene
0
2
4
6
8
10
12
3 days
7 days
14 days
21 days
28 days
a
a
a
a
a

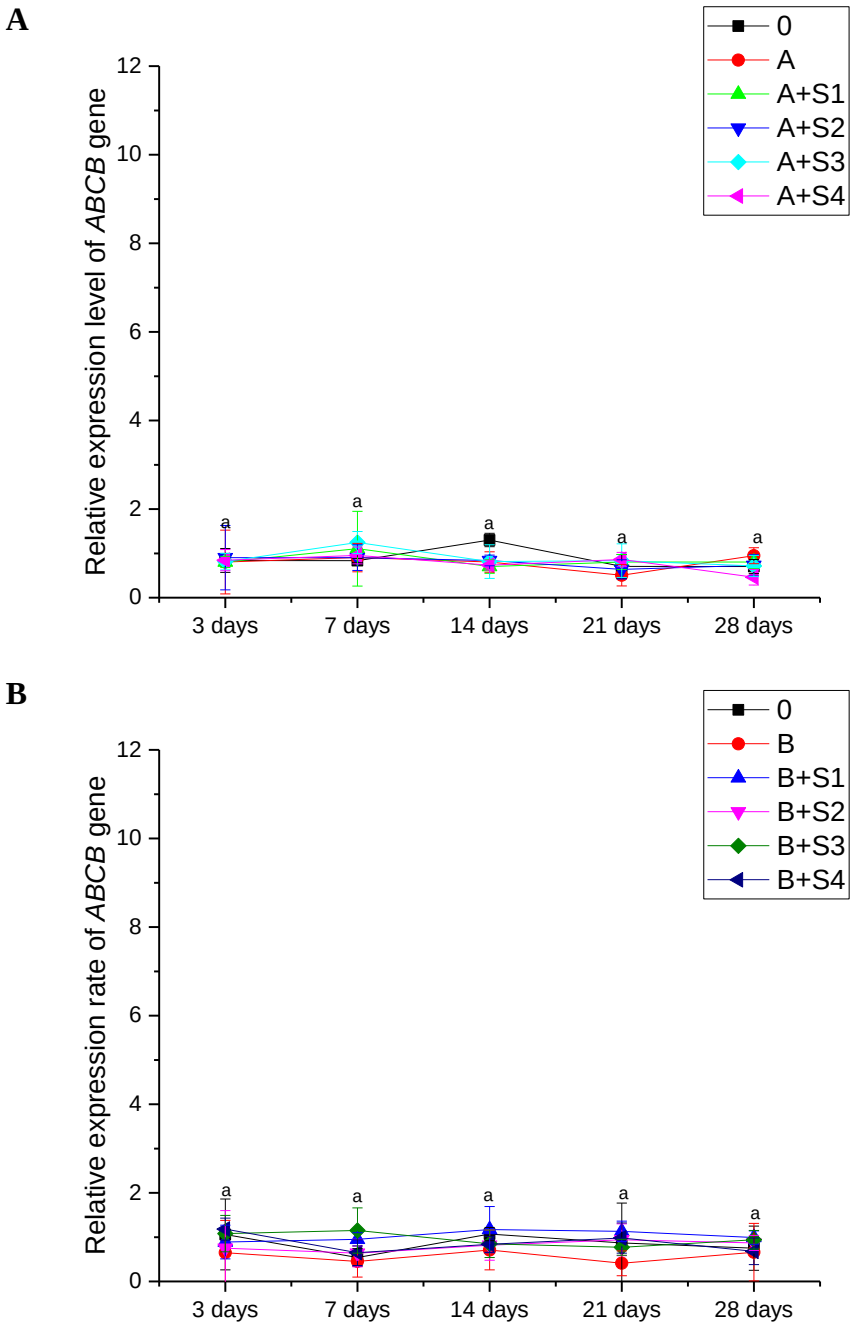


Figure 4. Relative expression level for *ABCC*, *ABCG* and *ABCB* gene in shoots of *S. alba*. [0 – organic, peat soil, A – soil contaminated with HM by industrial activities; S1 – municipal sewage sludge contaminated by HMs; S2 – uncontaminated, municipal sewage sludge; S3 – industrial sewage sludge after purification of mineral water and beverages, S4 - industrial sewage sludge after purification of drinking water with high iron content]. All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey Test ($p < 0.05$). Different letters "a", "b" "c", "d" indicate a significant difference according to two-way ANOVA with post-hoc Tukey's test $p < 0.05$.

4 DISCUSSION

Since plants are sessile organisms that cannot avoid adverse changes to the environment, they had to optimize their metabolism for the stress survival by implementing specific measures (Song et al., 2014). The ATP-binding cassette (ABC) proteins are present in all living kingdoms, and the evidence of ABC transporter's presence in plants came from the research of vacuolar uptake of glutathione conjugates in *Arabidopsis thaliana*. Evolutionary studies suggest that ABC transporters multiplied in plants during their first occurrence on dry land and allowed to adapt to abiotic stressors caused by terrestrial environmental conditions (Fabre et al., 2016). Hence, later studies focused primarily on the question of whether they are involved in the detoxification mechanisms in plants and various xenobiotics. Heavy metals and herbicides have been considered as targets for these processes (Yadav et al., 2014, Song et al., 2014). Several recent studies suggest that ABC transporters can be strictly involved in plants metal transport (Nguyen et al., 2014, Sasse et al., 2016, Do et al., 2018). It was also previously found that they are especially abundant in plants with even more than a hundred ABC proteins identified in *A. thaliana* and *Zea mays* (Toussaint et al., 2017). This extraordinary vast number of ABC transporters is an evolutionary adaptation of plants, strictly related to their settled vegetation, which makes them vulnerable to abiotic stress (Yedav et al., 2014). However, given that there are more than 100 different ABC transporter genes in model plant species like *A. thaliana* and rice, the data on their functional characterization is still limited (Song et al., 2014, Gupta et al., 2019). Moreover, research on the expression of these genes in plants grown in natural/contaminated soil is mostly not available, while in *in vitro* studies are also limited for most agriculturally important species (Nguyen et al., 2018).

One elucidated example of their involvement in plants response to stress was observed in several species of cereals fighting against rust disease (Sucher et al., 2016). Especially type G of ABC transporters had been shown to provide resistance against powdery mildew, leaf rust, and stripe rust in wheat plants (Rinaldo et al., 2017). Interestingly, this class of ABC transporters is only found in plants and fungi and is assumed to evolved recently in comparison to the rest of ABC transporters (Gupta et al., 2019).

The presented experiment was intended to explore the impact of soil contamination with HMs and its supplementation with sewage sludge on *S. alba* during assisted phytoremediation. Altogether, four unique types of SS had been applied into two soil

types. The primary point of the investigation was to evaluate the impact of post-industrial soil and its supplementation with SS on the expression levels of *ABC* transporters in shoots of *S. alba* so as to assess some impacts of complex pollution and soil supplementation with SS.

During the exposure to selected conditions, plants biomass and roots development were severely affected by heavy metal contamination, and the SS supplementation increased plants overall well-being, which is in accordance with recent findings by Dubey et al., 2018. These two parameters (biomass and roots) are also the universal toxicity biomarkers (Mahar et al., 2016). However, although differences are observed, it was not possible to distinguish between applied SS based on the biomass and roots length responses up-until 14 days of exposure.

Overall, lower metal accumulation was reported after application of sewage sludge in comparison to the contaminated soil without any supplementation but again the rate by which it was decreased varied severely between different types of SS applied. Concomitantly, in metal accumulation, similarly to the biomass and roots length, no difference was reported before14 days of exposure. In sorghum and *Arabidopsis thaliana* , a decrease in metal accumulation after soil fertilization with sewage sludge has been observed previously (Mulethya et al., 2017, Nissim et al., 2018). However, other studies reported an increase in HM accumulation following amendment with sewage sludge, especially on soils with more alkaline pH, when the dose of SS caused the pH to decrease (Agnihotri et al., 2019). In the present work, the supplementation with EU recommended SS doses did not affect the soil pH after 28 days of the experiment, but it needs to be noticed that the pH of tested soil was already around 5.5. This acidic pH may explain the high HMs plants accumulation on not fertilized soil, metals being more bioavailable, can be excessively up-taken by plants and can cause cellular damage (Gautan et al., 2017, Mohamed et al., 2018). A slightly acidic pH in post-industrial soil is thus a most crucial factor limiting the remediation of such areas. Moreover, in such soils there is a danger of Cd and Pb migration into underlying soil layers since they are highly mobile in acidic soils. Similar results were highlighted by Alvarenga et al., 2015, and Mohamed et al., 2019.

In the presented study we hypothesized that the expression of ABC transporters types C,

G, and B would be altered by soil supplementation with sewage sludge, thus changing the metal concentration in plants. Results showed a severe overexpression of ABC G and ABC C transporters, accompanied by an accumulation of Cd and Pb from post-industrial soil in shoots of *S. alba*. In previous studies, gene expression of several ABC transporters, such as *At-ATM3, At-PDR8,* or *At-PDR12,* in *Arabidopsis thaliana* were shown to be stimulated by Pb (Kang et al., 2017). Furthermore, transient expression of *A. thaliana* metallothioneins *At-MT2a* and *At-MT3* has been proven to enhance the resistance of *Vicia faba* guard cells to Cd. Similarly, the expression of *Brassica juncea BjMT2* gene in *A. thaliana* seedlings were shown to increase plants tolerance against cadmium (Kang et al., 2011, Grafe et al., 2019). Another study proposed that At-*PDR8* confers cadmium resistance in *A. thaliana* by pumping the metal out of the roots (Agnihotri et al., 2019). However, all above-mentioned studies which have been performed *in vitro,* and are informative from a mechanistic point of view. Therefore, to this day, there have been still no reports concerning the effects of heavy metals from a contaminated field collected soil on ABC gene expression in plants. In our study, the expression of ABC type G and C was significantly ($p < 0.05$) impacted by both, heavy metal contamination and the application of sewage sludge. It is the first research investigating the influence of natural soil and sewage sludge on the levels of expression of genes coding ABC transporters.

Members of the ABC type G subfamily are only found in fungi and plants and were found to play an essential role in various physiological processes (Garroum et al., 2016). To this date, ABCG transporters are believed to take part in the transport of phytohormones, defence against pathogens, transport of alkaloids and heavy metals detoxification. As an example, the transport of cytokinins in *Petunia hybrida* was shown to be directly mediated by ABCG proteins (Wang et al., 2015). At the same time, still very little is known regarding their functions, especially in real environmental conditions and in other plants than *A. thaliana* (Li et al., 2017). Nevertheless, a recent study observed that jasmonates could highly induce the expression of *ABCG* transporters in leaves of *Nicotiana* and *Arabidopsis* species (Xie et al., 2015). Another study found, that exogenous abscisic acid (ABA) stimulated the expression level of poplar *ABCG* transporter which was involved in lead (Pb) uptake, transport and detoxification (Wang et al., 2019). Moreover, in our work, the *ABCC* expression was more pronounced and occurred much faster than the expression of *ABCG* gene. It also differentiated different types of sewage sludge with more significance ($p < 0.05$). At the same time, the second

soil (B) - not contaminated with HMs but fertilized with sludge containing trace amounts of heavy metals also induced the expression of *ABCC* showing that such action can alter plants gene expression and that the *ABCC* is a sensitive biomarker in regards to even slight metal contamination. In the study by Wang et al., 2019, the expression of ABC type G transporter was proven to take part in Pb detoxification and that it can be strongly induced in both roots and shoots of poplar tree. Another similar study by Song et al., 2010 showed that *ABCC* transporters are immensely sensitive to arsenic in yeast. Further studies confirmed that the same transporters also take part in the detoxification of mercury and cadmium in *Saccharomyces cerevisiae*, a fungus unable to produce any glutathione-derived metal chelators known as phytochelatins (Agnihotri et al., 2019). In comparison, plants are well known to produce phytochelatins in response to lead, nickel, cadmium, mercury, and other metals. Such metal-phytochelatin complexes are beneficial since they are more stable than those, including glutathione. Thus, they can be more efficient in metal detoxification. Study by Brunetti et al., 2013 showed, that in *A. thaliana*, metal-phytochelatin complexes are taken to vacuoles via ABC transporters suggesting their direct involvement in metal detoxification. The same study also reported that, from all 8 subfamilies of ABC transporters ABC type C are the most abundant group in plant tonoplast and the *ABCC* expression in *A. thaliana* showed its involvement its overexpression after plants exposure to cadmium (Brunetti et al., 2015). Moreover, several studies in rice indicated the involvement of ABC type G genes to be also altered by drought and salt stress (Nguyen et al., 2018).

In recent years, apart from model plant species, when it comes to phytoremediation and phytoextraction of heavy metals, the use of different poplar trees is gaining more and more attention (Kubatova et al., 2016). Therefore, one study transferred *ABCG* transporter gene from *A. thaliana* into *Populus tomentosa* in order to explore the potential increase of metal uptake. After that, the plants were exposed to cadmium in an *in vitro* study for 24 h and the gene expression as well as the metal uptake were accessed in their tissues. Results showed, that the overexpression of the *ABCG* gene is effective in enhancing the Cd tolerance via decreasing the Cd content in plants, which indicates that the ABCG transporter can work as a cadmium extrusion pump to participate in Cd response. Hence, it could be a reasonable way to create heavy metal tolerant poplar trees via manipulating ABCG transporters (Wang et al., 2019. In our study, we also noticed strong induction of the expression of *ABCG* transporters but after plant exposure to real

soil i.e., contaminated by more than one metal, which can further support such claims of its function as Cd extrusion pump. Although we cannot directly link the overexpression of *ABCG* transporters in *S. alba* to Cd content in soil, it is probable that its overexpression is involved in Cd regulation. Thus, its overexpression could potentially be used to create more tolerant plants species for Cd stress. Moreover, an interesting interaction between metal uptake, oxidative stress and ABC transporters in poplar trees was seen in a study by Benyó et al., 2016, which explored the molecular differences between closely related species of *Populus deltoides* and *Populus canadensis*, which allow them to have a completely different outcome after exposure to metal stress (in this case copper and zinc). *P. deltoides* was shown to be clearly more effective at inducing the expression of several genes involved in metal detoxification such as metallothioneins, glutathione transferases, and ABC transporters, specifically ABC type C. The induction of such expression after metal exposure allowed *P. deltoides* to be more tolerant to metal stress, whereas *P. canadenis* showed significantly higher levels of cellular oxidative damage. Similarly to our study, in *P. deltiides*, the induction of expression of *ABCC* genes occurred relatively quickly and was significant after 7 days post-exposure to, in this case, copper and zinc. Furthermore, the described study had shown that both poplar species are efficient in stress acclimatization, but with different molecular bases behind it, which can be used in phytoremediation of metal-contaminated sites – species and specific lines with higher expression of ABC type C accumulated less metals (but still accumulated and sequestered them in the vacuoles which makes them suitable for phytoextraction). They sustained less oxidative damage, whereas plants with lower expression of ABC type C transporter could accumulate even more metal ions. However, it resulted in severe cellular damage, which could lead to process failure as plants won't survive in the environment (Benyo et al., 2016). Another recent study explored the transcriptomic changes in rice and the role of gene expression on the excess accumulation of Cd. Overexpression of a couple of ABC transporters in rice exposed to cadmium was shown to increase Cd sequestration during Cd stress (Fu et al., 2019) which we also saw on post-industrial soil contaminated mainly by cadmium and lead. At the same time, a transcriptomic study on *A. thaliana* also reported ABC type C transporters as cadmium extrusion pump conferring plants resistance to cadmium and lead (Parc et al., 2012). Moreover, ABC type C transporters had been identified as a significant phytochelatin-heavy metal complex transporter. Such HM-PC complexes are sequestered in the vacuoles following transport mediation by ABC transporters (Grafe et al., 2019). Similar tendencies were also confirmed in wheat, where

13 *ABC* transporter genes had been shown to be differently expressed after Cd exposure, changing Cd uptake, transport, and sequestration. However, it needs to remembered, that in addition to the ABC transporters, vacuolar compartmentalization of toxic heavy metals relies mainly on tonoplast energization and associated establishment of proton motive force caused by the H+ translocating activities of V-ATPase and transporters in the tonoplast (Ding et al., 2011, Wang et al., 2016). In total, ABC C and G transporters had been proven to take part in various cellular processes, including the osmotic homeostasis, nutrient uptake, the transport of hormones, pathogen resistance, and tolerance to heavy metals (Tang et al., 2014). For example, in wheat, which has a genome size greater than those of *Arabidopsis*, rice, and maize, ABC transporters had also been shown to play a crucial role in plants resistance to rust, glutathione-mediated detoxification pathway, grain formation, ripening, and enhancement of the resistance against fusarium head blight (Shibata et al., 2016, Rinaldo et al., 2017, Wang et al., 2018). Hence, further functional characterization of ABC transporters in plants other than model and the real-life scenario could bring new and vital knowledge for plant improvement not exclusively for phytoremediation purposes but also for the development of crops with improved stress resistance via molecular breeding and transgenic approaches.

5 CONCLUSIONS

The presented study provides evidence of interactions between the expression of ABC transporters in shoots of *S. alba* and soil contamination with HMs as well as between the expression of ABC transporters and soil supplementation with sewage sludge. We demonstrated that in order to truly evaluate the applicability of chosen sewage sludge for soil application, long term studies or strictly sensitive biomarkers are necessary. The expression of ABCC was the fastest to differentiate the influence of low/high-quality sewage sludge on plants and therefore, can be used as a biomarker for planning the assisted phytoremediation with waste products. Moreover, further functional characterisation of genes involved with metal transport and detoxification can be used to choose plant species for a specific remediation purpose and to create new and enhanced plants via selective breeding of lines and species able to store higher concentration of heavy metals or sustain less cellular damage during the exposure. Soil supplementation with sewage sludges which did not contained heavy metals turned out to be beneficial for plants development and overall phytoremediation efficiency by decreasing the metal concentration in plants tissues to a point in which the plants could actually survive and increase their biomass, ensuring the long-time success. However, the supplementation with sludges that contained trace metals did not provide any beneficial effects for plants survival. Hence, study provided insights into plants stress markers that can be used to assure safe application of sewage sludge into soils which could become a routine in the future as molecular analyses slowly are becoming cheaper and more broadly available. Moreover, further exploration of the functional characterization of genes involved in metal detoxification can be used for the identification species that are better equipped to acclimatize in real-life scenarios (for example on soil contaminated by anthropogenic sources).

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CHAPTER IV

Modeling of the assisted phytoremediation of metal contaminated soils

Despite still being a novel technology, phytoremediation has already entered the stage of modeling the biological and chemical processes for this purpose. There are several models available, with more currently being in development, that can be applied for this purpose. Such a growing variety of models is mostly a result of different outcomes that can be modeled. For example, we can model the phytoremediation by heavy metal accumulation in a selected part of the plant or by its overall distribution among all plant compartments. Thus, the main aim of the first part of Chapter IV was to present and critically compare available models for HMs contaminated soil phytoremediation to point out their main advantages, limitations, to explore their applicability in given circumstances.

In the second part, the data obtained as a result of the previous two experiments were used to model and optimize the removal of cadmium from contaminated post-industrial soil via *Sinapis alba* L. by comparing two modeling approaches: Response Surface Methodology (RSM) and Artificial Neural Networks (ANN). Thus model could be used for the prediction of cadmium removal during assisted phytoremediation with sewage sludge. Moreover, such an approach could also be used to determine the dose of sewage sludge that will ensure the highest process efficiency.

In this part, the results are presented as the two following publications:

1. Jaskulak, M., Grobelak, A., & Vandenbulcke, F. (2020). Modelling assisted phytoremediation of soils contaminated with heavy metals – main opportunities, limitations, decision making and future prospects. Chemosphere, 126196. doi:10.1016/j.chemosphere.2020.126196

2. Jaskulak, M., Grobelak, A., Vandenbulcke, F. (2020). Modeling and optimizing the removal of cadmium by *Sinapis alba* L. from contaminated soil via Response Surface Methodology and Artificial Neural Networks during assisted phytoremediation with sewage sludge. International Journal of Phytoremediation, 22(12), 1321-1330. doi:10.1080/15226514.2020.1768513

Modelling assisted phytoremediation of soils contaminated with heavy metals – main opportunities, limitations, decision making and future prospects*

Marta Jaskulak[1,2], Anna Grobelak[1], Franck Vandenbulcke[2]

[1]*Institute of Environmental Engineering, Faculty of Infrastructure and Environment, Czestochowa University of Technology, Czestochowa, Poland;*

[2]*University of Lille, Laboratory of civil engineering and environment (LGCgE), Environmental Axis, F-59650 Villeneuve d'Ascq, France;*

Abstract:

The heavy metals (HMs) soils contamination is a growing concern since HMs are not biodegradable and can accumulate in all living organisms causing a threat to plants and animals, including humans. Phytoremediation is a cost-efficient technology that uses plants to remove, transform or detoxify contaminants. In recent years, phytoremediation is entering the stage of large-scale modelling via various mathematical models. Such models can be useful tools to further our understanding and predicting of the processes that influence the efficiency of phytoremediation and to precisely plan such actions on a large-scale. When dealing with extremely complicated and challenging variables like the interactions between the climate, soil and plants, modelling before starting an operation can significantly reduce the time and cost of such process by granting us an accurate prediction of possible outcomes. Research on the applicability of different modelling approaches is ongoing and presented work compares and discusses available models in order to point out their specific strengths and weaknesses in given scenarios. The main aim of this paper is to critically evaluate the main advantages and limitations of available models for large-scale phytoremediation including, among others, the Decision Support System (DSS), Response Surface Methodology (RSM), BALANS, PLANTIX and various regression models. Study compares their applicability and highlight existing gaps in current knowledge with a special reference to improving the efficiency of large-scale phytoremediation of sites contaminated with heavy-metals. The presented work can serve as a useful tool when choosing the most suitable model for the phytoremediation of contaminated sites.

KEYWORDS: phytoremediation, soil contamination, heavy metals, dynamic modelling, Response Surface Methodology (RSM), regression models

Highlights

- Comparison of the main advantages and limitations of models for phytoremediation
- Dynamic modelling is necessary for simulation of real-life scenarios
- Many existing models lack proper validation to be used in large-scale remediation
- Mathematical modelling can predict remediation feasibility and financial viability

1 INTRODUCTION

The rapid increase in soil contamination with heavy metals in recent decades causes a major threat to the environment and human health (Muthusaravanan et al., 2018). The removal of heavy metals (HMs) from soils requires effective technological approaches and cost-efficient solutions, especially since HMs are considered a unique group of pollutants and they cannot be broken down into safe, non-toxic forms (Hou et al., 2017, Cameselle et al., 2019). The areas which have been subjected to soil contamination by HMs have dramatically risen in past years, mostly as a result of industrialization and the discharge of waste and wastewater. Among them, cadmium, lead, nickel and mercury were shown to appear as the most frequent pollutants in soil samples throughout the world (Mahar et al., 2016, Oconnor et al., 2019, Jaskulak and Grobelak, 2019b). Due to the growing health concerns associated with heavy metals and their broad distribution in soils and water, more attention has been directed to identify the sources of such pollution and to remediate contaminated sites (Duan et al., 2017). The use of physical and/or chemical methods to deal with heavy metal contaminated soils includes the use of ion exchange, reverse osmosis, chemical reduction, precipitation and evaporation (Gong et al., 2018). Modern techniques that allow for a reduction in the total concentrations of hazardous substances include processes that promote the desorption and subsequent removal in the liquid phase. Such actions include soil washing techniques, as well as electrokinetic methods and heat treatments (Habibul et al., 2019). All of them can be successfully applied to the process of decontamination, but they require a lot of external resources and in most cases are too expensive for large-scale use (Sharma et al., 2014). Due to those reasons, in recent decades, more and more attention has been given to phytoremediation processes in which plants absorb and transform contaminants in order to detoxify the site and clean-up polluted environments (Yadava et al., 2018). It is worth mentioning that such methods can also be applied to other contaminants - not solely to heavy metals but also to pesticides, explosives and crude oil. Overall, phytoremediation can take advantage of natural processes and it requires less equipment and labor than other technologies (Burges et al., 2017, Jaskulak et al., 2019a). The contamination of waterbodies is also currently a significant challenge especially in emerging economies, resulting in over a billion people affected by water contamination. Phytoremediation of waterbodies refers to the application of aquatic plants for the remediation of pollutants in water (Ekperusi et al., 2019). Aquatic species like water hyacinth (*Eichhornia crassipes*) and duckweed

(*Lemna minor*) had been shown to be extraordinarily adapted for remediation of most contaminants from shallow waters and wastewaters (Oporto et al., 2006, Neag et al., 2018, Ergen and Tunca, 2018). In parallel to identifying the best aquatic plants species for remediation purpose, several studies focused on the development of models to optimize the phytoremediation of specific contaminants encountered in waterbodies and wastewaters. Such approach allows not only to predict the overall efficiency of the process but also to formulate the most optimal conditions/procedures such as the number of plants to be used and their optimal placement to ensure the best possible outcome (Ergen and Tunca, 2018, Alvarez-Vazquez et al., 2019).

The implementation of mathematical models in environmental science can significantly help to evaluate different scenarios in order to make a more precision decision and achieve the best effect for researched areas. Besides, it can also avoid human biases and errors, especially when discussing such complex systems as plant-soil interactions (Chirakkara et al., 2016). Thus, several mathematical models using different approaches have been used in the past twenty years to understand plant-soil interactions, including their possible application for planning large-scale phytoremediation operations. As a result, various mathematical algorithms have been implemented to improve the efficiency assessment of various processes in the field of environmental sciences (Luo et al., 2016, Yadava et al., 2018).

Phytoremediation, despite still being a novel technology, has already entered the stage of modelling of biological and chemical processes for this purpose. Currently, there are several models available, with more currently in development, that can be applied for this purpose (Urbaniak et al., 2016). Such a continually growing variety of models is mostly as a result of different outcomes that can be modeled, for example, we can model the phytoremediation by heavy metal accumulation in a selected part of the plant or by its overall distribution among all plant compartments (Hasanuzzaman et al., 2017). Thus, the main aim of this review is to present and critically compare available models for HMs contaminated soil phytoremediation in order to point out their main advantages, limitations, to explore their applicability in given circumstances.

2 PHYTOREMEDIATION MODELS – CLASSIFICATION AND DECISION MAKING

One of the issue associated with the phytoremediation of sites contaminated by high concentrations of heavy metals, and that have been left untreated for many years, is not solely the toxicity of such substances, but also the consistently decreasing soil quality due to the lack of vegetation, which increases soil phytotoxicity (Aleixandre-Benavent et al., 2017, Kovacs et al., 2017). One of the most commonly used technologies to battle such problem is so-called 'assisted' phytoremediation, referring to the application of various soil amendments that can diminish the occurring phytotoxic problem by improving the physical and chemical characteristics of soil, increasing soil buffering and its water holding capacity, as well as the availability of nutrients (Anning et al., 2018). At the same time, in order to keep the phytoremediation the lowest cost consumable on a large-scale, discussed actions often include the supplementation of soil with waste products such as sewage sludge or composts (Alaribe et al., 2015). Most of the phytoremediation processes currently carried out in the world are primarily aimed to stop or minimize soil erosion, desertification and the transfer of toxic substances toward the reservoirs of groundwaters. Hence, such actions act mostly on the mobile and bioavailable forms of a given contaminant (Fellet et al., 2011). In addition, it needs to be taken into consideration that phytoremediation technologies are extremely site-specific due to various contaminants that can be evenly mixed together on a given site, as well as due to a number of soil properties which can unavoidably affect plant-soil interactions (Kovacs et al., 2017). Thus, there are several questions that have to discussed and answered during the decision making stage of the phytoremediation process that include among others: 1) the phytotoxicity of any given site to the chosen plant species, 2) metal concentration and its bioavailability within the top layer in which roots can grow, 3) the rate of metal uptake by roots, 4) the rate of metal translocation via xylem to shoots, 5) cellular tolerance of given plants to metals and other abiotic stressors occurring on the site (such as a low content of organic carbon or a low capacity to hold water due to lack of vegetation) (Figure 1) (Polasky et al., 2011, Oconnor et al., 2019).

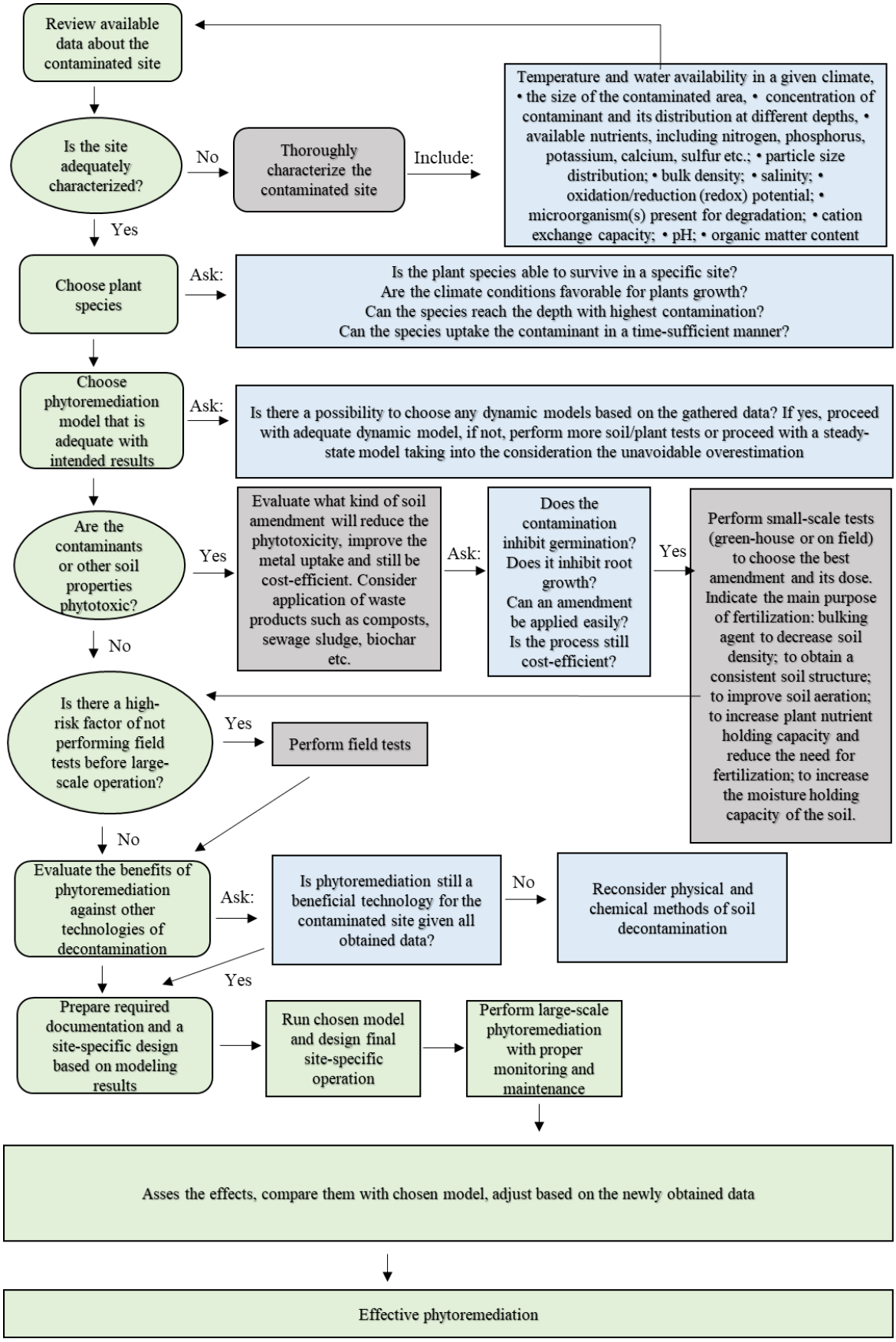
Review available data about the contaminated site
Is the site adequately characterized?
No
Thoroughly characterize the contaminated site
Include:
Temperature and water availability in a given climate, • the size of the contaminated area, • concentration of contaminant and its distribution at different depths, • available nutrients, including nitrogen, phosphorus, potassium, calcium, sulfur etc.; • particle size distribution; • bulk density; • salinity; • oxidation/reduction (redox) potential; • microorganism(s) present for degradation; • cation exchange capacity; • pH; • organic matter content
Yes
Choose plant species
Ask:
Is the plant species able to survive in a specific site?
Are the climate conditions favorable for plants growth?
Can the species reach the depth with highest contamination?
Can the species uptake the contaminant in a time-sufficient manner?
Choose phytoremediation model that is adequate with intended results
Ask:
Is there a possibility to choose any dynamic models based on the gathered data? If yes, proceed with adequate dynamic model, if not, perform more soil/plant tests or proceed with a steady-state model taking into the consideration the unavoidable overestimation
Are the contaminants or other soil properties phytotoxic?
Yes
Evaluate what kind of soil amendment will reduce the phytotoxicity, improve the metal uptake and still be cost-efficient. Consider application of waste products such as composts, sewage sludge, biochar etc.
Ask:
Does the contamination inhibit germination? Does it inhibit root growth? Can an amendment be applied easily? Is the process still cost-efficient?
Yes
Perform small-scale tests (green-house or on field) to choose the best amendment and its dose. Indicate the main purpose of fertilization: bulking agent to decrease soil density; to obtain a consistent soil structure; to improve soil aeration; to increase plant nutrient holding capacity and reduce the need for fertilization; to increase the moisture holding capacity of the soil.
No
Is there a high-risk factor of not performing field tests before large-scale operation?
Yes
Perform field tests
No
Evaluate the benefits of phytoremediation against other technologies of decontamination
Ask:
Is phytoremediation still a beneficial technology for the contaminated site given all obtained data?
No
Reconsider physical and chemical methods of soil decontamination
Yes
Prepare required documentation and a site-specific design based on modeling results
Run chosen model and design final site-specific operation
Perform large-scale phytoremediation with proper monitoring and maintenance
Asses the effects, compare them with chosen model, adjust based on the newly obtained data
Effective phytoremediation

Figure 1. Scheme of phytoremediation decision making (boxes shown in green indicate the general stages of phytoremediation planning, boxes shown in gray indicate additional steps that can provide better assumptions for process efficiency, boxes shown in blue indicate crucial questions that can help with planning a large-scale operation).

The model selection for phytoremediation planning depends mostly on the objectives of the operator, but also on the main advantages and drawbacks of such models and overall the comprehension level of given results (Yadava et al., 2018). Modelling phytoremediation is a tremendously specific process mostly due to some unavoidable assumptions as well as some limitations, and the complicated nature of interactions. Therefore, specific models differ in their level of sophistication and extent of possible applicability (Baltrėnaitė et al., 2016). Different models can also be chosen by different stakeholders, for example, an environmental researcher can choose a different model than the environmental officer or the owner of the contaminated site, depending on their experience and primary objectives. As an example, the researcher can be focused on the concentration of a selected chemical in plant tissues and use a model that involves mechanisms of the uptake, translocation and final accumulation of such a substance. A model operator from the food industry would be mostly focused on models that give a detailed forecast of contaminant concentration in plants parts used as a food source which can predict the risk of a selected contaminant entering the food chain. An environmental protection officer would look for models that could assess the ecological risks posed by the contaminated site, whereas the owner of such a site would preferably use models that can predict the time of decontamination, as well as its total cost.

Currently, models for the assessment of chemical uptake into plants are widely applied tools for human exposure risk assessment, particularly in designing and testing new pesticides in addition to being used as a risk assessment for soil and air pollution. Overall, a variety of models had been created in recent years in order to predict element uptake from air or soil into plants (Table 1). They can be divided into steady-state or dynamic models (Figure 2). The first group provides a forecast for an infinite period of time while the second group is more useful in assuming the timeframe needed before the modeled variables will reach the target values (Baltrėnaitė et al., 2014). Depending on the main objectives, the stakeholderhas to choose a model with an appropriate level of details suitable for planning such a process. Steady-state models are the most commonly applied,

mainly due to their simplicity since they require only a few data points (Chen et al., 2012). However, most of the time, the emission patterns are not steady, for example during pesticide application or soil supplementation with organic fertilizers including compost, manure and sewage sludge which is a common practice during assisted phytoremediation. Hence, in these scenarios, steady-state approaches are not appropriate and they require the use of more complex, dynamic simulations. Moreover, almost all environmental conditions can be characterized as dynamic processes that change in cycles: daily (day/night, temperature) and annually (temperature, humidity) (Figure 3) (Eid et al., 2018). At the same time, it is worth mentioning that the major disadvantage of complex models is that the input data is often incomplete and the final data can be a rough estimation. Therefore, simpler models have the advantage of a smaller need for preliminary data input, but in that case, proper modelling requires a more theoretical basis including a detailed understanding of processes occurring in the selected site (Janani et al., 2019). Moreover, in order to evaluate the phytoremediation process of a specific regional site, most operators choose relatively simple models with a basic description of processes occurring on such site. Such steady-state models of differential equation systems have an advantage in being much more straightforward and require substantially less effort, programming and smaller sets of data (Kumar et al., 2019). At the same time, it is important to remember that there are always consequences to selected simplifications such as specific processes not being taken into consideration (for example the pH change, changes in humidity, content of nutrients or lack thereof, etc.). Thus, to avoid such consequences and gain more insight into our model choice, it is often recommended to perform modelling with a couple of different models using various parameters and compare the given results. By doing this, we can also gain valuable insights into the differences in the sensitivity of selected parameters and the overall relevance of different processes in the systems (Pandey et al., 2015). Thus, in recent years, there has been an ongoing dispute regarding whether steady-state models are sufficient enough or if only dynamic models should be further developed and implemented on contaminated sites (Janani et al., 2019). For example, Undeman et al., 2009, used the dynamic behavior of elements in the soil-plant-air system and found, that during the rapid growth of plants, the steady-state approach can be sufficient and valid, at least under certain conditions. However, that study did not take into consideration the ripening of plants and thus, a dynamic decrease in their growth after the first, fast growth period (Undeman et al., 2009).

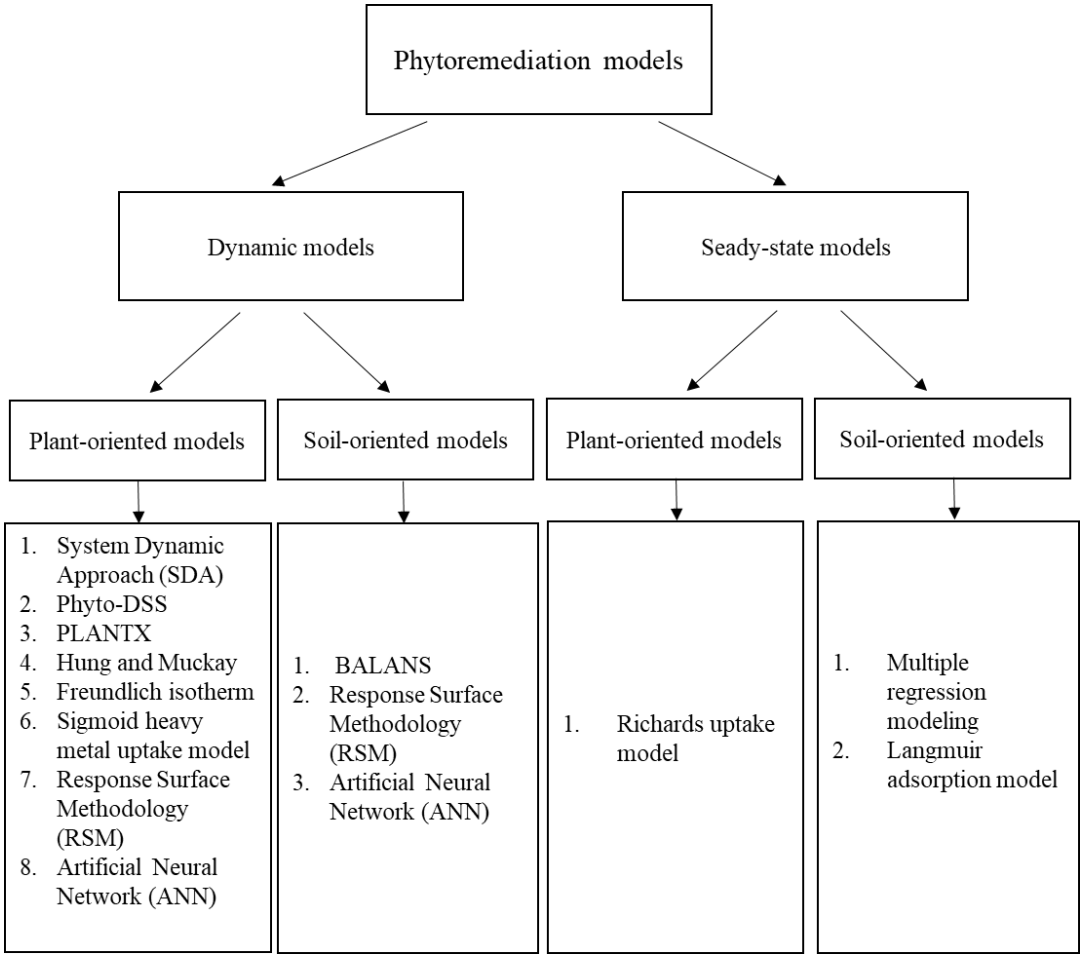

Figure 2. Basic classification of models applicable for phytoremediation with examples of the most commonly used models for that purpose.

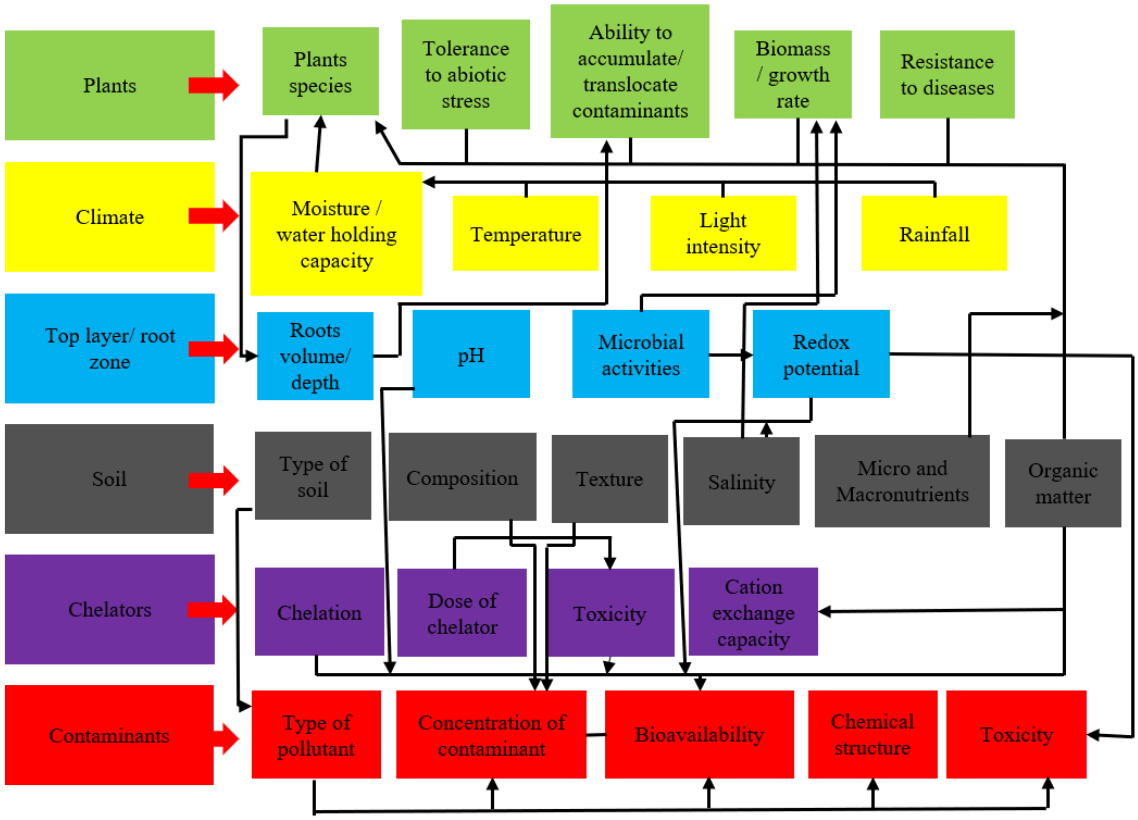

Figure 3. Interactions between factors influencing phytoremediation that should be taken under consideration before choosing a specific model for any given scenario.

Table 1. Comparison between different types of input data in dynamic and steady-state models, all aimed at the determination of the reduction of contaminant content in soil via contaminant concentration in plant biomass after a specific time.

Name	Average input	Reference
PLANTX	• continuous and/or pulse input into any compartment, • degradation, ageing, leaching, run-off and plant uptake, resulting in loss from soil, • uptake of the transpiration water into roots optionally: diffusion into roots, growth dilution, degradation and metabolism in roots, stem, leaves and fruits, translocation from roots to the stem, and from the stem to leaves and fruits with the transpiration stream, loss from leaves and gain for fruits by phloem flux, loss from stem, leaves and fruits to air, gaseous and particle deposition from air to soil, stem, leaves, fruits.	Ouyang et al., 2002
Phyto-DSS	• metal concentration in soil, • soil density, • plants roots distribution, • soluble concentration of contaminant in roots, • soluble concentration of contaminant in soil, • temperature, • moisture, • pH, • microbial activity, • concentration of competing ions,	Cano-Reséndiz et al., 2011

	• soil bulk density, • root density, • concentration of selected component in soil solution,	
BALANS	• number of metals i simulated in the model, • the initial concentration of metal in soil, • soil density, • the depth of the ploughed soil, • the content of metal in soil before the amendment, • the mass of soil amendment, • concentration of metal in amendment, • the annual yield of plants, • the portion of plants annually removed from the field, • the concentration of metal in plants, • the mass of metal removed with yield,	Baltrėnaitė et al., 2016
Freundlich model	• concentration of contaminant in soil, • sorption capacity, • strength of sorption,	Yaashikaa et al., 2019
MINTEQA2 /PRODEFA2	• concentration of dissolved metal, • concentration of total metal,	Titaha et al., 2018
Hung and Mackay	• molar mass of a contaminant, • contaminant concentration in soil solution, • total concentration of contaminant, • soil density,	Baltrėnaitė et al., 2007

CTSPAC (coupled transport of water, heat and solutes in the soil –plant – atmosphere continuum)	• soil type, • soil porosity, • hydraulic conductivity, • thermal conductivity of sand, • thermal conductivity of clay, • thermal conductivity of water, • thermal conductivity of organic matter, • temperature at groundwater level, • initial soil water content, • initial soil temperature, • initial concentration of contaminant, • continuous and/or pulse input into any compartment, • degradation, ageing, leaching, run-off and plant uptake, resulting in loss from soil, • uptake into roots with the transpiration water.	Ouyang et al., 2002
SDA (System dynamic approach)	• the water flow rates between selected plant compartments (root/stem/leaves/fruits), • the contact areas between compartments, • the conductance between compartments, • the water potentials in the compartments, • the hydrostatic pressure or pressure potential, • the diurnal factor characterizing daily variations of hydrostatic potential, • the osmotic potential, • the water density, • the gravitational acceleration, • the height of the roots, stems, or leaves referenced to a datum,	Zang et al., 2010

	• the rate of contaminant uptake, • the contaminant concentration, • the reflection coefficients (the ease with which given contaminant crosses the membrane between plant compartments), • the transformation loss of contaminant, • the fraction of contaminant in vapor phase.	
Response surface methodology	• the initial concentration of contaminant in soil, • the final concentration of contaminant in plant compartments, • the amount of added soil amendment, • the concentration of contaminant in soil amendment, • time of plants growth.	Mohammadi et al., 2019
Artificial neural networks (ANNs)	• the concentration of contaminant in soil, • sampling time, • aeration rate.	Titaha et al., 2018
Langmuir adsorption model	• The amount of absorbed ions by sorbent, • The required metal concentration for the formation of one layer, • The initial and remaining concentrations of dissolved metals,	Yaashikaa et al., 2019

3 BASIC ELEMENT UPTAKE MODELLING – THE MAIN OPPORTUNITIES AND LIMITATIONS

The efficiency of metal phytoextraction relies on the plant species and the bioavailability of contaminants in the soils (Kuppusamy et al., 2017). Plants that are able to tolerate high concentrations of heavy metals and accumulate them (hyperaccumulators) are considered suitable for phytoextraction (Dinh et al., 2018). The bioavailability of the contaminant, namely the mobile fractions in soils that are available for uptake, are primarily conditioned by specific soil properties which can determine the distribution of metals between the solid and liquid soil phases. In general, the soil pH, the content of clay and organic matter regulate the uptake of heavy metals to plants tissues via adsorption-desorption mechanisms (Hechmi et al., 2013, Kuppusamy et al., 2017). The phytotoxicity of a given metal or mixed contamination also has to be taken into consideration before starting any restoration processes. Thus, feasibility tests should be based not only on the experimental data such as plants growth rate and accumulation of metals but also provide a mathematical uptake model that will account for changing conditions during the process (Chen et al., 2017). The whole purpose of modelling is to predict the time and the efficiency of toxic substance transfer from the soil into plants (Reddy et al., 2017). Currently, the evaluation of phytoextraction efficiency using feasibility tests is generally based on the ratio of HM in plant above-ground tissues to the total concentration in soil (C_{shoot}/C_{soil}). Such a ratio is often called the 'phytoextraction coefficient' and is used to evaluate the effectiveness of plants in removing metals from the soil in a short period of time. However, it does not take into account the metal availability in soil, the phytotoxicity of a given scenario, nor the reduced growth of the plants after initial germination and adolescence (Maqbool et al., 2018). Therefore, in recent years, several models of plant uptake had been developed, most of which are based on more complex equations describing the behavior of both inorganic and organic contaminants in soil and plants tissues (Eid et al., 2018). The specific aims of such models vary; some of them are intended for determination of potential, hazards to human health, others are made to address the theoretical aspects of soil remediation or to assess the interactions between the contaminants and the environment (Paul et al., 2017). For practical and large-scale phytoremediation, it is commonly preferred to use a simple model that can potentially be applied immediately and that can swiftly provide the essential data in order to plan field remediation. This is the reason why it usually ends up being a simple, linear model

(Shafaghat et al., 2012). However, such an approach leads to a significant overestimation in the efficiency of the treatment, a factor that can only add more uncertainties to an already intricate design, which unavoidably leads to a higher risk of not achieving the desired effects, especially for more complex cases of contamination (Oconnor et al., 2019). On the contrary, dynamic, non-linear models can offer much more reliable results at a cost for their higher complexity (Ma et al., 2016). Several studies showed that, the dynamic uptake models offer several performance advantages for acute exposure in continually changing environmental conditions. Usually, such models use numerical iterations in order to solve differential equations. This iteration requires short time steps to avoid errors and to meet stiff metrics. Most numerical models are implemented in FROTRAN or C although they can also be used in spreadsheet formats which are easier to use, learn and distribute to different specialist interested in phytoremediation (Baltrėnaitė et al., 2016, Paul et al., 2017).

In coming sections, we individually discuss the most commonly used models for phytoremediation, including their functions, advantages and limitations for any given circumstances. Examples of research that successfully implemented selected models as feasibility tests during phytoremediation planning can be found in Table 2. The specific findings and the comparison between research found in Table 2 are also discussed in a more detailed manner in the continuation of the manuscript.

Table 2. Examples of successful application of selected models for assessment of phytoremediation efficiency

Model	Plant species	Contaminant	Soil type/location/additional information regarding contamination	Soil amendment	Reference
Multiple regression modelling	*Pistia stratiotes*	Mix of heavy metals: Mn, Cd, Cu, Fe, Pb, and Zn	Paper mill effluent contaminated with several HMs	None	Kumar et al., 2019
Hung and Mackay	*Pinus sylvestris, Betula pendula, Alnus glutinosa*	Cu, Ni, Zn, Mn and Pb	Experimental site, field tests on natural soil contaminated with HMs	Industrial wastewater sludge	Baltrënaitë et al., 2007
Freundlich	*Brassica pekinensis* L.	Zn Pb	Natural agricultural soil contaminated with Zn and Pb due to past mining and smelting operations	None	Zhang et al 2016
CTSPAC	Hybrid: *Populus deltoiddes* × *Populus nigra,*	1,4-dioxane	Sandy soil, artificially spiked	None	Ouyang et al., 2002
Sigmoid heavy metal uptake model	*Helianthus annuus*	Cd Ni Pb Zn	Artificial contamination via spiking of natural soil 600 mg/kg for Cd, 150 mg/kg for Ni, 2,000 mg/kg for Pb, and 99 500 mg/kg for Zn, respectively.		Zhao et al., 2019

Comparison between several empirical models	*Raphanus sativa*	Pb	Agricultural soil contaminated by Pb due to smelting activities	None	Zhang et al., 2018
Langmuir adsorption model	*Sorghum bicolor*	Cr	Artificially spiked soil with (25, 50, 100, and 150 mg/L of Cr(VI))	bacteria *Pannonibacter phragmetitus*	Yaashikaa et al., 2019
Freundlich model	*Sorghum bicolor*	Cr	Artificially spiked soil with (25, 50, 100, and 150 mg/L of Cr(VI))	bacteria *Pannonibacter phragmetitus*	Yaashikaa et al., 2019
Modified Freundlich model	*Brassica juncea* *Lupinus albus* *Helianthus annuus*	As	Industrial site located in Tuscany, Italy. The site had been used for various industrial productions and contaminated with from various inorganic contaminants	Dipotassium phosphate	Pedron et al., 2017
Response Surface Methodology (RSM) and an Artificial Neural Network (ANN)	*Sorgum bicolor*	Pyrene	Natural soil artificially spiked with pyrene (150, 225, and 300 mg kg-1)	indole acetic acid (IAA) and bacterial species: *Pseudomonas aeruginosa*	Mohammadi et al., 2019
Response Surface Methodology (RSM) and an Artificial Neural Network (ANN)	*Ludwigia octovalvis*	As	Natural soil spiked with arsenic	None	Titaha et al., 2018
Response surface methodology (RSM) and	*Paspalum scrobiculatum* L.	Petroleum hydrocarbon (TPH)	Garden soil artificially spiked by diesel	None	Sanusi et al., 2016

artificial neural networks (ANNs)					
Response surface methodology	*Phragmites australis*	Fe, Mn, Cu, and Ni	Plants grown on contaminated urban waste leachate	None	Mojiri et al., 2015
Artificial neural networks	*Chrysopogon zizanioides (L.)*	Chemical Oxygen Demand (COD) and biodegradable organic matter (BOD) removal from palm oil mill secondary effluent (POMSE)	POMSE samples were collected from a nearby palm oil mill in Labu, Negeri Sembilan, Malaysia	None	Darajeh et al., 2016
Artificial neural networks	*Brassica napus*	Cerium oxide nanoparticles and Cd	Artificially spiked soil	None	Rossi et al., 2019
Response surface methodology (RSM) and artificial neural networks (ANNs)	*Ophiopogon japonicus*	Zn	Artificially spiked soil	EDTA	Janani et al., 2019

Numerical simulation using HYDRUS-2D based on Richards uptake model	Willow hybrid Salix × smithiana Willd.	Pb, Cd, Zn, Cu	Soil contaminated by HMs due to past smelting activities	None	Trakal et al., 2013
Response surface methodology (RSM)	*Melastoma malabathricum L.*	Pb	Artificially spiked soil	None	Selamat et al., 2017
Combined solubility-uptake model	*Pteris vittata*	As	Twenty-one UK soils contaminated with arsenic (As) from a wide range of natural and anthropogenic sources.		Shelmerdine et al., 2009
Multiple regression modelling	*Cucumis sativus* L.	Several HMs	Agricultural soil supplemented with sewage sludge	Municipal sewage sludge	Eid et al., 2018
System Dynamic Approach (SDA)	*Oryza sativa*	Cd Pb Cu Zn	Dongguan, Shangyu County, located at the northern part of Zhejiang Province in southeastern China. Rice production area near an abandoned lead–zinc mine	6 different extracting agents: Mehlich 1, Mehlich 3, EDTA (ethylenediaminetetraacetic acid), DTPA–TEA (diethylenetriaminepentaacetic acid–triethanolamine), ammonium acetate	Zang et al., 2010

				(NH4OAc), and calcium chloride ($CaCl_2$)	
System Dynamic Approach (SDA)	*Populus fastigiata*	2,4,6-trinitrotoluene (TNT)	Artificially spiked soil	None	Ouyang et al., 2007
Phyto-DSS	*Aloe vera* and poplar species	Mix of heavy metals with highest concentrations of Ag, Hg and Sb	La Cata mine tailings in Guanajuato, México	None	Cano-Reséndiz et al., 2011
Phyto-DSS	Hybrids: *Populus deltoides* × *P. yunnanensis*, *P. deltoides* × *P. nigra and Salix matsudana* × *Sinapis alba*	Cd	The soil used was Manawatu silt loam, artificially spiked with cadmium	chelating agents (0.5 and 2 g kg−1 EDTA, 0.5 g kg−1 DTPA and 0.5 g kg−1NTA Soils were fertilized with an OsmocoteT M slow release fertilizer at a rate recommended by the manufacturer	Cano-Reséndiz et al., 2011

3.1 Models focused on plants

3.1.1 System Dynamic Approach (SDA)

Several mathematical algorithms have already been implemented as tools to increase phytoremediation efficiency or to better our understanding regarding specific soil processes (Rizwan et al., 2017). A diverse range of diffusion laws and statistical correlations, aimed primarily to understand the phenomena in a complete way, had been researched, generated and intensively specialized for specific purposes (Oh et al., 2014). One example is the implementation of System Dynamic Approach (SDA), which provides a differential equation set, defined by specific models for specific physiological responses of plants (Table 3). However, adding references like plant metabolic response to given contaminants can sometimes add excessive complexity to the model, given the number of total parameters needed for a prediction. The time and cost of this kind of action can be justified in some cases of complex contamination, while in other cases, it is advised to not surpass 30 total variables (soil and plant) per model (Ouyang et al., 2002, Rizwan et al., 2017). SDA was successfully implemented for large-scale phytoremediation in several previous experiments. One example is the study of Zhang et al., 2010 where the system dynamic approach was used to predict the uptake of cadmium, lead, copper and zinc in shoots of rice grown on a contaminated site close to an abandoned lead and zinc mine. The described study applied six different chelating agents in experimental fields and found that the chelation with NH_4OAc and $CaCl_2$ were the most suitable extractants for predicting the bioavailability of heavy metals for rice grown on post-mining soil, with several problems causing plants abiotic stress, including mixed metal contamination (Table 2) (Zhang et al., 2010). In another study, the SDA was successfully used to predict the necessary time to extract 2,4,6-trinitrotoluene (TNT) via *Populus fastigiate*. However, this study was performed in artificial conditions of spiked soil and not on a large-scale (Ouyang et al., 2007).

3.1.2 Decision Support System model - DSS

One example of a model designed for the assessment of plants uptake (for instance, heavy metals uptake) is a Phyto Decision Support System – Phyto-DSS. In its original form, it was strictly intended for the evaluation of phytoextraction including the overall

absorption and distribution of selected metals in plants tissues (Cano-Reséndiz et al., 2011). The use of this model can also provide insights into the economic feasibility of the phytoextraction process and compare it to other land reclamation methods (Table 3). Phytoextraction is always associated with a vast range of site-specific uncertainties, such as changes in the type of contaminant, its concentration, seasonal changes in the physical or chemical properties of soil, changes in climate conditions and plant physiological response to abiotic stress, which all contribute to the efficiency of such process (Shahid et al., 2017). Therefore, in order to improve phytoremediation planning, DSS models seem to be the most suitable method of precisely assessing the time necessary for the phytoextraction and to evaluate different possible scenarios including the supplementation of soil with selected properties (Maco et al., 2018). The Phyto-DSS was created in 2000 at the Instituto de Recursos Naturales y Agrobiología de Sevilla, Spain. Subsequently, the Phyto-DSS was developed at HortResearch, Palmerston North, New Zealand (2001 - 2004) and the Swiss Federal Institute of Technology, Zurich, Switzerland (2005 – 2007). In summary, the DSS model is based on the processes involved in the transfer of metals through transpiration flow and, as a consequence, water transfer through plant tissues (Cano-Reséndiz et al., 2011). It is mostly focused on metal concentration in soil and its fluctuation after the addition of plants, all while taking into consideration the soil density, plant root distribution and the root-absorption factor (Table 1). Since soil solution is taken up via the roots and transported into different plants tissues, any component absorbed by the roots will accumulate in plants tissues. It is also possible for such components to migrate to other tissues and begin to accumulate in different places. The DSS model also depends on the fact that the metal translocation from the roots to the shoots of plants is driven by plant water uptake (Shahid et al., 2017). Overall, the DSS can predict the amount of component (M) that will be removed by plants, taking into account its transpiration rate (T) over a selected period of time (t). Also, the amount of component is a function of the component concentration in soil solution (C) due to the fact that the total amount of metal that accumulates in the plant is proportional to the metal concentration in soil solution and not to the metal concentration in bulk soil alone (Table 1) (Cano-Reséndiz et al., 2011).

It has been well established that the total concentration of metal that accumulates in plant tissues does not simply equal the concentration of metal in soil solution times the volume of transpired water since metals have to be translocated to the shots via apoplastic or

symplastic pathways, where they can be percolated and/or filtrated. Due to this reason, the DSS defines the root absorption factor (f) which represents the metal concentration divided through the root xylem/soil solution system and other different factors that can influence the metal uptake in the roots (Table 3). Factors like temperature, moisture, pH, microbial activity and the concentration of competing ions can also severely affect the value of f. This issue is even more complicated due to the fact that root absorption factor can also change depending on the metal concentration in the soil solution (Cano-Reséndiz et al., 2011, Shi et al., 2017).

Furthermore, in the DSS model, plant species have been divided into three main groups depending on their capability to store metal in their shoots in relation to metal concentration in soil. For toxic and non-essential elements including lead, cadmium and nickel, plants that have low values of f are considered to be 'excluders'. Most plant species belong to that first group (Zhao et al., 2019). The second group consists of plant species that have a close to constant f value for a wide range of metal concentrations. They are considered 'indicators' due to the almost linear relationship to metal concentration in soil. In the third group, there are plants which are able to tolerate high concentrations of metals in their shoots and/or possess active uptake mechanisms for such metals. Plants in this group are known as 'hyperaccumulators' (Wang et al., 2015). For hyperaccumulators and excluders, the value for f is constant only for a narrow concentration rate. Above a certain threshold, the protective mechanisms will break down and metal will flood into the plant causing high toxicity symptoms, reduced biomass and even plant death. Alternatively, the f values and overall phytoextraction process can be enhanced by selective breeding, gene manipulation or by increasing the concentration of available contaminants for example by the use of chelates (Bell et al 2014). Overall, the plant species-specific f can be assessed by measuring the plant's water use, dry biomass of shoots and the metal concentration in the soil solution. This is why the DSS model adds a constant known as the 'decay constant K' (Table 3) (Cano-Reséndiz et al., 2011).

The distribution of contaminants in soils is never uniform throughout its depths and the highest concentrations are typically found close to the surface layer of the soil. The DSS model considers soil to be divided into three zones: 1) contaminated zone – usually the surface layer - maximum concentration of contaminant; 2) intermediate zone – in which the mixing of contaminated and uncontaminated soils occurs; 3) uncontaminated soil –

most often deeper layers of soil that are unaffected by the contamination at any given time (Anning et al., 2018).

The uptake of contaminant by plants causes changes in soil concentration of such contaminant (mg/kg) at specific depth d which can also be taken into consideration and calculated in DSS model (Table 3).

Overall, the DSS model is relatively simple but provides general information about the whole process of contaminant uptake by plants. A study by Canales-Pastrana & Paredes (2013) successfully used the DSS model aiming to evaluate internal interaction of plants metabolism and a contaminant via Stella Professional software (Canales-Pastrana et al., 2013). Another study by Cano-Resendiz et al. (2011) used dynamic model Phyto-DSS in order to predict element uptake in plants specifically silver, mercury and antimony via *Aloe vera* and poplar species grown on post-mining site with a long record of contamination. The study showed that revegetation would require a soil amendment to improve the tailings nutrient status and water retention properties. Small-scale pot or field studies are needed to assess the adequacy of the chosen model (Cano-Resendiz et al., 2011). Thus, the main drawback of using the DSS model is that environmental conditions such as drought, extreme colds and rapid changes in temperature are not taken into consideration. Moreover, the f factor for a pollutant uptake can be different for plants grown on different soil types, mostly due to the presence of various ions and pH values (Canales-Pastrana et al., 2013). Another significant drawback is that, after entering the root, the DSS model assumes that the contaminant will be translocated and stored in plants shoots, but many researchers demonstrated that metals stored in shoots could be relocated back to the roots which would consequently result in an overestimation of the component that is uptaken. Finally, this model is imperative for the fact that takes into consideration the moisture distribution in soil, which is almost never constant in the natural environment and influences the uptake of metals by roots (Wang et al., 2015). Given these limitations, the DSS model can actually indicate a shorter time of remediation than that which would occur in a natural scenario. Therefore, it is advised to apply caution and not mislead potential clients. Overall, in using the DSS model, the precise input data is required to predict future biomass and metal accumulation. The environmental conditions must be considered very precisely because the DSS model is strongly susceptible to changes in water content (Canales-Pastrana et al., 2013, Wang et al., 2015).

One of the main advantages of DSS approach is that, the economic viability of phytoextraction in DSS model can be easily compared to the best alternative technology and the cost of complete inaction (Table 3) (Canales-Pastrana et al., 2013).

The final step of DSS approach is the comparison of selected technology to the cost of no activity. Such cost is determined mostly by specific legislation but also as a loss of income from land. In one study, Phyto-DSS was applied to model phytoremediation of sites amended with sewage sludge and planted with pine trees. The study was focused on the comparison of the phytoremediation effectiveness to the efficiency of other, physical, and chemical methods of soil remediation. It was calculated that the phytoremediation of 2 ha land would cost approximately 17 000 EUR whereas the combined physical and chemical remediation would cost over 160 000 EUR (Baltrėnaitė et al., 2007).

3.1.3 PLANTX

Another model that can be used in phytoremediation planning is the PLANTX model which considers the dynamic transfer of compounds from soil to plants and air, and also the metabolism of contaminants, including their final accumulation in stems, leaves or fruits. It is mostly useful for organic contaminants (Essaid et al., 2015). The transfer of contaminants in the atmosphere-plant-soil system was modeled using PLANTX by Trapp and McFarlane (Table 1). It considered the following: 1) the dynamic transfer of contaminant from soil and air to plants and 2) the metabolism of anthropogenic contaminants including their accumulation in plant tissues (roots, leaves, stems and fruits). Overall, the model is based on the pollutant diffusion across soil solution and soil pores, the transfer of contaminants to roots via transportation and the total distribution of contaminants in plants tissues (Baltrėnaitė et al., 2016). Hence, it was shown to predict the concentration of pollutants in plants, but it is was not predominately designed for metals, but rather for organic pollutants. However, in one study, the PLANTX approach was used to predict the uptake of metals and other inorganic pollutants from the soil into plants, including their concentration in roots, leaves, stems and fruits. This selected model took into consideration the following input data: continuous or pulse uptake into any plant tissue, contaminant leaching and degradation, uptake to roots via transpiration water and the diffusion into roots, degradation and metabolism in roots, stems and fruits,

translocation of the contaminant from roots to stems, leaves and fruits, loss of leaves and fruits by phloem flux and loss from shoots to air (Table 1) (Baltrėnaitė et al., 2016).

3.1.4 Response Surface Methodology and Artificial Neural Network

One of imperative steps to ensure the quality of each phytoremediation is to optimize the parameters that are applicable to the process (Mojiri et al., 2015, Janani et al., 2019). Nowadays, one of the most important prediction and optimization strategies is the use of statistical and mathematical models, such as Response Surface Methodology (RSM) and Artificial Neural Network (ANN), which are being implemented in a growing number of studies. RSM is a model based on the statistical methodology used for designing experiments, modelling, evaluating the effects of variables and their interactions and determining the optimal points of a process (Janani et al., 2019). A neural network is a series of algorithms that endeavors to recognize underlying relationships in a set of data through a process that mimics the way the human brain operates. Neural networks can adapt to changing inputs; thus, the network generates the best possible result without the need to redesign the output criteria (Mojiri et al., 2015). The concept of neural networks, which has its roots in artificial intelligence, is swiftly gaining popularity in the prediction of complex systems. A neural network is set up to perform specific tasks such as identifying patterns, estimating and approximating functions and categorizing information during a training process (Liu et al., 2019). As a nonlinear computational modelling technique that has generated increasing interest, both RSM and ANN have only recently been applied to optimize phytoremediation studies. Their main advantage is that they are able to act in a manner similar to the human brain and both try to imitate human intuition in reaching conclusions and making decisions when confronted by irrelevant, noisy, complex and partial information (Sanusi et al., 2016). The study by Mohammadi et al., 2019 showed that RSM could be successfully used to predict the uptake of pyrene by sorghum during assisted phytoremediation with indoleacetic acid (IAA) and *Pseudomonas aeruginosa* as soil amendments. However, as with most similar research, experiments were performed on artificially spiked soil with only singular contamination, so further evaluation of model efficiency in real-life conditions is required to prove its usefulness for a broad scale application (Mohammadi et al., 2019). In the experiments of Titah et al., 2018, researchers compared Response Surface Methodology (RSM) and an Artificial Neural Network (ANN) as models for arsenic uptake via the plant *Ludwigia*

octovalvis. The results obtained showed that both explored models could be used in the prediction of As uptake, giving only a minor overestimation (approximately 3.5% overestimation of arsenic uptake for RSM and approximately 1.8% for ASS in comparison to experimental data). Overall, both models were appropriate for the optimization of arsenic removal with ANN demonstrating significantly higher predictive and fitting ability than RSM (Titah et al., 2018). Sanusi et at al., 2016 also compared RSM to ASS approach but in this case, it was done in order to choose the best model for the prediction of petroleum hydrocarbon intake by tropical plant species *Paspalum scrobiculatum* L. Validation analysis showed the predicted values by RSM and ANN were close to the validation values, whereas the ANN showed the lowest deviation, 2.57%, compared to the RSM. This finding suggested that the ANN ensures a better prediction and fitting ability in comparison to the RSM for non-linear regression analysis (Sanusi et al., 2016). Another study explored the phytoremediation of heavy metals from waste water treatment plant effluent by *Phragmites australis*. RSM was implemented as a useful tool in the design of experiments, statistical analysis and optimization of the parameters. Moreover, the work of Selamat et al., 2017, used RSM to assess the bioaccumulation of lead in *Melastoma malabathricum* L. The difference between the validation value and the predicted value was within 6.7%, indicating that RSM was able to predict the optimum lead bioaccumulation precisely with only a minor error (Selamat et al., 2017).

Overall, artificial neural networks (ANNs) have been widely used in problem solving due to their reliable, robust and salient characteristics in capturing the nonlinear relationships between variables in complex systems. In a study by Darajeth et al., 2016, ANN was applied for the modelling of Chemical Oxygen Demand (COD) and biodegradable organic matter (BOD) removal from palm oil mill secondary effluent (POMSE) by vetiver system. Results showed that the ANN has excellent potential in the prediction of COD and BOD removal from POMSE with a residual standard error (RSE) of less than 0.45% (Darajeh et al., 2016).

Work by Rossi et al., 2019, focused on the determination of the synergistic effects of Cd and CeO_2NPs on the physiological parameters of Brassica and its accumulation in plant tissues and explored the underlying physiological/phenotypical effects that drive these specific changes in plant accumulation using Artificial Neural Network (ANN) as an

alternative methodology to modelling and simulating plant uptake of Ce and Cd. The study showed that ANN identified key physiological factors affecting the plant uptake of co-occurring Cd and CeO_2NPs. Specifically, these results showed that fresh root weight and the net photosynthesis rate are both parameters governing Ce uptake in plant leaves and roots, while fresh root weight and the F_v/F_m ratio are parameters affecting Cd uptake in leaves and roots. Overall, ANN is an efficient and effective approach to determining model plant uptake of co-occurring CeO_2NPs and Cd (Rossi et al., 2019).

3.1.5 Multiple regression models

The study by Trakal et al., 2012, investigated the possibility of phytoremediation of complex contamination with mixed metals, including cadmium, lead, zinc and copper, caused by smelting operations. The root uptake model mentioned earlier, which was based on Richards' equation, was applied for a prediction study. However, for cadmium and zinc, it was found that the model severely underestimated the concentration of metal uptaken by willow tree tissue, whereas for lead it overestimates those values (Trakal et al., 2012). Since Richards' equation was applied in other studies where it was applicable to artificially spiked soil with single metal contamination, it clearly demonstrates the importance of testing models in real-life conditions in which there are most often multiple sources of abiotic stress for plants. In another experiment, researchers investigated different regression models topredict heavy metal uptake in cucumbers grown on soil fertilized with sewage sludge containing trace amounts of mixed heavy metals (Eid et al., 2018). Regression analysis indicated that soil HM, pH and OM contents were good predictors for HM concentrations in cucumbers. The regression models for root Co, Cr, Fe and Zn were described by high model efficiency values that explain 48–58% variability. The best regression models for cucumber stems were for Cu, Mn, Ni and Zn that are characterized by high R2 and model efficiency values. For cucumber fruits, R2 values ranged from 54 to 82%, with leading models for Cr, Pb, Cd, Cu, Ni and Co in the fruit. Such model can be potentially beneficial for risk assessment studies on sewage sludge utilization in agriculture (Eid et al., 2018). In a study by Kumar et al., 2019, multiple regression modelling was used to predict the uptake of several heavy metals in tissues of *Pistia stratiotes* grown on paper mill effluent contaminated with Cd, Cu, Fe, Pb, Mn, and Zn. Multiple regression modelling studies were successfully implemented to develop models for predicting the heavy metal uptake. *Pistia stratiote* was found a

suitable plant for the phytoextraction of these metals while the multiple regression modelling has been shown to be a reliable tool for modelling the phytoextraction process. For prediction modelling studies, the pH and heavy metal concentration were found to be the best predictors for developing multiple regression models. The developed models were reliable with high validity performance, efficiency, and minimum errors when tested by several model validation tools (Kumar et al., 2019).

3.1.6 Other models

Another model called CTSPAC (Full name: Model for coupled transport of water, heat and solutes in the soil-plant-atmosphere continuum) was developed in the early 1990s and consists of two separate sub-models: one for soil and one for plants (Ouyang et al., 2002). When it was first developed, it provided a fundamental increase in our understanding of plant-uptake kinetics. However, for more than a decade, there was no effort devoted to its implementation in the investigation of the phytoremediation of contaminants. Its soil submodel consists of three time-dependent equations for the flow and transport of water, chemicals and heat through the soil. The movement of water is described by the Richards' equation focusing on the effects of daily cycles of water infiltration, evaporation, root uptake and leaf transpiration. The transport of heat is described simultaneously by heat conduction in solid/liquid/air phases, whereas the transport of chemicals is described by simple models for convection, dispersion/diffusion, sorption, degradation and root uptake. The second part of the model, the plant submodel, is primarily focused on the compartmentalization of chemicals in specific plants parts with similar tissue function and structure, including root uptake, transport of chemicals via phloem, xylem and accumulation in shoots and fruits. CTSPAC model was used for a prediction of 1,4-dioxane uptake via poplar hybrid *Populus deltoiddes* × *Populus nigra,* and was successful in its prediction with only minor differences to experimental data. However, the study used only artificially spiked sand instead of natural soil and the model was not evaluated in a real field conditions scenarios (Ouyang et al., 2002). Another example of successful implementation of models to phytoremediation are studies by Boersma et al. who integrated both: the transport of the pollutant and its transformation into one, mathematical model known as CTSPAC, which combined two separate models representing soil and plants (Boesma et al., 1991). A similar approach was undertaken by Ouyang, who researched the problem of selection and application of modelling to predict

the transfer of 1,4 dioxane to poplars (Ouyang et al., 2007). Moreover, such an approach was also discussed by Baltrenaite and Buthis, who researched the transfer of inorganic contaminants to higher plant species through the implementation of the Hung & Mackay model which can be used to predict the uptake of contaminants from soil (Baltrenaite et al., 2007). It can also evaluate the whole circle of contamination transfer including the translocation from soil to roots, through stem to leaves, from leaves to air, air to leaves, leaves to stem and finally from stems to roots. The uptake of the selected contaminant is based on select balancing factors which can describe the distribution of pollutants and the rate of plants metabolism (Table 1). Additionally, the time of plant growth is also evaluated. In this model, the parameters taken into the consideration are as follows: 1) leaf surface area; 2) growth and metabolism – duration of exposure to contaminants, the growth rate of all organs; 3) metal characteristics – concentration of pollutants, its molecular mass; 4) physio-chemical analysis of plants – the density of leaves and roots, transpiration rate (Baltrenaite et al., 2007). Hung & Mackay model was successfully implemented for a prediction of heavy metal uptake in *Pinus sylvestris, Betula pendula* and *Alnus glutinosa* grown of HMs contaminated soil with supplementation with sewage sludge. (Baltrenaite et al., 2007). In 2019, Zhao et al., developed a simple sigmoid heavy metals uptake model which uses plants' bioaccumulation factor (BAF), biomass and heavy metal contamination rate. It was shown to be a useful uptake model for sunflowers grown on soil contaminated with Cd, Ni, Pb and Zn, with only a minor overestimation in the model in comparison to experimental data (less than 20%). However, the model was tested only on artificially spiked soil with single metal contamination and therefore needs more evaluation before large-scale use (Zhao et al., 2019).

3.2 Models focused on soil

The creation of comprehensive mathematical models for the migration of metals and their concentration in soil as a heterogenic system with variable composition is disputed due to the complexity of those strictly interlinked processes and the vast quantities of input data necessary for these models (Chirakkara et al., 2016). Overall, the migration of metals depends on several conditions including granulometric soil composition, its filtration capacity, pH, metal sorption, the composition of soil phases (percentage of clay, organic matter, iron, etc.), climatic conditions (e.g., moisture) and the geochemical structure of the terrain (Shelmerdine et al., 2009). Given that these are not easily determined

parameters and yet are necessary for the characterization of metal migration, the basic parameters of modelling metal migration in soil are frequently insufficient (Essaid et al., 2015). Consequently, the application of mathematical models for a comprehensive description of metal migration needs to take into consideration the full, complicated nature of these processes (Stritsis et al., 2014).

3.2.1 MINTEQA2/PRODEFA2 model

The creation of comprehensive mathematical models for the migration of metals and their concentration in soil as a heterogenic system with variable composition is often disputed due to the complexity of those strictly connected processes and the vast quantities of input data necessary for these models. Thus, one of the complex models used for the determination of metal migration is MINTEQA2/PRODEFA2, which has shown to be able to precisely investigate the interactions between soil and metals (Allison et al., 1991). Overall, the MINTEQA2 was previously used as a versatile and quantitative tool for the prediction of the equilibrium behavior of heavy metals in different environments. The data needed for the prediction includes chemical analysis of samples especially the total concentration of all components of interest as well as other measurements of the environment system such as the pH and pressure. However, it needs to be remembered that the MINTEQA2 model can only be used if the studied chemical system is at or will reach equilibrium. This is a severe limitation in natural systems. Moreover, the model was also shown to have lacks of internal consistency of the data in the available databases (Christensen et al., 1999, Xu et al., 2020).

3.2.2 BALANS

The BALANS model was developed in Tomsk State University (Russia) to simulate the processes involved in the natural attenuation of soils from metals. These processes mostly include metal uptake and its removal through the harvesting of plants and the removal of metals as a consequence of its natural migration in the soil (Oconnor et al., 2018). The total amount of metals bioaccumulated in crops is estimated in this model as biomass removed and metal concentration in the biomass. The natural removal of metals from contaminated soils is calculated using annual migration rates after taking into the consideration the climatic zone and the type of soil (Table 1). The program uses annual

metal leaching rates for lead, chromium, copper, zinc and nickel chosen from a vast range of literature which can divide soils into selected typed including podzols and albeluvisols (Baltrėnaitė et al., 2016). The main advantages of the BALANS model includes a clear difference between metals removed by phytoremediation and metals removed by naturally occurring soil processes such as leaching. Unfortunately, the BALANS model by default cannot take into account the influence of various forms of metals found in amendments (i.e. biochar and sewage sludge) on the geochemical migration intensity in the soil. This can be taken into consideration only if an operator inputs the respective data (Urbaniak et al., 2016, Oconnor et al., 2018).

3.2.3 Freundlich model

The Freundlich model can be used to assess the uptake of heavy metals from contaminated soil, characterized by areas with significantly different concentrations of contaminant. The model is based on both the total and available concentration of the contaminant (Table 1) (Pedron et al., 2017). It supports the non-linearity of the uptake and takes into consideration differences among plant species which helps during the selection process of the most suitable plants for a given area. At the same time, it is not as complicated as other dynamic models, causing it to be easier to implement and assess the efficiency of large-scale phytoremediation (Yaashikaa et al., 2019). The Freundlich-like model was used by Zhang et al., 2016 looking for Improving the prediction of metal uptake by Chinese cabbage (*Brassica pekinensis* L.). The described experiment was performed on real, agricultural soil with Zn and Pb contamination due to mining and smelting operations near-by. Results had shown that the root uptake of Cd, Cu and Zn was non-linear whereas for Pb and Ni it was linear. Thus, it was noticed that the complexity of metal uptake mechanisms makes linear predictions extremely difficult and that models derived from a singular and direct approach are often not predictable and full of overestimations which is why more complex, dynamic models seem to have numerous advantages for such processes (Zhang et al., 2016). In another study by Zhang et al., 2018, researchers tested several empirical models in order to adequately predict the uptake of lead by radish plants. Overall, the study found that the best prediction for Pb uptake was granted when models included CEC, pH and the content of dissolved organic matter as its main factors (Zhang et al., 2018). Similarly, in another experiment, a Freundlich-like model was used to predict the uptake of arsenic from plants grown on a contaminated

site, characterized by areas with varied contaminant concentrations across the remediated area (Pedron et al., 2017). The results, based both on total and bioavailable concentrations, support the non-linearity of the uptake and highlight the differences between plant species, thus helping in the selection of the best class of plants (Pedron et al., 2017, Zhang et al., 2019). Several other models can be used to predict the soil-to-plant transfer of elements, but often they are rather complex but, a positive feature of the Freundlich-like model is that it is easy to handle and can be used to predict the efficiency of a field-scale phytoremediation procedure. With this model, a more realistic prediction of the potential of the technology can be obtained, since the use of linear soil-to-plant transfer functions may overestimate the uptake. However, during the study by Yaashikaa et al., researchers investigated the prediction of chromium uptake by sorghum after its inoculation with the plant-promoting growth bacteria *Pannonibacter phragmites*. Two models were chosen for the uptake assumption: the Freundlich model and the Langmuir adsorption equation. The aforementioned Langmuir equation includes the following data: the maximum rate of chromium uptake (pmol/cell/h), the half-saturation constant (μM), the ambient Cr(VI) ion concentration (μM), the mass of Cr(VI) ion adsorbed per unit mass of plant (mg/g), the maximum monolayer adsorption capacity (mg/g), the Langmuir adsorption constant (L/mg) and Ce is the equilibrium Cr(VI) ion concentration (mg/L). In this case, a Langmuir isotherm fitted the plant Cr(VI) uptake rate with slightly better precision in comparison to the Freundlich model (Yaashikaa et al., 2019).

Table 3. Main mathematical equations behind selected models showing the output data capabilities of each approach.

Model	Basic mathematical explanation	Legend	Plant species	Reference
System Dynamic Approach (SDA)	**1. Water uptake and transport** $Q_{root} = A_{Soil}^{Root} L_{Soil}^{Root} (\varphi^{root} - \varphi^{soil})$ $Q_{stem} = A_{Root}^{Stem} L_{Root}^{Stem} (\varphi^{Stem} - \varphi^{Root})$ $Q_{leaf} = A_{stem}^{leaf} L_{stem}^{leaf} (\varphi^{leaf} - \varphi^{stem})$ **2. Water potentials** $\varphi^{root} = P^{root} f_d - \Pi^{root} + \rho g h^{root}$ $\varphi^{stem} = P^{stem} f_d - \Pi^{stem} + \rho g h^{stem}$ $\varphi^{leaf} = P^{leaf} f_d - \Pi^{leaf} + \rho g h^{leaf}$ **3. The rates of contaminant uptake by roots from soil and transport from roots to stems and leaves** $M_{rate}^{root} = Q_{water}^{root} C_{soil} \delta^{root} \beta^{root}$ $M_{rate}^{stem} = Q_{water}^{stem} C_{root} \delta^{stem} \beta^{stem}$	Q - the water flow rates between compartments ($cm^3\ h^{-1}$), A - the contact areas between compartments (cm^2), L - the conductances between compartments ($cm\ h^{-1}\ MPa^{-1}$), φ - the water potentials (MPa) in the compartments P - the hydrostatic pressure or pressure potential (MPa), f_d - the diurnal factor characterizing daily variations of hydrostatic potential, P - the osmotic potential (MPa), ρ - the water density ($g\ cm^3$), g - the gravitational acceleration, h is the height of the roots, stems, or leaves referenced to a datum (cm), M - the rate of contaminant uptake ($mg\ h^{-1}$), C – the contaminant concentration ($mg\ cm^3$), δ - the reflection coefficients (the ease with which given contaminant crosses the membrane between compartments),	*Populus deltoiddes* × *Populus nigra*,	Ouyang, Y., (2007).

	$M_{rate}^{leaf} = Q_{water}^{stem} C_{stem} \delta^{leaf} \beta^{leaf}$ $M_{rate}^{vp} = Q_{water}^{leaf} C_{leaf} \delta^{leaf} \beta^{leaf} f_v$	β - the transformation loss of contaminant, f_v - the fraction of contaminant in vapor phase.		
Phyto-DSS	**1. The contaminant uptake** $M \propto \int_0^t T\, dt$ $M \propto [C]$ **2. Root absorption factor** $f = \frac{[Cr]}{[C]}$ **3. Root absorption factor with decay constant** $f(C) = \frac{f_1 C_1}{C_1 + K(C - C_1)}$ (4) $f \cong \frac{MB}{TC}$ (5) **4. The uptake of contaminant by plants causes changes in soil concentration of such contaminant (mg/kg) at specific depth d** $\Delta[M]_z = \frac{1}{\rho_z} \int_0^t R_z T C f \; dt$ (6) **5. The economic viability of phytoextraction**	M – the overall amount of selected component (mg/kg), t – selected period of time (usually in days), C – component concentration in soil solution (mg/kg), f – root absorption factor, Cr – soluble concentration of contaminant in roots (mg/L), C – soluble concentration of contaminant in soil (mg/L), f(C) – root absorption factor at specific concentration of component in soil (mg/L), f_1 – measured root absorption factor at component concentration C_1 (mg/L), K – decay constant (0 < K < 1), M - component concentration in the above-ground dry plant biomass (mg/kg), B - whole above-ground dry biomass (kg), T - the total water use (L), C - concentration of component in soil solution (mg/L), $\Delta[M]_z$ – change in component concentration at certain depth (mg/kg), ρ_z – soil bulk density at depth z (g cm^{-2}),	*Aloe vera* and poplar species	Cano-Reséndiz et al., 2011

$$V = A * \left(\int_0^t (C1 + C2 - P1\ x\ V1)\ dt + \sum_{x=1,2,3\ldots t} \int_0^x (C1 + C2 - P1\ x\ V1)\ dt\ x \frac{I}{100} \right)$$

R_z – root density (root mass at depth z) / (total mass of roots),
T – total water use (L/day),
f – calculated root absorption factor,
A – contaminated area (ha),
C1 – cost of planning ($ ha^{-1}),
C2 – cost of production ($/ha),
P1 – biomass that can be sold (t/ha),
V1 – value of biomass ($/t),
I – interest rate (%),

6. **Comparison to alternative technologies**

$$V_a = A * \left(C \left(1 + \frac{I}{100}\right)^{t1} - L \left(1 + \frac{I}{100}\right)^{(t2-t1)} \right)$$

t1 – time necessary for different technology to remediate site (years),
t2 – time for phytoremediation of site (years),
C – cost of different technology ($ ha^{-1}),
I – interest rate (%),
L – profit from the land ($).

PLANTX / CTSPAC	**1. Available concentration of metal in soil:** $A = \left(\frac{-c/\alpha + (f/\alpha)S}{-e + S}\right) * S$	*A* - the available concentration of metal M^{n+} (mg/L) in the soil solution, *S* - the metal concentration in trees (mg/kg), *α* - is the coefficient of absorption (L/kg/year), *c*/*α*, *f*/*α*,*e* - can be fitted by experimental results in order to establish the relationship between *A* and *S*.	*Hybrid: Populus deltoiddes* × *Populus nigra,*	Boersma et al., 1988, Boersma et al., 1991
Hung and Muckay	**1. Concentration of contaminants in leaves**	B_{er} - the uptake ratio of the contaminant from soil to roots,	*Pinus sylvestris*,	Baltrėnaitė et al., 2007

$$C_{leaves} = \left(\frac{B_{er} * B_{rs} * B_{sl} * K_{lw} * C_s}{K_{ew}} + \frac{B_{al} * K_{tw} * C_a}{K_{aw}}\right) * \frac{M_i}{\rho_l}$$

2. Concentration of contaminant in stems

$$C_{stem} = \left(\frac{B_{er} * B_{rs} * B_{sl} * K_{stw} * C_s}{K_{ew}} + \frac{B_{al} * B_{ls} * K_{stw} * C_a}{K_{aw}}\right) * \frac{M_i}{\rho_{st}}$$

3. Concentration of contaminants in roots

$$C_{stem} = \left(\frac{B_{er} * K_{rw} * C_s}{K_{ew}} + \frac{B_{al} * B_{ls} * B_{sr*} K_{rw} * C_a}{K_{aw}}\right) * \frac{M_i}{\rho_r}$$

B_{rs} - the uptake ratio of the contaminant from roots to stem,
B_{sl} - the uptake ratio of the contaminant from stem to leaves,
B_{al} - the uptake ratio of the contaminant from air to leaves,
B_{ls} - the uptake ratio of the contaminant from leaves to stem,
B_{sr} - the uptake ratio of the contaminant from stem to roots,
C_s - the concentration of the contaminant in the soil,
C_a - the concentration of the contaminant in the air,
K_{ew} - the equilibrium partition coefficient of the contaminant between soil and water,
K_{lw} - the equilibrium partition coefficient of the contaminant between leaves and water,
K_{stw} - the equilibrium partition coefficient of the contaminant between stem and water,
K_{aw} - the equilibrium partition coefficient of the contaminant between air and water,
K_{rw} - the equilibrium partition coefficient of the contaminant between roots and water,
M_i - the molecular weight of the contaminant,
ρ_l, ρ_{st}, ρ_r - the density of plant leaves, stem, and roots, respectively.

Betula pendula, Alnus glutinosa

Multiple regression modeling	**1. Basic model of metal uptake in plants** $\Delta Y = \sum (C_t - C_0)/n$ **2. The multiple linear regression model including two independent variables** $Y = \beta + \beta_i * X_1 + \beta_j * X_2$	ΔY - the calculated metal uptake (mg/kg DW), C_t – concentration of the contaminant after the treatment (mg/kg DW), C_0 – concentration of contaminant before the treatment (mg/kg DW), β - the regression constant, β_i, β_j, X_1, X_2 - the estimated coefficients (β) for independent variables, ant their concentration (X) (mg/L). Usually referring to soil pH and metal concentration.	*Pistia stratiotes*	Kumar et al., 2019
Freundlich isotherm	**1. The amount of adsorbed metal ions** $\rho = KC^{\frac{1}{n}}$ **2. Its linear relation:** $log\rho = logK + \frac{1}{n} logC$	ρ - the amount of absorbed ions by sorbent (mg/g), C, C_o - are initial and remaining concentrations of dissolved metals (mg/L), K - the equilibrium constant of Freundlich represents absorption power (mg/g), n - the equilibrium constant of Freundlich which represents binding energy of metal and adsorbent,	*Sorghum bicolor* *Brassica juncea* *Lupinus albus* *Helianthus annuus*	Yaashikaa et al., 2019 Pedron et al., 2017

Langmuir adsorption model	**1. The amount of adsorbed metal ions** $\rho = (\rho_m * K_l * C)/(1 + K_l * C)$ **2. Its linear relation:** $C/\rho = 1/(\rho_m * K_l) + (C/\rho_m)$	ρ - the amount of absorbed ions by sorbent (mg/g), ρm - the required metal concentration for the formation of one layer (mg/g), C, C_o - are initial and remaining concentrations of dissolved metals (mg/L), K_L - the equilibrium constant in Langmuir dependent on absorption energy (mg/L),	*Sorghum bicolor*	Yaashikaa et al., 2019
Sigmoid heavy metal uptake model	**1. Total heavy metal uptake in plant** $M_{HM\ plant} = \frac{M_{max}}{1 + e^{-k_M(t-t_{0.5M})}} * C_{HM\ soil} * BAF_{HM}$	M_{max} - the maximum plant biomass (g), k_M - constant that determines the curvature of the sigmoid growth pattern, t – time (days), t0.5 is the inflection time at which the growth rate reaches the half of maximum value, BAF – bioaccumulation factor of a given metal, $M_{HM\ plant}$ – concentration of heavy metal in plant, $C_{HM\ soil}$ – concentration of heavy metal is soil.	*Helianthus annuus*	Zhao et al., 2019

4 VALIDATION OF PREDICTION MODELS

All models need to be validated against experimental data before their application in real conditions scenarios. The quality of the models is usually evaluated by the correlation coefficient (r), the model efficiency (ME) (Equation 1) and the mean normalized average error (MANE) (Equation 2) (Wang et al., 2015).

$$ME = 1 - (\sum \frac{(C_{model} - C_{measured})^2}{(C_{model} - \acute{C})^2} \quad (1)$$

$$MANE = \sum(|C_{model} - C_{measured}|^2 / C_{measured})/n \quad (2)$$

Where: C_{model} is the predicted concentration of given contaminant by the chosen model;

$C_{measured}$ is the measured concentration of contaminant;

Ć is the mean of measured contaminant concentration;

n – is the total number of observations;

5 CONCLUSIONS AND FUTURE PROSPECTS

The use of mathematical models in environmental studies can improve the evaluation of different outcomes in order to make an objective decision about the most suitable process for the selected contaminated site. Over the last three decades, several mathematical approaches have been used to better understand the interaction between soil and plants which can then be applied for modelling the process of phytoremediation. Contaminant absorption and accumulation models can play a key role in understanding processes occurring in soils and plants, which can subsequently allow to manage contaminated sites. The main obstacle facing linear analytical models is their lack of complexity of suitable mathematical solutions, which often include only a few input types. Dynamic modelling is necessary for the simulation of real-life scenarios with non-constant input. However, in the case of constant input, steady-state models can yield consistent results, but only for the time period when plant growth is exponential (during the initial growth phase). While the results of modelling studies provide useful insights into the modelling of plant uptake processes, the number of available case studies is highly limited. Many existing models have been poorly validated with limited data and can be solely applied to certain described soil and plant conditions. In order to achieve the desired efficiency of phytoremediation, an interesting but not often explored direction is to combine the physicochemical properties of contaminated land with mathematical models and programming tools. As shown in our Review of the literature, there are several gaps in the way of considering the complex behavior of soil metal contamination into the modeling of metal uptake by plants. Thereby, an emerging area of phytoremediation research is to develop more sophisticated models based of biogeochemical processes. Information could be obtained by building new models based on new empirical advances. For instance, experiments measuring in various scenarios, biomarkers allowing an evaluation of plants' physiological processes and analytical data on changes in metal bioavailability in time. Creating simulations based on such data should allow addressing the interactions between plants soil and climate into our models. which can then be rapidly scaled-up to, for example, vast post-industrial contaminated lands. Ideally, by creating new improved and validated models for phytoremediation, the process itself will not need extensive laboratory or small-scale experiments before starting the process each time, which will reduce the time and labor required. Moreover, the application of machine learning and

mathematical modeling techniques can highlight the linear and nonlinear characteristics of metal extraction via plants and lead to an increase in our prediction capabilities.

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Modeling and optimizing the removal of cadmium by *Sinapis alba* L. from contaminated soil via Response Surface Methodology and Artificial Neural Networks during assisted phytoremediation with sewage sludge*

Marta Jaskulak[a,b*], Anna Grobelak[a], Franck Vandenbulcke[b]

[a] *Institute of Environmental Engineering, Faculty of Infrastructure and Environment, Czestochowa University of Technology, Czestochowa, Poland;*

[b]*University of Lille, Laboratory of civil engineering and environment (LGCgE), Environmental Axis, F-59650 Villeneuve d'Ascq, France;*

Abstract:

The study was aimed to model and optimize the removal of cadmium from contaminated post-industrial soil via *Sinapis alba* L. by comparing two modelling approaches: Response Surface Methodology (RSM) and Artificial Neural Networks (ANN). The experimental design was done using the Box-Behnken Design method. In the RSM model, the quadratic model was shown to predict the closest results in comparison to our experimental data. For ANN approach, a two-layer Feed-Forward Back-Propagation Neural Network model was designed. The results showed that sewage sludge supplementation increased the efficiency of the *Sinapis alba* plant in removing Cd from the soil. After 28 days of exposure, the removal rate varied from 10.96% without any supplementation to 65.9% after supplementation with the highest possible (law allowed) dose of sewage sludge. The comparison proved that the prediction capability of the ANN model was much higher than that of the RSM model (adjusted R-square: 0.98, standard error of the Cd prediction removal: 0.85 ± 0.02). Thus, the ANN model could be used for the prediction of heavy metal removal during assisted phytoremediation with sewage sludge. Moreover, such approach could also be used to determinate the dose of sewage sludge that will ensure highest process efficiency.

KEYWORDS: phytoremediation, sewage sludge; modelling; Response Surface Methodology (RSM); Artificial Neural Networks

1 INTRODUCTION

In the past few decades, soil contamination by heavy metals (HMs) had become one of the most critical environmental issues due to its direct threat to all members of the food chain (Anning *et al.* 2018). The primary cause of such contamination derives from industrial activities as well as from other anthropogenic sources (Almasi *et al.* 2018, Sivarajasekar *et al.* 2019). Hence, safe and efficient treatments for soil contamination with HMs are of utmost importance and the restoration of soils contaminated with high concentrations of metalloids is a major global concern (Dubey *et al.* 2018). In addition, they can have toxic effects not solely on soil organisms but can also easily transfer into vegetative cover and enter the food chain (Muthusaravanan *et al.* 2020). Thus, in recent years there has been growing concerns about the long term threat that consuming contaminated crops poses to human health (Yang *et al.* 2017, Bagheri *et al.* 2020). Recent evidences suggest that exposure to pollutants such as heavy metals can interrupt the epigenetic factors of the genome. Such changes are shown to be responsible for the etiology of several cancers and other diseases. Moreover, exposure to heavy metals can interrupt with epigenetic factors including with changes in miRNA expression levels, and DNA methylation (Sharavanan *et al.* 2020).

Currently, there are several chemical and physical methods of dealing with this issue, including processes like chemical soil washing, solidification and electroremediation. However, each method has its limitations, and many of them are too expensive to be used in real-life scenarios, especially when the area of contamination is large (Muthusaravanan *et al.* 2018). On the contrary, phytoextraction, which consist to remove metals via plant uptake, can offer a cost-effective alternative to those treatments. Overall, it is a rising green technology with many advantages that include being an environmentally friendly, inexpensive and less destructive solution in comparison to conventional chemical methods (Roy *et al.* 2015, Sarwar *et al.* 2017).

The process of phytoextraction requires that the selected species can absorb metals in its roots and translocate them to the above-ground tissues (Almasi *et al.* 2018). Plants can absorb environmental contaminants including metals from the rhizosphere through their roots. Water and solutes enter plants root systems through its epidermis and are, depending on plant species, accumulated in roots or transported to aerial tissues via xylem/phloem (Bagheri *et al.* 2019). Various plant species had been studied in the past for the uptake of mercury, copper, nickel, lead, cadmium, chromium and zinc (Ouyang *et*

al. 2002, Roy *et al.* 2015, Ouyang *et al.* 2002). Species able to cumulate relatively large quantities of metals in their shoots are commonly called hyperaccumulators, and they are pivotal in the remediation of contaminated sites since many of them are able to tolerate high contamination and other stressors often occurring on contaminated sites like droughts and with low concentrations of organic matter and micronutrients (Darajeh *et al.* 2016). However, since contaminated sites are often left unused for decades, they develop several secondary problems, including the loss of organic matter due to lack of vegetation and progressive desertification. Such issues make the restoration of land even more challenging (Roy *et al.* 2015). Thus, if plants cannot survive and thrive in the remediated area, the soil needs to be supplemented with fertilizers before starting any phytoremediation attempts (Nissim *et al.* 2018). Since it is crucial that the process stays cost-effective, the use of waste products, including various composts and sewage sludge, is currently most often implemented (Eid *et al.* 2018). Moreover, since the quantity of wastewater generated in developed countries is continually increasing and the remaining sewage sludge has to be disposed or treated in some manner, the use of sewage sludge as a fertilizer is becoming more common practice. It was shown before that this management strategy improves soil properties and increases plant productivity (Sarwar *et al.* 2017). Therefore, the re-use of biosolids like sewage sludge is proposed as an alternative solution for improvement in organic matter content and soil quality at low costs (Kumar *et al.* 2019).

In recent years, machine learning is starting to be applied in environmental studies due to the possibility to obtain precise prediction of several chemical and biological processes (Fan *et al.* 2017). Especially machine learning models capable of prediction of various bioconcentration factors in fish are getting more and more attention. In phytoremediation, process optimization is performed in order to seek and identify the ideal solution for certain conditions which inevitably leads to improving the overall efficiency of the process (Souza *et al.* 2018, Balasubramani *et al.* 2020). Overall, thorough optimization of assisted phytoremediation is a prerequisite, particularly for scaled-up applications in order to minimize process costs and maximize metal removal efficiency (Burges *et al.* 2017, Kumar *et al.* 2019). Classical methods of optimization in which only one factor changes at a time not only requires a lot of data collection but is also often incomparable to complex real-life scenarios since it disregards the interactive effects between certain variables. Hence, the use of complex approaches in which the variables are optimized

simultaneously is always more precise than single factor optimization (Šereš *et al.* 2018, Jaskulak *et al.* 2020). Moreover, the use of such strategies can substantially reduce the number of experiments required to predict the adequate process conditions or its efficiency in time (Movafeghi *et al.* 2016). In recent years, response surface methodology (RSM) and artificial neural networks (ANN) approaches have been implemented, often together for optimization and modeling of different processes, including environmental studies (Kausar *et al.* 2017, Eid *et al.* 2018).

The RSM approach is a collection of mathematical techniques useful for modeling and analyzing problems in which a response of interest is altered by more than one variable, providing ways in which interactions between several variables can be studied simultaneously (Martínez-Álvarez *et al.* 2015). Thus, the RSM uses a combination of experiments according to an experimental design to generate several mathematical models of different orders (including linear, nonlinear, quadratic, cubic, etc.), to find the optimum point for a particular set of response variables (Chen *et al.* 2018). On the other hand, ANN is a nonlinear computational modeling method that is gaining more and more interest but has only recently been applied to environmental research (Fan *et al.* 2018). One of the most desirable traits of ANN is its ability to imitate human intuition by searching for general conclusions when encountered with a lot of irrelevant, complex and incomplete information (Cao *et al.* 2017). Such proficiency in performing accurate generalizations of nonlinear processes makes it remarkably useful as a modeling tool (Sanusi *et al.* 2016).

The overall aim of this study was to model the assisted phytoremediation of a contaminated soil during sewage sludge application as a necessary soil amendment and *S. alba* L. growth. For this study *S. alba* was chosen due to its high resistance to abiotic stress and ability to accumulate cadmium in its above-ground parts (Matraszek *et al.* 2016). Previous studies had shown that *S. alba* plants are able to tolerate and accumulate relatively large amounts of several heavy metals. Such characteristic depends on the efficiency of HM uptake, translocation from roots to shoots and intracellular compartmentalization, which is widely seen in *Brassicaceae* plants, and associated with an elevated content of several defense proteins such as antioxidants, and metal chelators like metallothioneins. However, even when tolerant to HM, a prolonged exposition to high doses, as well as low quality of contaminated lands, still causes an adverse influence

on *S. alba*, which is why soil supplementation is often implemented for phytoremediation to ensure plants growth (Kuramshina *et al.* 2018).

Two model approaches were applied to the same set of data in order to asses and compare their applicability: the response surface methodology (RSM) and artificial neural networks (ANN). Both, RSM and ANN were used to describe the uptake of cadmium from contaminated soil affected by past smelting operations. Due to the severity of soil contamination and occurring desertification, sewage sludge was applied in two different doses to ensure plant survival. *S. alba* L. was selected for this particular study considering its capabilities to survive in a heavy-metal contaminated site and be resistant to other abiotic stressors like drought. Moreover, the use of fast-growing native plant species is highly beneficial for phytoremediation of HMs (Burges *et al.* 2017). Since the use of RSM and ANN approaches for phytoremediation of metals in real contaminated soil instead of lab-made contamination had not been reported in the literature, the ultimate goal of this study was to investigate the possible capabilities of RSM and ANN to model and optimize such processes and to explore their utility for planning large-scale operation.

2 MATERIALS AND METHODS

2.1 Soil and plants laboratory experiments

Soil contaminated by cadmium due to past smelting operations was used in this study. In accordance with the World Reference Base for Soil Resources (WRB) experimental soil belongs to podzols and is described as sandy loam containing approximately 15% clay, 20% silt, and 65% sand. In addition, soil was characterized in previous studies (Jaskulak *et al.* 2019, 2019) by low fertility, low content of organic matter, and reduced microbial activity. Topsoil (0 – 30 cm) was taken up from three separate contamination zones on the site which differed in their content of cadmium including one contamination-free zone (GPS for contamination zone: 50°30′N 18°56′E). Before starting the experiments, soil was dried and sifted. Dewatered municipal sewage sludge was obtained from a wastewater treatment plant in the Silesia region in Poland. Sludge was transported to the Faculty of Infrastructure and Environment (Czestochowa University of Technology, Czestochowa, Poland), where the experiment started immediately. The physical and chemical properties were assessed both before and after the research for all treatments. The dose of sewage sludge was applied to the soil in doses corresponding to 0.5 and 1 maximum EU nitrogen standards (170 kg of nitrogen/year/ha) (EUR-Lex - 31991L0676 e EN). The overall experiment consisted of 9 experimental treatments performed in triplicates. The specific treatments were as follows: 1) three soils without any supplementation, 2) three soils supplemented with half of the EU maximum recommended dose of sewage sludge, 3) three soils with the maximum recommended dose of sewage sludge. Each pot (H - 25 cm, a - 12 cm, b – 25 cm) contained 4 kg of soil and 96.5 or 48.25 g of sewage sludge (full/half of the dose). Cation exchange capacity (CEC) was determined by standard Sumner *et al.* 1996 method with HACH spectrophotometer (HATCH DR/4000V, USA). The contaminated zone was characterized by CEC at approximately 14.1 ± 0.32 cmolc/kg, whereas on contamination-free zone at 15.5 ± 0.32. A standard pH meter was used for pH determination (Cole Parmer Model No. 59002e00). The pH values of all treatments were measured in distilled water and 1 M solution of KCl following ISO 10390:2005. Total organic carbon was measured using a Multi N/C H1300 Analitykjena (PN-ISO 10694:2002). The content of total organic nitrogen was established using the Kjeldahl method (PN-ISO11261:2002) (Karczewska and Kabala 2008), while the concentrations of heavy metals were determined by ICS-OES (ICP-OES; Spectro apparatus- Arcos multi view, Germany) after

digestion in a microwave digestion system according to EPA method 3051. The content of total phosphorus was measured following the Egner method (Karczewska and Kabala 2008). Prepared growth media were left in the phytotron chamber for seven days before the planting of seeds. Twenty seeds of *S. alba* were sown approximately two cm deep. Commercially available, certified and high-quality seeds of *S. alba* were used (Dary-Podlasia, Poland). Subsequently, plants were incubated for 28 days in a growth chamber (Biogenet FS360, Poland) under controlled conditions: photoperiod: 16 h light 8 h dark, temperature during the day 21 °C, night 18 °C, light intensity 4000 lx (photosynthetic LED light), watering every 3 days according to plants requirements. Plants were harvested after 14, 21 and 28 days of the experiment and dried. The concentrations of metals in the shoots biomass were determined by ICS-OES after digestion in a microwave digestion system according to EPA method 3051. The experimental design and the results of soil physicochemical analyses are presented in Table 1.

Since supplementation of natural soil with sewage sludge is a complex and multi-variable change, in our study we initially focused on three independent factors that were previously shown to influence metal removal to the highest extent – the change in soil pH, the content of organic carbon and the content of nitrogen. However, since all soils had quite low pH (5.39 ± 0.06), similar to applied sewage sludge (5.58 ± 0.14), and our experimental data showed no statistical changes in soil pH for all experimental treatments, we focused on the remaining two variables (the content of organic carbon and the content of nitrogen) and the time of Cd exposure (data not shown).

Table 1. BBD design matrix for the chosen variables with the observed and predicted responses for Cd removal [Cd soil – content of cadmium in soil at the beginning of the experiment, Cd plant – content of cadmium in plants shoots after corresponding timepoint of exposure to given soil, TOC – total organic carbon]

Run number	**Cd soil [mg kg^{-1}]**	**Cd plant [mg kg^{-1}]**	**TOC [g kg^{-1}]**	**N [mg kg^{-1}]**	**Sampling day**	**Cd removal [%]**	**RSM predicted removal [%]**	**ANN predicted removal [%]**
1	1.12 ± 0.2	0.21 ± 0.03	19.8 ± 1.2	841.77 ± 23.14	21	18.54	32.83	19.24
2	1.12 ± 0.2	0.23 ± 0.08	19.8 ± 1.2	841.77 ± 23.14	28	20.16	35.89	12.59
3	1.12 ± 0.2	0.14 ± 0.10	19.8 ± 1.2	841.77 ± 23.14	14	12.64	31.64	13.46
4	1.12 ± 0.2	0.09 ± 0.02	9.6 ± 1.5	658.04 ± 15.11	21	8.28	19.80	7.55
5	1.12 ± 0.2	0.31 ± 0.04	26.4 ± 1.6	1012.25 ± 33.1	28	27.84	41.55	26.43
6	1.12 ± 0.2	0.18 ± 0.07	26.4 ± 1.6	1012.25 ± 33.1	14	15.73	23.86	16.78
7	1.12 ± 0.2	0.36 ± 0.11	26.4 ± 1.6	1012.25 ± 33.1	21	31.82	41.54	35.84
8	1.12 ± 0.2	0.07 ± 0.05	9.6 ± 1.5	658.04 ± 15.11	14	5.86	14.59	8.39
9	1.12 ± 0.2	0.11 ± 0.06	9.6 ± 1.5	658.04 ± 15.11	28	9.78	17.58	10.28
10	14.22 ± 0.3	6.09 ± 0.21	26.4 ± 1.6	1012.25 ± 33.1	21	42.84	44.63	43.21
11	14.22 ± 0.3	6.48 ± 0.18	26.4 ± 1.6	841.77 ± 23.14	28	45.55	46.22	47.18
12	14.22 ± 0.3	5.66 ± 0.29	26.4 ± 1.6	1012.25 ± 33.1	14	39.80	42.55	43.67
13	14.22 ± 0.3	3.67 ± 0.33	9.6 ± 1.5	658.04 ± 15.11	21	25.81	29.81	26.45

14	14.22 ± 0.3	4.74 ± 0.14	19.8 ± 1.2	841.77 ± 23.14	28	33.36	38.95	34.69
15	14.22 ± 0.3	1.85 ± 0.11	9.6 ± 1.5	658.04 ± 15.11	14	12.99	22.54	14.23
16	14.22 ± 0.3	4.53 ± 0.34	19.8 ± 1.2	841.77 ± 23.14	14	31.84	34.95	32.66
17	14.22 ± 0.3	4.65 ± 0.12	19.8 ± 1.2	841.77 ± 23.14	21	32.72	39.16	30.52
18	14.22 ± 0.3	2.54 ± 0.08	9.6 ± 1.5	658.04 ± 15.11	21	17.88	15.28	19.20
19	20.80 ± 0.8	11.95 ± 0.24	19.8 ± 1.2	841.77 ± 23.14	28	57.41	61.46	57.92
20	20.80 ± 0.8	10.65 ± 0.36	19.8 ± 1.2	841.77 ± 23.14	21	51.21	60.54	50.69
21	20.80 ± 0.8	11.18 ± 0.15	26.4 ± 1.6	1012.25 ± 33.1	21	53.74	55.63	54.74
22	20.80 ± 0.8	8.89 ± 0.12	19.8 ± 1.2	841.77 ± 23.14	14	42.73	48.18	44.18
23	20.80 ± 0.8	2.28 ± 0.17	9.6 ± 1.5	658.04 ± 15.11	14	10.96	22.31	8.78
24	20.80 ± 0.8	13.70 ± 0.37	26.4 ± 1.6	1012.25 ± 33.1	28	65.88	69.85	66.44
25	20.80 ± 0.8	9.96 ± 0.23	26.4 ± 1.6	1012.25 ± 33.1	14	47.87	51.27	48.32
26	20.80 ± 0.8	3.08 ± 0.19	9.6 ± 1.5	658.04 ± 15.11	21	14.82	15.50	15.65
27	20.80 ± 0.8	4.26 ± 0.27	9.6 ± 1.5	658.04 ± 15.11	28	20.50	24.61	20.91

2.2 Modeling cadmium removal via RSM

The Box-Behnken (BBD) method, which is a second-order model for a three-level incomplete factorial design, was used to design experiments and model the uptake of Cd from contaminated soil. The BBD design was also applied in order to assess the influence of sewage sludge supplementation on the uptake of cadmium by *S. alba* plants. The chosen variables and their coded levels are presented in Table 1. Adequacy checking for the model was conducted to determine whether the approximating model would give inaccurate results. Four different high-degree polynomial models such as linear, 2F1, quadratic and cubic models were applied and fitted to the experimental results to represent the relationship between the variables and the response (cadmium removal rate). Several tests, namely the sequential model sum of squares, model summary statistics and lack-of-fit tests, were performed to determine the adequacy of the selected approach. Based on the sequential model sum of squares, the quadratic model was significant ($p < 0.05$) compared to other models. Thus, the quadratic model was chosen as the best model. A quadratic model was developed to fit the coefficients acquired through multiple regression analysis. Overall, the relationship between the chosen variables was expressed using the aforementioned quadratic model as follows (Equation 1):

$$\mathrm{RR} = b_0 + b_1A + b_2B + b_3C + b_4D + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{34}CD + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{44}D^2 \quad (1)$$

Where: ‘RR’ is the removal rate of metal, A, B, C, D are the effects of independent parameters, (initial concentration of cadmium, content of TOC, content of nitrogen and sampling day, respectively), AB, AC, BC, BD, CD are the interactions of independent variables, A^2, B^2, C^2, D^2 are the square forms of independent variables, b0 is the BBD model constant, b1, b2, b3, b4 are linear coefficients of the model, b12, b13, b14, b23, b24 are the interaction coefficients between chosen parameters and b11, b22, b33, b44 are the second-order coefficients of the model. In total, the Box-Behnken design for 4 factors consisting of 27 points was used, as it is the classical optimal plan for quadratic model adjustment.

2.3 Modeling cadmium removal via ANN

For the ANN approach, MATLAB Neural Network Toolbox (MATLAB Deep Learning Toolbox version R2019a) was used. The feed-forward backpropagation multilayer perceptron ANNs (FFBPNNs) was chosen as a neural network since it is currently the most widely used network in the fields of ecotoxicology and environmental studies. FFBPNNs includes more than one input and output layer, and each of them consists of several neurons in which the number of neurons in the hidden layer is vital for the particular response. Thus, weights can be assigned in order to determinate the relationship between layers (Movafeghi *et al.* 2016). The network was trained via laboratory data from the experiment mentioned previously. In order to achieve the most appropriate response, the error between the predicted and the actual experimental response variable was returned to the network in ways that modified the weights. Since underfitting and overfitting can potentially occur in the ANN model, the early stopping approach was used as a solution. In brief, the input dataset was randomly divided into three parts: training, validation and test samples by proportions of 70%, 15% and 15%. In the next step, the dataset was applied to estimate the network weights, but at the same time, the calculated errors in the validation step were monitored and expected to decrease during the training. If the calculated error increased after a certain number of training repetitions, the training was stopped to make appropriate adjustments. To test its performance, the remaining 15% of the experimental data that the network have not train with was used, and the determination coefficient (R^2) of the experimental data was used as a suitable parameter for detecting overfitting in the network. The best structure of the neural network was a network that had the highest correlation coefficient (R) and the lowest error values while predicting the response (Sanusi *et al.* 2016). Since choosing the optimal number of neurons in the hidden layer is extremely important to prevent overfitting in the network, there are several approaches for choosing the number of neurons in the hidden layer while designing an ANN model. Some of them include the following relations and were chosen to the presented study (Equations 2 and 3):

$$\frac{2(i+o)}{3} < n < i(i+o) - 1 \qquad (2)$$

$$0.5i - 2 < n < 2i + 2 \qquad (3)$$

Where 'i' is the number of inputs, 'o' is the number of outputs and 'n' is the number of hidden layer neurons. Regardless of the method used to select the number of neurons in the hidden layer, it is highly advised by previous research always to choose the network that works most appropriately during the testing of given datasets with the smallest possible number of neurons in the hidden layer. Moreover, while testing the hidden neurons it is crucial to keep other parameters constant since any change at this point will create a new neural network with a different error surface which in the end would severely complicate the selection of the number of neurons in the hidden layer (Titah *et al.* 2018).

2.4 Validation of RSM and ANN models

All models need to be validated against experimental data before their application in real-life scenarios. In this study, model validation was performed by the correlation coefficient (r), the Mean Square Error (MSE) (Equation 4) and the Mean Absolute Error (MAE) (Equation 5) (Darajeh *et al.* 2016). Alternatively, other recent experiments showed an advantages of model validation via cross-checking (Fan *et al.* 2018, Amdoun *et al.* 2019) .

$$MSE = 1 - (\sum \frac{(C_{model} - C_{measured})^2}{(C_{model} - \acute{C})^2} \quad (4)$$

$$MAE = \sum(|C_{model} - C_{measured}|^2 / C_{measured})/n \quad (5)$$

Where: C_{model} is the predicted concentration of given contaminant by the chosen model;

$C_{measured}$ is the measured concentration of contaminant;

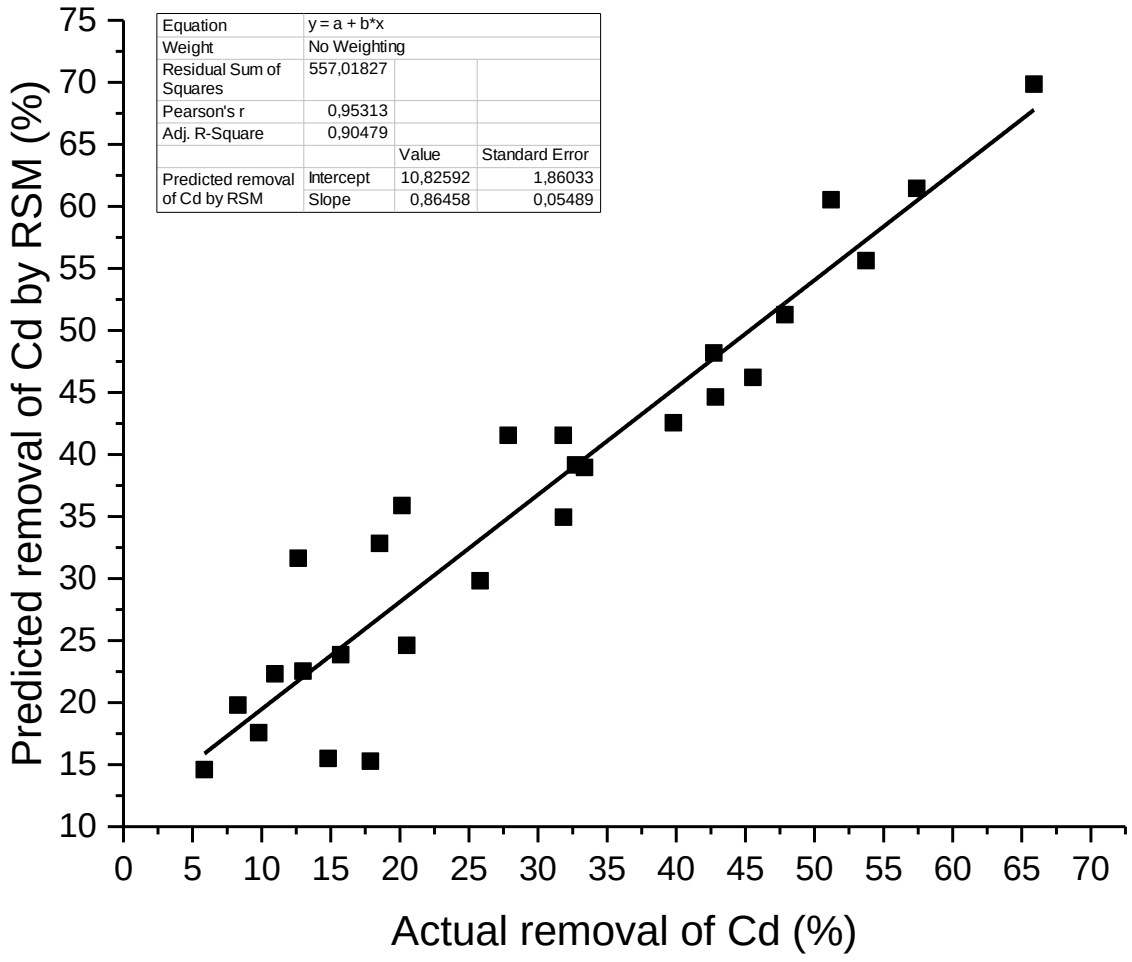
$\acute{C}$ is the mean of measured contaminant concentration;

n is the total number of observations;

3 RESULTS AND DISCUSSION

3.1 General observations, plant germination, biomass and roots length

S. alba was grown on soil contaminated with Cd. Due to the severe and quite old origin of contamination and thus, the desertification of the studied soil, to enhance the survival of plants, sewage sludge supplementation was used. For the comparison of different models that could predict the effects of such action, the impact of sewage sludge supplementation was investigated as supplementation with total organic carbon (TOC) and nitrogen. Initially, the results of such supplementation on soil pH and by that, on the bioavailability of the heavy metals were also supposed to be taken into consideration. However, due to the nature of studied soil and sewage sludge, the pH did not change significantly by supplementation with SS. The results were modeled using both the RSM and ANN approaches, and the modeling outputs can be observed in Figures 1, 2 and 3.



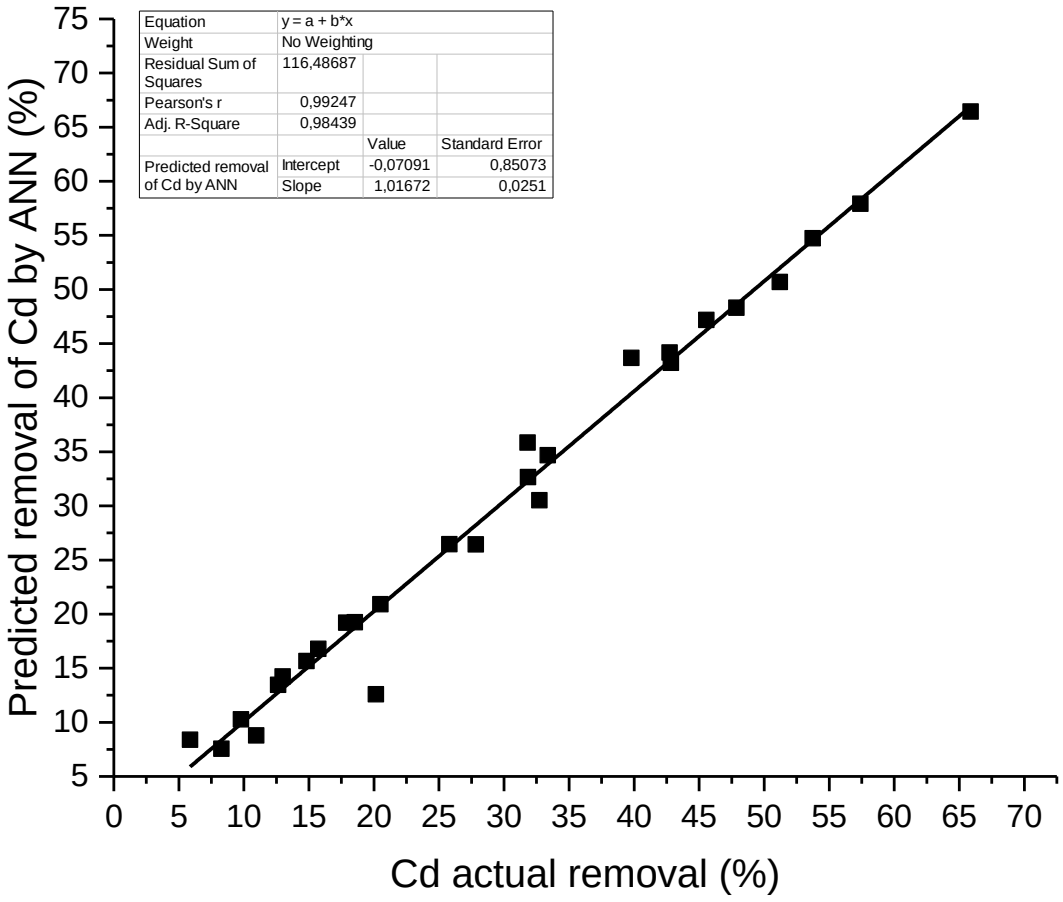
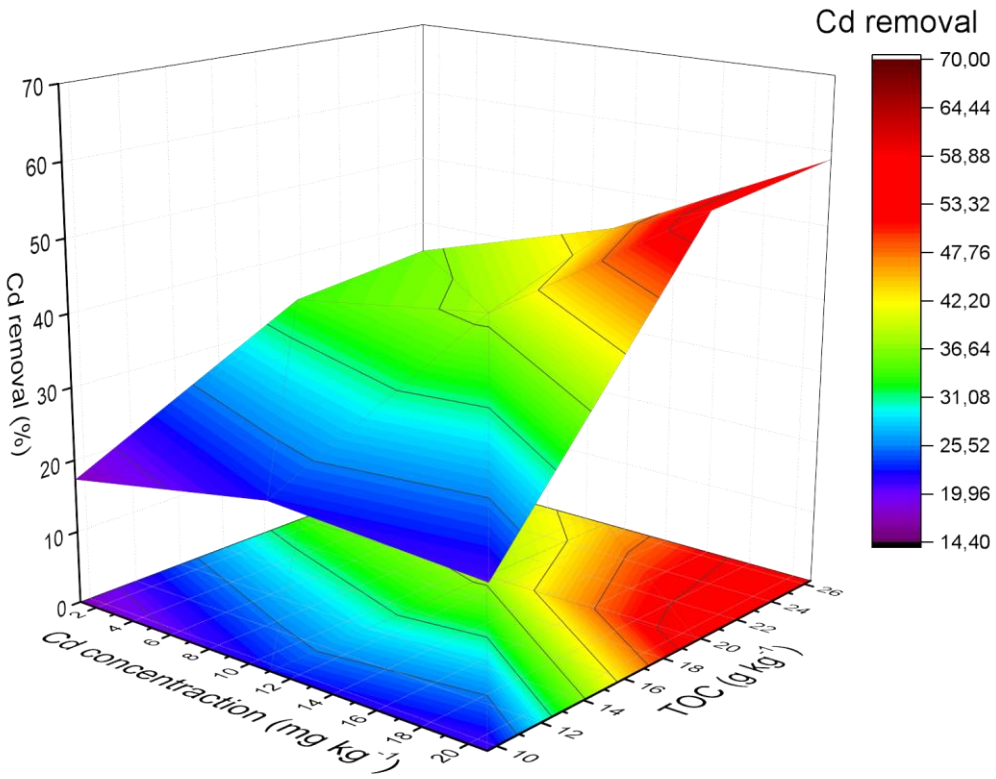
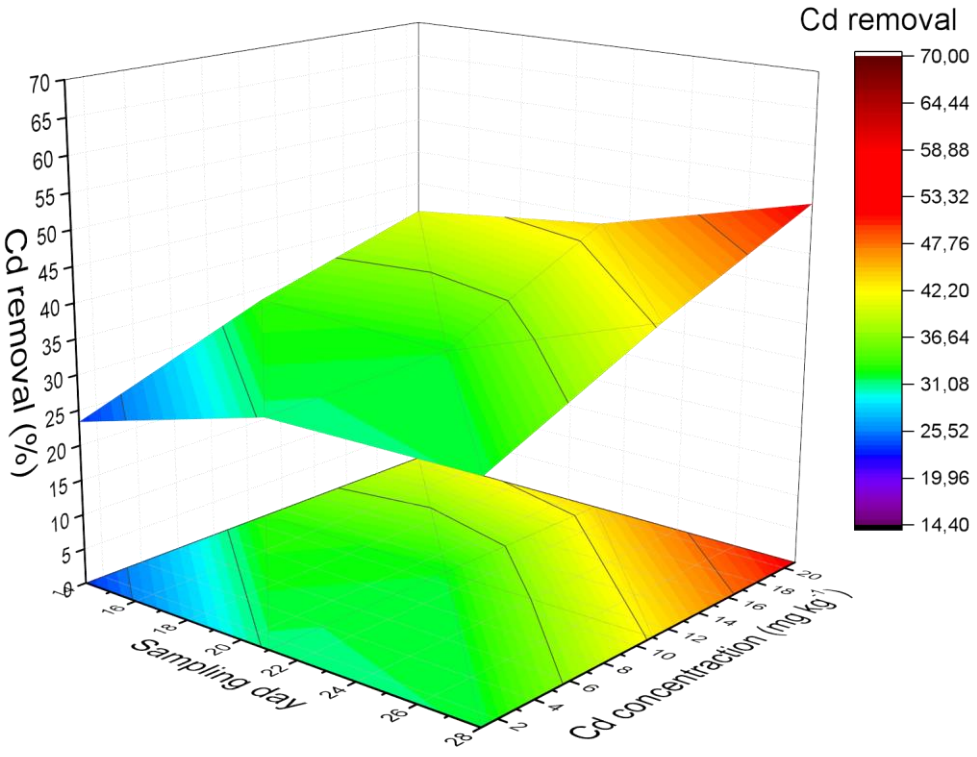


Figure 1. Comparison of the Cd removal rate for the actual experimental results and the predicted results by RSM and ANN models

A


Cd removal
70,00
64,44
58,88
53,32
47,76
42,20
36,64
31,08
25,52
19,96
14,40
Cd removal (%)
Cd concentration (mg kg-1)
TOC (g kg-1)

B


Cd removal
70,00
64,44
58,88
53,32
47,76
42,20
36,64
31,08
25,52
19,96
14,40
Cd removal (%)
Sampling day
Cd concentration (mg kg-1)

C

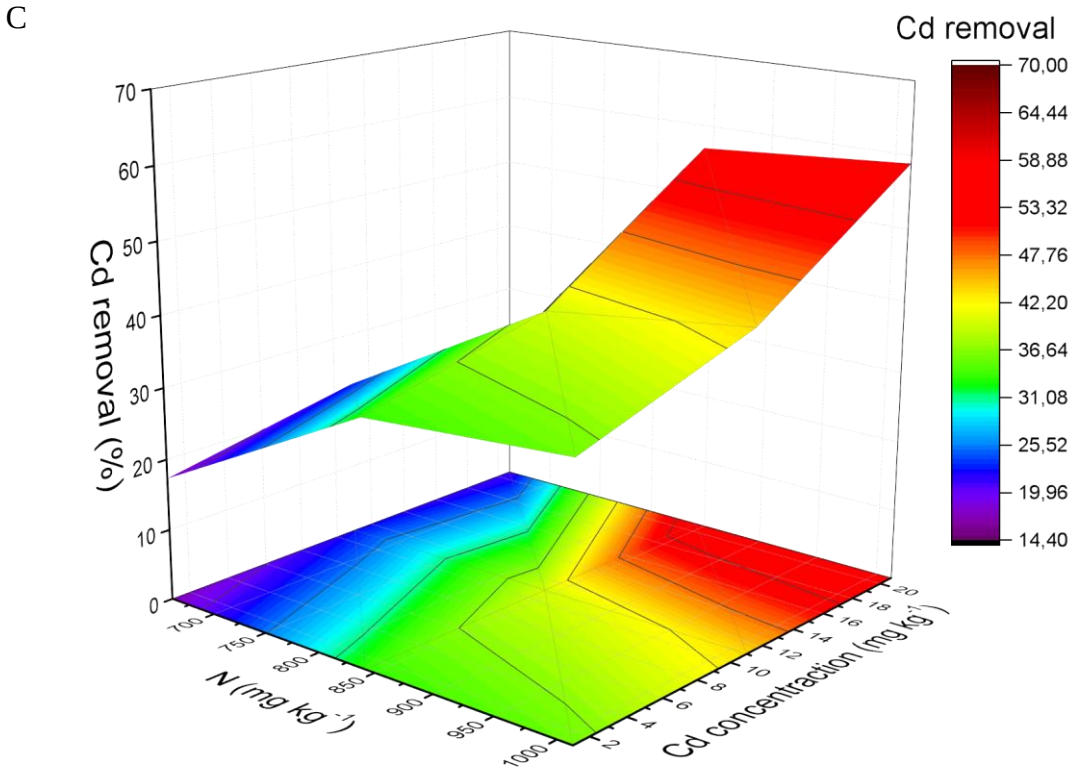
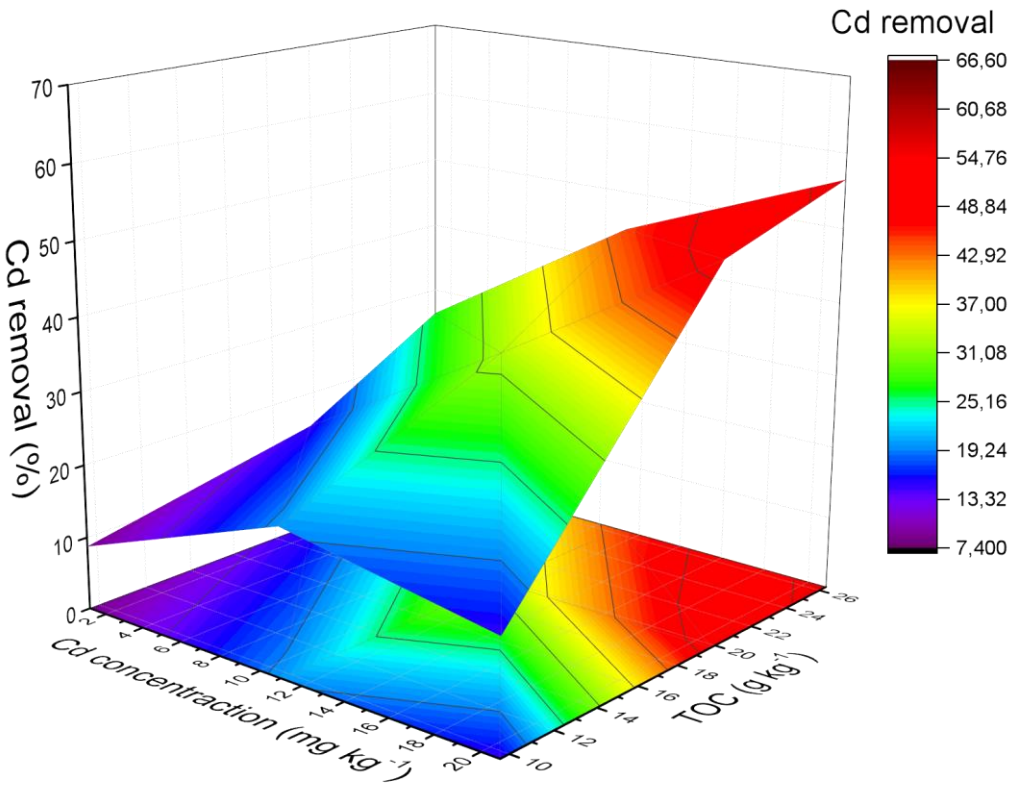
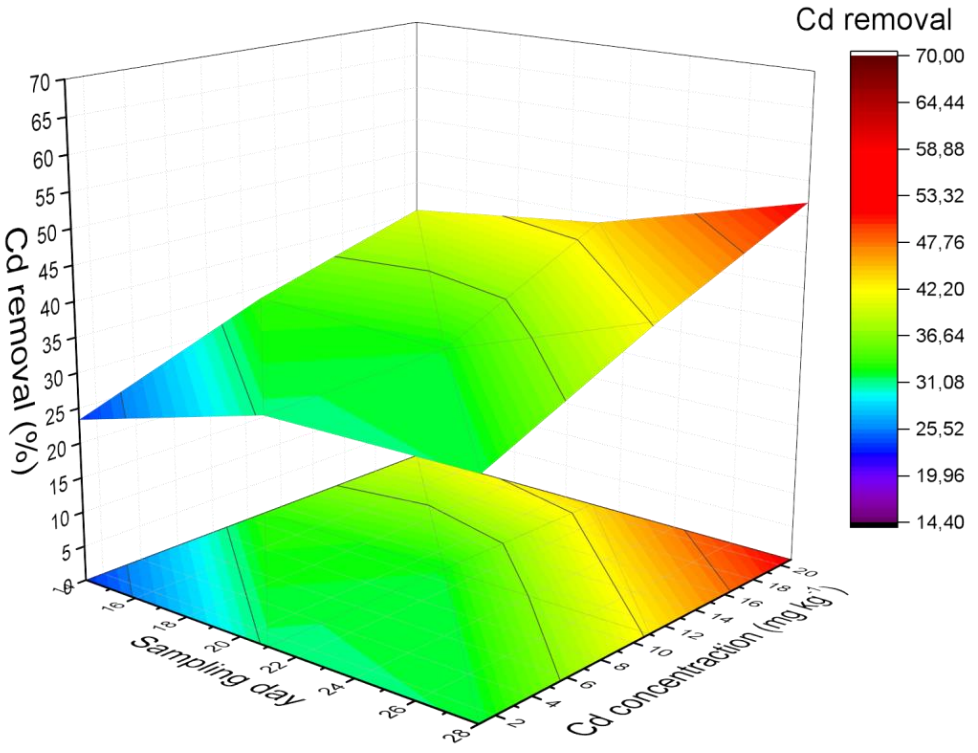


Figure 2. RSM response surface for; A - TOC; B - Sampling day; C – N in soil versus the initial concentration of Cd

B


Cd removal
66,60
60,68
54,76
48,84
42,92
37,00
31,08
25,16
19,24
13,32
7,400
Cd removal (%)
Cd concentration (mg kg-1)
TOC (g kg-1)


Cd removal
70,00
64,44
58,88
53,32
47,76
42,20
36,64
31,08
25,52
19,96
14,40
Cd removal (%)
Sampling day
Cd concentration (mg kg-1)

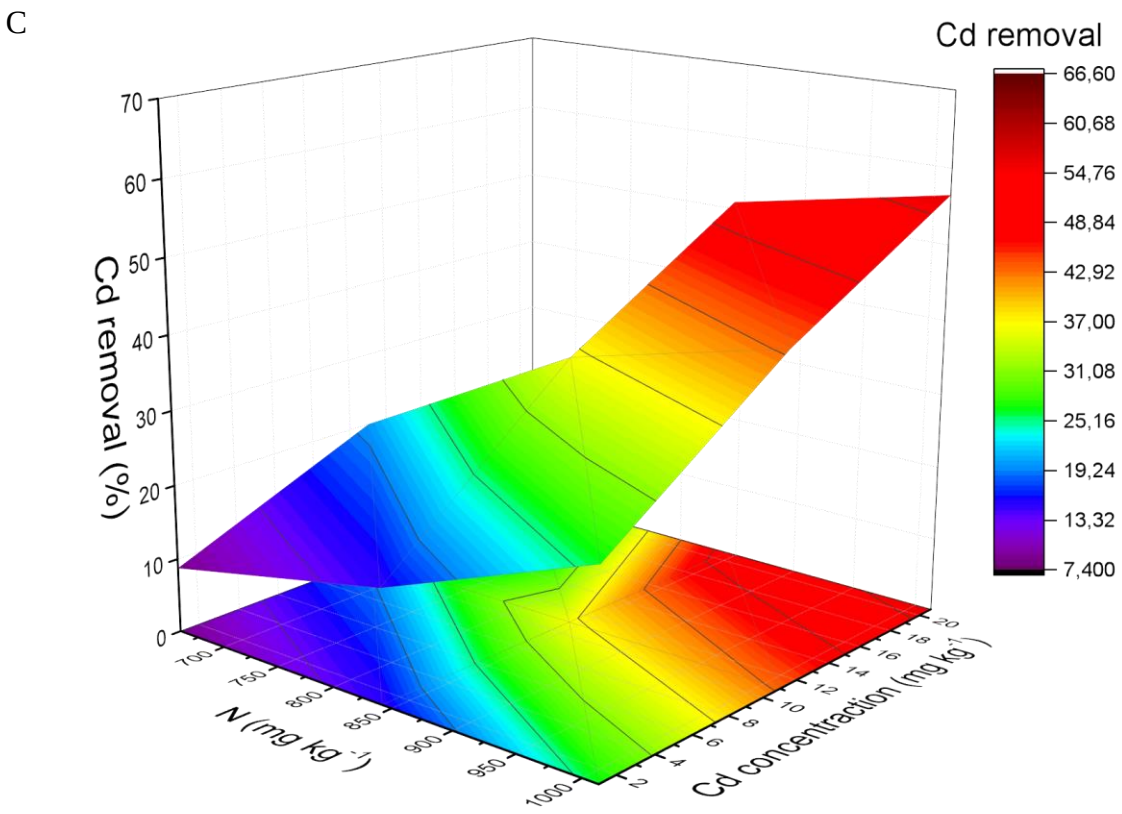


Figure 3. ANN response surface for; A- TOC; B- Sampling day; C- N versus the initial concentration of Cd

In comparison to the soil from the non-contaminated zone, the contamination severely reduced the germination index, plants biomass and roots length (Table 4 – supplementary materials). The supplementation of soil with sewage sludge reduced the direct effects of metal toxicity on plants in all experimental treatments. However, for germination and biomass there were no statistically significant changes between two selected doses of sewage sludge. Overall, the germination index on the most contaminated soil raised from 13% without any supplementation to 42% with sewage sludge. The plants biomass increased four-fold for the same treatments and the roots length was seen to increase more than three-fold. The obtained results are in agreement with several other studies dealing with HMs contamination, as it was extensively shown before the contamination with HMs and especially with cadmium inhibits proper development of plants roots what we also observed (Roy *et al.* 2015, Anning *et al.* 2018, Nissim *et al.* 2018). Overall, the supplementation of soil with sewage sludge had a positive effect on Cd removal from the

soil, and the response was also dose-dependent with the highest Cd removal after the highest dose of sewage sludge supplementation (Table 4 – supplementary materials).

3.2 Prediction of cadmium removal via RSM

All experiments were performed based on the RSM models' proposals and a non-linear quadratic equation was fitted to the obtained results by the multiple regression method. After the selection of variables and elimination of statistically non-significant factors ($p > 0.05$), the final equation of cadmium removal in RSM model was as follows (Equation 6):

$$\text{Cd removal rate [\%]} = 73.90 - 10.68 * A + 4.32 * B + 5.25 + C + 10.90 * D - 3.72 * A * C - 4.20 * B * C - 25.88 * A \quad (6)$$

Where: 'A' was the initial concentration of given metal (mg kg^{-1}), 'B' was the content of organic carbon (g kg^{-1}), 'C' was the content of nitrogen (mg kg^{-1}) and 'D' was the sampling day. The comparison of the experimental results to the prediction via a chosen and optimized model have been presented in Figure 1 and Table 1.

In our study, the supplementation of contaminated soil with sewage sludge severely increased the removal of Cd (Figure 1, Table 1). From the standpoint of the phytoextraction of contaminants, it is an advantageous action. However, other studies show contradictory results. In several papers when soil supplementation with sewage sludge was applied, the uptake of metals by plants decreased after supplementation (Almasi *et al.* 2018, Nissim *et al.* 2018). At the same time, in different experiments, such bioaccumulation was showed to increase in a similar matter to our study (Pulford *et al.* 2002, Gautam *et al.* 2017). Overall, the increase in Cd removal after sewage sludge supplementation could be caused by a couple of factors. We eliminated the element of change in soil pH since the pH did not change significantly after the supplementation. In previous studies, the increase in soil pH was showed to be responsible for decreasing the accumulation of metals in plant biomass due to the decrease in metal mobility (Almasi *et al.* 2018). Our results allowed us to find that in contaminated soil, without any supplementation, plants did not accumulate higher concentrations of Cd due to the severe inhibition of root development from the contamination, whereas after the supplementation with sewage sludge which is rich in organic matter and nitrogen, the

roots did grow allowing for the much higher accumulation of Cd. The removal rate varied from 10.96% in soil with the highest contamination without any supplementation and after 14 days of exposure to 65.88% in the same soil supplemented with the highest dose of sewage sludge after 28 days of exposure (Table 1). In comparison to the experimental data, the RSM model was able to predict Cd removal for the sludge-assisted phytoremediation most precisely. The univariate Analysis of Variance (ANOVA) was used in order to examine the significance of differences between different groups. The results are presented in Table 2. The RSM approach requires a lack of fit to be analyzed through ANOVA and F-test. While the original model should be significant and smaller p-values are more appropriate, the lack of fit should not be significant. This means that the observational errors are not directed, substantial, and systematic. It can even be assumed that the selected factors are suitable for analyzing the response variable (Selamat *et al.* 2017). In our experiment, the results of the lack of fit have been presented in Table 2. Considering the p-value above 0.05, the errors were not significant, and the chosen modeling approach was adequate (Table 2). Overall, the F-value was 22.51 and the p-value was lower than 0.05, which indicates that the second-order regression equation of the model for the prediction of Cd extraction was significant at a suitable level. It should be taken into consideration that in the RSM approach, the components of the model must be significant (Titath *et al.* 2018). Hence, parameters with p-values above 0.05 have been extracted from the model (AB, AD, BD, CD, B2, C2 and D2). Overall, there was a sensible consensus between the R-squared (0.953), adjusted R-squared (0.904) and expected R-squared (0.925) coefficients. Moreover, AdeqPrecision, which is the signal-to-noise ratio measurement, was also checked for the RSM approach (Table 2). Usually, the model is adequate if the AdeqPresicion value is above 4, and in this experiment, it was 14.56, indicating an appropriate signal (Table 2). The findings, therefore, reflected the capacity of the model to predict the process of Cd removal during the sludge-assisted phytoremediation. Similar utility of the implementation of RSM modeling in environmental studies was observed by Chen *et al.* 2018 for the biodegradation of polycyclic aromatic sulfur heterocycles. Moreover, in a study by Darajeh *et al.* 2016, *Chrysopogon zizanioides* was used to decrease the chemical and biological oxygen demand (COD, BOD) form palm oil mill secondary effluent in floating wetland. Results of the RSM modeling were similar to ours, close to experimental data which suggested that such action can be used to model the large-scale process. In another study RSM approach was used to optimize the bioaccumulation of lead in *Melastoma malabathricum*.

The model was shown to be accurate in its prediction in comparison to experimental data. However, study was performed in *in vitro* conditions and without soil which highly reduced the number of possible variables as well as its applicability in real-life scenarios (Selamat *et al.* 2017). In another study, kinetic model was developed to predict the purification of wastewater by *Eichhornia crassipes* using the various forms of nitrogen as a model input. Results showed that in total, the content of nitrogen was removed by 63% but the denitrification contributed 73.8% of the removed nitrogen. Such models were previously shown to be successful in the assessment of water contamination and water treatment, but studies on soil contamination still require more research (Mayo *et al.* 2017).

Table 2. ANOVA for response surface quadratic model (A, B, C, D are the effects of independent parameters, (initial concentration of cadmium, content of total organic carbon, content of nitrogen and sampling day, respectively)

Source	**Sum of squares**	**Mean square**	**F-value**	**p-value**	
Model	805608,6441	115086,9	22.51	<0.0001	significant
A	2614,3236	2614,3236	30.22	<0.0001	significant
B	1934,28	1934,28	17.85	<0.0001	significant
C	799813,262	799813,262	11.32	<0.0001	significant
D	1246,777	1246,777	9.25	0.0068	significant
AC	176.84	176.84	5.56	0.0391	significant
BC	195.22	195.22	5.71	0.0422	significant
A^2	27338.75	27338.75	106.54	<0.0001	significant
Residual	1065.66	42.11			
Lack of fit	1048.45	49.52	6.24	0.0921	Not significant
Pure error	22.68	7.82			
R^2	0.9107				
Adjusted R^2	0.8864				
Predicted R^2	0.8652				
Adeq Precision	14.26				

3.3 Predicting cadmium removal by sewage-sludge assisted phytoremediation using the ANN approach

For the presented experiment, in order to choose the best ANN structure, the 4:4:1 network topology was initially assessed against nine different training algorithms and the most suitable one was selected based on its performance. The experimental results shown in Table 1 were used as network inputs and the (Levenberg–Marquardt LM) algorithm was proved to have the highest correlation coefficient for all datasets, as well as the lowest MSA and MAE among all tested training algorithms (Table 3). After that, based on equations 3 and 4, the use of 4-10 neurons were proposed in the hidden layer and assessed with the chosen algorithm (Levenberg–Marquardt LM), to choose the most optimal number of neurons in the hidden layer. For our datasets, the implementation of eight neurons was observed to produce the best R and the lowest MSE and MAE (Table 5 – supplementary materials).

Table 3. Comparison between training algorithms available in MATLAB for Multilayer Neural Network

Training algorithm	**MATLAB acronym**	**R (test data)**	**MSE**	**MAE**
BFGS Quasi-Newton	BGF	0.918	0.00088	0.0195
Levenberg-Marquardt*	LM	0.992	0.00014	0.0098
Resilient Backpropagation	RP	0.974	0.00548	0.0389
Scaled Conjugate Gradient	SCG	0.746	0.00564	0.0531
Conjugate Gradient with Powell/Beale Restarts	CGB	0.626	0.00698	0.0632
Fletcher-Powell Conjugate Gradient	CGF	0.488	0.00475	0.0224
Polak-Ribiére Conjugate Gradient	CGP	0.890	0.00054	0.018

One Step Secant	OSS	0.492	0.00612	0.0548
Variable Learning Rate Backpropagation	GDX	0.782	0.00098	0.0254

*** Levenberg-Marquardt algorithm was chosen due to lowest MSE and MAE**

The final results of the prediction by the trained neural network are presented in the Table 1, and the correlation between the experimental and predicted results is presented in Figure 3. In total, the results predicted via the ANN approach were strictly consistent with the experimental results. Moreover, the ANN approach was shown to predict the actual removal rate better than the RSM approach, especially for a lower concentration of Cd where the RSM model showed some over and underestimations (Figure 1). Based on Figures 2 and 3, the ANN approach was observed to be able to predict the Cd removal rate with much higher precision. In contrast, the RSM model showed some over and underestimations, especially when the Cd concentration was low. Similarly, in a study by Sanusi *et al.* 2016, researchers investigated RSM and ANN approach for the optimization of pilot-scale total petroleum hydrocarbon (TPH) degradation by *Paspalum scrobiculatum* L. during which, the ANN model was shown to give more accurate response. Due to its complexity, the process of assisted phytoremediation was not often researched in the past. However, a study by Janani *et al.* 2019 focused on the optimization of zinc phytoaccumulation in *Ophiopogon japonicus* grown on Zn-contaminated soil enriched with EDTA. Study also compared RSM and ANN approaches. After optimization of ANN model, the prediction of Zn phytoaccumulation efficiency was found to be 88.23% accuracy in comparison to experimental data. In addition, the same experimental data was used as an input onto Random Forest (RF) model in order to develop a monitoring system in real-time. The performance and flexibility of the RF model was found to be suitable for real-time monitoring of phytoextraction of Zn by *Ophiopogon japonicus* in soils enriched with EDTA. Interestingly, ANN was also recently used to assess the uptake of cerium oxide nanoparticles and Cd uptake by *Brassica napus*, a species closely related to *S. alba* used in our study. The ANN approach was shown to be able to predict the physiological changes in plants as well as metal uptake with high efficiency. However, the study was conducted *in-vitro*, which significantly reduced the number of possible variables (Rossi *et al.* 2019). Another study, on the phytoextraction of arsenic by *Ludwigia octovalvis* also noticed better adjustment of ANN

model in comparison to RSM with a higher R^2 (0.97) close to 1.0 and very small Average Absolute Deviation (AAD) (0.02) and Root Mean Square Error (RMSE) (0.004). Nevertheless, the study was performed on spiked soil and needs more evaluation to be used in large-scale operations (Titath *et al.* 2018). Moreover, similarly to our results, study by Roohi *et al.* 2019 used ANN approach to model the phytoremediation ability of *Bromus tomentellus* exposed to soils contaminated with Zn and Cr and supplemented with municipal waste compost and biochar. The obtained results also have shown that ANN approach can be used for prediction of optimal soil treatment that will reduce the toxicity and to obtain a given target of Cr and Zn in plant tissues from given set of soil properties. An interesting solution for future studies could be to combine both ANN-RSM models into one approach, which could potentially improve prediction capability by taking advantages from both models. A recent study by Amdoun *et al.* 2019 explored such possibility for the prediction of Cr removal by hairy roots. Although both models (RSM and ANN) provided decent quality predictions for the chosen parameters within the design range, the ANN model was shown to be superior over the RSM for data fitting and estimation capabilities which is with an agreement with our study and previous studies focused mostly on water contamination (Hamid et al. 2016, Rosi *et al.* 2019). Other experiments concerning the modeling of metal removal from contaminated water showed that ANN approach could usually be more precise because the assumption of quadratic non-linear correlation limits the RSM approach, whereas the ANN approach can overcome that since it can inherently capture complex and non-linear process. Moreover, unlike RSM, the ANN was a more accurate prediction model, but it did not allow easy separation of the effects of the two chosen factors and interactions between them. An ANN-RSM approach combined the accuracy of the ANN model with the ability of the RSM model to statically separate the effects of chosen factors and their interactions (Amdoun *et al.* 2019).

4 CONCLUSIONS

The presented study was the first attempt to model Cd extraction during sewage sludge assisted phytoremediation via RSM and ANN approaches. The change in soil content of organic carbon and nitrogen were chosen as valid for modeling. Overall, the supplementation of sewage sludge significantly increased the removal rate, which was the highest after 28 days of exposure to contaminated soil supplemented with the highest dose of sewage sludge. The results showed that the ANN model with the LM training algorithm and eight neurons in the hidden layer could predict the removal rate with much higher accuracy than the RSM model. Therefore, the ANN approach with proper process optimization can be applied to plan effective metal phytoextraction on a large-scale. Moreover, the ANN approach can also be a useful tool in assessing the optimal dose of sewage sludge required for the highest efficiency of the process. The comparison between the RSM and ANN approach proved that the prediction capability of the ANN model was higher than that of the RSM model (for RSM: adjusted R-square: 0.90; standard error of the prediction of Cd removal 1.86 ± 0.05, whereas for ANN: adjusted R-square was higher: 0.98, and the standard error of the prediction of Cd removal was lower: 0.85 ± 0.02). Thus, the ANN model could be used for the prediction of heavy metal removal during assisted phytoremediation with sewage sludge.

Appendix: supplementary data:

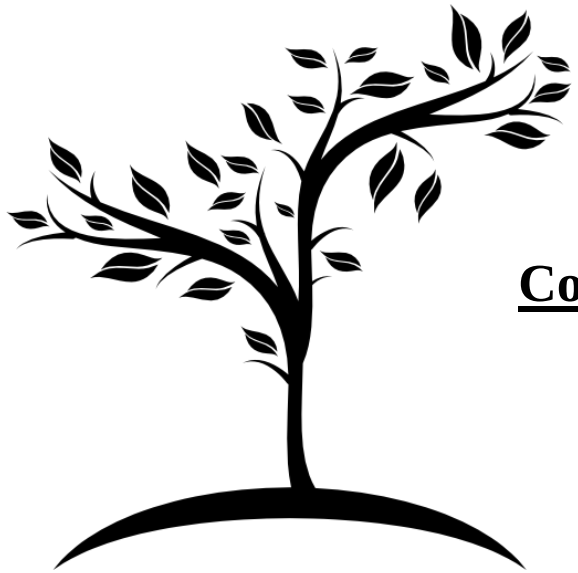
Table 4. Germination index, plants biomass and roots length after 28 days of exposure [A, B, C – soils with Cd concentrations: 1.12, 14.22, 20.80 mg kg^{-1} respectively, A+0.5SS, B+0.5SS, C+0.5SS – soils with half of a full dose of sewage sludge supplementation based on the EU norm (EUR-Lex - 31991L0676 e EN), A+SS, B+SS, C+SS – soils with full dose of sewage sludge supplementation (EUR-Lex - 31991L0676 e EN). All results are expressed as means ± standard deviation, n = 3. Means within a column followed by the different letters are significantly different according to Tukey test ($p < 0.05$).]

Treatment	Average germination index [%]	Biomass [g]	Root length
A	96a	7.45 ± 0.4a	15.24 ± 1.5a
B	43b	3.2 ± 0.3b	5.27 ± 1.6b
C	13c	2.0 ± 0.2c	1.81 ± 1.1c
A+0.5SS	96a	9.1 ± 0.4d	19.7 ± 0.8d
B+0.5SS	73d	7.7 ± 0.1a	11.4 ± 0.5e
C+0.5SS	41b	3.5 ± 0.3b	5.21 ± 0.b
A+SS	97a	9.5 ± 0.8d	19.1 ± 0.4d
B+SS	75d	3.9 ± 0.5b	11.5 ± 0.2e
C+SS	42b	3.8 ± 0.3b	6.84 ± 0.b

Table 5. Comparison between the use of different number of neurons in the hidden layer

Number of Neurons in hidden layer	R (test data)	MSE	MAE
4	0.788	0.00546	0.0632
5	0.851	0.00035	0.0211
6	0.983	0.00022	0.0145
7	0.987	0.00019	0.0098
8	0.997	0.00006	0.0081
9	0.961	0.00045	0.0138
10	0.954	0.00208	0.0314

CHAPTER V



Conclusions and perspecitves

5.1 Main conclusions

The main scientific goal of the work was to determine the effects of soil additives (including sewage sludge and manures) on changes in the expression of selected genes in plants (including genes encoding: metallothioneins, phytochelatins, metal transporters, RuBiSCO), in order to analyze the effectiveness of assisted phytoremediation process, as well as the safety assessment of the use of waste products in terms of potential genotoxic effects. The planned research contributed to a better understanding of the mechanisms of the stress response of plants after exposure to heavy metals, silver nanoparticles, as well as selected soil additives of different specifics and content of micropollutants. The obtained results illustrated the effects of the use of sewage sludge on the reduction of oxidative stress and the limitation of DNA damage in plants exposed to heavy metals (both in the case of contamination created in laboratory conditions, contamination present on soils contaminated by the metallurgical industry and on agricultural soil). The obtained results can be used in processes related to the remediation of metal contaminated sites. The use of selected biomarkers led to the selection of optimal process conditions guaranteeing an increase in plant yield, which in turn had a significant impact on the effectiveness of the assisted phytoremediation process.

Overall, the performed studies resulted in the following conclusions:

- Basic indicators of environmental toxicity - including in particular the germination index, are sensitive markers only in model plants. In the selected plants from the *Fabaceae* family, which are widely used in the phytoremediation process, the germination index is not a sensitive indicator to assess the degree of environmental toxicity nor to assess the impact of a given soil additive on plant growth and development;

- Presented study provided insights into plants stress markers that can be used to assure safe application of sewage sludge into soils. Research showed that ecotoxicological markers such as the level of DNA damage, the content of chlorophyll and the expression of *rbcL* and *mt* could provide a more accurate description of the influence of specific sewage sludge on plants metabolism.

- The study showed the effects of sewage sludges on the level of genotoxic effects caused by heavy metals as well as on *MT* and *ABCC* and *ABCG* expression. As such, a significant increase in the expression levels of those genes was observed in plants grown under metal stress;

- With proper supplementation of degraded soil with sewage sludge, the level of DNA damage and *MT* expression significantly decreased;

- *ABC* and *MT* expression are sensitive biomarkerm for the early prediction of a phytoremediation outcome;

- The activity of guaiacol peroxidase, the content of phenolic substances, chlorophyll, proteins and the expression of genes encoding RuBisCO and metallothioneins show a different stress response in individual plant species, while maintaining the same upward or downward tendencies. This means that the presented indicators can be used as stress markers regardless of the taxonomic affiliation of a given species, in contrast to the germination index and root length, which can only be effectively used for model plants.

The obtained data deepened our understanding of the molecular mechanisms of heavy metal tolerance in plants and the safety of using sewage sludge as a fertilizer on degraded soils. A number of genes related to metal binding and transport and stress signaling have been recognized as significantly altered expression genes upon exposure to heavy metals/sludge. Overexpression of some of them at the early stage of phytoremediation (including metallothioneins, after 3 days of exposure) was a factor determining the long-term survival of plants in a given area, which can be used in planning phytoremediation on a large scale, in order to select an appropriate soil additive and or its survival rate.

5.2 Recent work and future perspectives

Currently, we perform an analysis from the last finished experiment titled: "Analysis of transcriptomic and physiological changes in leaves of *Brassica napus* L. after the exposure to heavy metals, with particular emphasis on the application of sewage sludge on degraded soils"

The primary project objective is to evaluate the transcriptomic changes in *B. napus* after exposure to heavy metal contaminated soil and its supplementation with sewage sludge. Moreover, the project aims to identify the mechanisms in which sewage sludge supplementation is influencing plants on the whole transcriptome level to assess the overall, safety of such actions. RNA-seq technique will be used to explore transcriptome of *B. napus* and to identify changes in gene expression in response to field-tested and complex metal contamination as well as the long-term impact of sewage sludge application on changes in metabolic pathways in *B. napus*. Obtained data will help in furthering our understanding of the molecular mechanisms of heavy metal tolerance in plants and the safety to use sewage sludge as a fertilizer in agricultural and degraded soils. Significantly differentially expressed genes (DEGs) will be recognized as associated with binding, transport, metabolic pathways, and signaling. Hence, the project will offer new information regarding biological changes and molecular mechanisms related to metal stress response and the influence of soil sewage sludge application on plants.

The project consist of a first study dealing with the influence of long-term sewage sludge application on plants metabolism on a transcriptomic level. The study will also illustrate the ways in which sewage sludge application reduces plants oxidative stress and limits the damage caused by HMs toxicity. The main aim of the project will allow for an in-depth assessment of the interactions between elements: soil contamination, sewage sludge application, plants response, which will have a significant impact on the planting of large-scale phytoremediation operations.

REFERENCES

[1] Adebesin, F., Widhalm, J. R., Boachon, B., Lefèvre, F., Pierman, B., Lynch, J. H., Alam, I., Junqueira, B., Benke, R., Ray, S., Porter, J. A., Yanagisawa, M., Wetzstein, H. Y., Morgan, J. A., Boutry, M. (2017). Emission of volatile organic compounds from petunia flowers is facilitated by an ABC transporter. Science, 356, 1386–1388.

[2] Adiloglu, A. (2017). The effects of manganese on the remediation of the heavy metal contaminated soil by using the dock (*Rumex patientia* L.) plant. Desalination and Water Treatment 93, 335-338.

[3] Agnihotri, A., Seth, Ch. (2019). Chapter 11 - Transgenic *Brassicaceae*: A Promising Approach for Phytoremediation of Heavy Metals. Transgenic Plant Technology for Remediation of Toxic Metals and Metalloids. Academic Press, 239-255. doi:/10.1016/B978-0-12-814389-6.00011-0.

[4] Alaraidh, I.A., Alsahli, A.A., Abdel Razik, E.S. (2018). Alteration of antioxidant gene expression in response to heavy metal stress in *Trigonella foenum-graecum* L., South African Journal of Botany 115, 90-93.

[5] Alaribe, F.O., Agamuthu, P. (2015). Assessment of phytoremediation potentials of *Lantana camara* in Pb impacted soil with organic waste additives. Ecol Eng 83, 513–520.

[6] Alavi, S. M., Arvin, M. J., Kalantari, K. M. (2014). Salicylic acid and nitric oxide alleviate osmotic stress in wheat (*Triticum aestivum* L.) seedlings. J. Plant. Interact. 9 (1), 683-688.

[7] Aleixandre-Benavent, R., Aleixandre-Tudó, J., Castelló-Cogollos, L., Aleixandre, J. (2017). Trends in scientific research on climate change in agriculture and forestry subject areas (2005–2014). J. Clean. Prod. 147, 406–418.

[8] Ali, H., Khan, E., and Sajad, M. A. (2013). Phytoremediation of heavy metals – Concepts and applications, Chemosphere, 91, 869–881.

[9] Allen, R.G., Tresini, M. (2000). Oxidative stress and gene regulation. Free Radical Biology and Medicine 28, 463–499.

[10] Allison, J.D., Brown, D.S., Novo-Gradac, K.J. (1991). MINTEQA2/PRODEFA2, a geochemical assessment model for environmental systems: version 3.0 user's manual, Report EPA/600/3-91/021. United States Environmental Protection Agency. Athens, Georgia, USA.

[11] Almaroai, Y.A., Usman, A.R. A., Ahmad, M., Kim, K.R., Vithanage, M. and Ok, Y.S. (2013). Role of chelating agents on release kinetics of metals and their uptake by maize from chromated copper arsenate-contaminated soil. Environ. Technol. 34: 747755

[12] Almasi A, Mohammadi M, Mosavi SA, Eghbali S. (2018). Phytoremediation potential of sewage sludge using native plants: *Gossypium hirsutum* L. and *Solanum lycopersicum* L. International Journal of Environmental Science and Technology. 16(10):6237–6246. doi:10.1007/s13762-018-2030-2

[13] Alscher, R. G. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53 (372), 1331-1341.

[14] Alvarenga, P., Farto, M., Mourinha, C., & Palma, P. (2016). Beneficial Use of Dewatered and Composted Sewage Sludge as Soil Amendments: Behaviour of Metals in Soils and Their Uptake by Plants. Waste and Biomass Valorization, 7(5), 1189-1201. doi:10.1007/s12649-016-9519-z

[15] Alvarenga, P., Mourinha, C., Farto, M., Santos, T., Palma, P., Sengo, J., Morais, M.-C., Cunha-Queda, C. (2015). Sewage sludge, compost and other representative organic wastes as agricultural soil amendments: benefits versus limiting factors. Waste Management, 40, 44–52

[16] Alvarez-Vázquez, L.J., Martínez, A., Rodríguez, C., Vázquez-Méndez, M.E., Vilar, M.A. (2019). Mathematical analysis and optimal control of heavy metals phytoremediation techniques. Appl. Math. 73, 387-400.

[17] Amdoun R, Benyoussef EH, Benamghar A, Khelifi L. (2019). Prediction of hyoscyamine content in *Datura stramonium* L. hairy roots using different modeling approaches: Response Surface Methodology (RSM), Artificial Neural Network (ANN) and Kriging. Biochemical engineering journal. 144:8-17. doi:10.1016/j.bej.2019.01.002

[18] An, Y.J. (2004). Soil ecotoxicity assessment using cadmium sensitive plants. Environmental Pollution 127, 21–26.

[19] Anjum, N. A., Umar, S., & Iqbal, M. (2014). Assessment of cadmium accumulation, toxicity, and tolerance in *Brassicaceae* and *Fabaceae* plants — implications for phytoremediation. Environmental Science and Pollution Research, 21(17), 10286–10293. doi:10.1007/s11356-014-2889-5

[20] Anning AK, & Akoto R. (2018). Assisted phytoremediation of heavy metal contaminated soil from a mined site with *Typha latifolia* and *Chrysopogon zizanioides*. Ecotoxicology and Environmental Safety. 148:97–104. doi:10.1016/j.ecoenv.2017.10.014

[21] Araujo, A.S.F., Monteiro, R.T.R. (2005). Plant bioassays to assess toxicity of textile sludge compost. Scientia Agricola 62, 286-290.

[22] Aravind, P., Prasad, M.N. V., Malec, P., Waloszek, A., and Strzalka, K. (2009). Zinc protects *Ceratophyllum demersum* L. (free-floating hydrophyte) against reactive oxygen species induced by cadmium. J. Trace Elements Med. Biol. 23: 50–60.

[23] Arnon, D. I. (1949). Copper Enzymes In Isolated Chloroplasts. Polyphenoloxidase In Beta Vulgaris. Plant Physiol. 24 (1), 1-15.

[24] Asada, K. (2006). Production and Scavenging of Reactive Oxygen Species in Chloroplasts and Their Functions: Figure 1. Plant Physiology, 141(2), 391-396. doi:10.1104/pp.106.082040

[25] Astolfi, S., Zuchi, S., Neumann, G., Cesco, S., Sanita′ di Toppi L., and Pinton, R. (2011). Response of barley plants to Fe deficiency and Cd contamination as affected by S starvation. J. Exp. Bot. 63: 1241–1250.

[26] Azab, E., Hegazy, A. K., El-Sharnouby, M. E., & Abd Elsalam, H. E. (2016). Phytoremediation of the organic Xenobiotic simazine by p450-1a2 transgenic *Arabidopsis thaliana* plants. International Journal of Phytoremediation, 18(7), 738–746. doi:10.1080/15226514.2015.1133559

[27] Bagheri M, Al-Jabery K, Wunsch D, Burken JG. (2019). A deeper look at plant uptake of environmental contaminants using intelligent approaches, Science of the Total Environment. 651:561-569.

[28] Bagheri M, Al-Jabery K, Wunsch D, Burken JG. (2020). Examining plant uptake and translocation of emerging contaminants using machine learning: Implications to food security, Science of the Total Environment. 698:133999.

[29] Baker NR. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol 59:89–113.

[30] Balasubramani K, Sivarajasekar N, Naushad M. (2020). Effective adsorption of antidiabetic pharmaceutical (metformin) from aqueous medium using

graphene oxide nanoparticles: Equilibrium and statistical modelling, Journal of Molecular Liquids. doi:10.1016/j.molliq.2019.112426

[31] Balcom, I. N., Driscoll, H., Vincent, J., & Leduc, M. (2016). Metagenomic analysis of an ecological wastewater treatment plant's microbial communities and their potential to metabolize pharmaceuticals. F1000Research, 5, 1881. doi:10.12688/f1000research.9157.1

[32] Balen, B., Tkalec, M., Sikic', S., Tolic', S., Cvjetko, P., Pavlica, M., and Vidakovic'-Cifrek, Z. (2011). Biochemical responses of *Lemna minor* experimentally exposed to cadmium and zinc. Ecotoxicol. 20: 815–826.

[33] Balseiro-Romero, M., Gkorezis, P., Kidd, P. S., Van Hamme, J., Weyens, N., Monterroso, C., & Vangronsveld, J. (2017). Use of plant growth promoting bacterial strains to improve *Cytisus striatus* and *Lupinus luteus* development for potential application in phytoremediation. Science of The Total Environment, 581-582, 676–688. doi:10.1016/j.scitotenv.2016.12.180

[34] Baltrėnaitė, E., Baltrėnas, P., Butkus, D., Lietuvninkas, A. (2014). The Method of Dynamic Factors in Bioindication and Phytoremediation. Phytoremediation. 21–30.

[35] Baltrėnaitė, E., Butkus, D. (2007). Modelling Of Cu, Ni, Zn, Mn And Pb Transport From Soil To Seedlings Of Coniferous And Leafy Trees. J. Environ. Eng. Landsc. 15(4), 200–207.

[36] Baltrėnaitė, E., Lietuvninkas, A., Baltrėnas, P. (2016). Modelling the Balance of Metals in the Amended Soil for the Case of 'Atmosphere–Plant–Soil' System. Environ. Model. Assess. 21(5), 577–590.

[37] Bayçu, G., Gevrek-Kürüm, N., Moustaka, J., Csatári, I., Rognes, S. E., & Moustakas, M. (2016). Cadmium-zinc accumulation and photosystem II responses of *Noccaea caerulescens* to Cd and Zn exposure. Environmental Science and Pollution Research, 24(3), 2840–2850. doi:10.1007/s11356-016-8048-4

[38] Bell, T.H., Joly, S., Pitre, F.E., Yergeau, E. (2014). Increasing phytoremediation efficiency and reliability using novel omics approaches. Trends Biotechnol. 32(5), 271–280.

[39] Benavides, M.P., Gallego, S.M., and Tomaro, M.L. (2005). Cadmium toxicity in plants. Brazilian J. Plant Physiol. 17: 21–34.

[40] Benisrael, M., Wanner, P., Aravena, R., Parker, B. L., Haack, E. A., Tsao, D. T., & Dunfield, K. E. (2019). Toluene biodegradation in the vadose zone of a poplar phytoremediation system identified using metagenomics and toluene-specific stable carbon isotope analysis. International Journal of Phytoremediation, 1–10. doi:10.1080/15226514.2018.1523873

[41] Benyó, D., Horváth, E., Németh, E., Leviczky, T., Takács, K., Lehotai, N., Gallé, Á. (2016). Physiological and molecular responses to heavy metal stresses suggest different detoxification mechanism of *Populus deltoides* and *P. canadensis*. Journal of Plant Physiology, 201, 62–70. doi:10.1016/j.jplph.2016.05.025

[42] Bergmann, H., Machelett, B., Lippmann, B., & Friedrich, Y. (2001). Influence of heavy metals on the accumulation of trimethylglycine, putrescine and spermine in food plants. Amino Acids, 20(3), 325–329. doi:10.1007/s007260170048

[43] Bernard, F., Dumez, S., Brulle, F., Lemière, S., Platel, A., Nesslany, F., Vandenbulcke, F. (2015). Antioxidant defense gene analysis in *Brassica oleracea* and *Trifolium repens* exposed to Cd and/or Pb. Environmental

Science and Pollution Research, 23(4), 3136–3151. doi:10.1007/s11356-015-5636-7

[44] Bernard, F., Dumez, S., Lemière, S., Platel, A., Nesslany, F., Deram, A., Cuny, D. (2018). Impact of cadmium on forage kale (*Brassica oleracea* var. *viridis* cv "Prover") after 3-,10- and 56-day exposure to a Cd-spiked field soil. Environmental Science and Pollution Research. doi:10.1007/s11356-018-1636-8

[45] Boersma, L., Lindstrom, F.T., Childs, S.W. (1991). Model for steady state coupled transport in xylem and phloem. Agronomy Journal 83, 401–415.

[46] Boersma, L., McFarlane, C., McCoy, E.L. (1988). Model of couple transport of water and solutes in plants. Spec. Report 818. Agric. Exp. Sta., Oregon State University, Corvallis, Oregon, USA, p. 109.

[47] Bolan, N., Kunhikrishnan, A., Thangarajan, R., Kumpiene, J., Park, J., Makino, T., Scheckel, K. (2014). Remediation of heavy metal(loid)s contaminated soils – To mobilize or to immobilize? Journal of Hazardous Materials, 266, 141–166. doi:10.1016/j.jhazmat.2013.12.018

[48] Bolan, N.S., Makino, T., Kunhikrishnan, A., Kim, P.J., Ishikawa, S., Murakami, M., and Kirkham, M.B. (2013). Cadmium contamination and its risk management in rice ecosystem. Adv. Agron. 119: 183–273.

[49] Bourioug, M., Alaoui-Sehmer, L., Laffray, X., Benbrahim, M., Aleya, L., Alaoui-Sossé, B. (2015). Sewage sludge fertilization in larch seedlings: Effects on trace metal accumulation and growth performance, Ecological Engineering, Volume 77, Pages 216-224.

[50] Bradstreet, R. B. (1940). A Review of the Kjeldahl Determination of Organic Nitrogen. Chemical Reviews, 27(2), 331–350. doi:10.1021/cr60087a002

[51] Brulle, F., Bernard, F., Vandenbulcke, F. (2014). Identification of suitable qPCR reference 533 genes in leaves of *Brassica oleracea* under abiotic stresses. Ecotoxicology, 23(3), 459–471. doi:10.1007/s10646-014-1209-7

[52] Brunetti, P., Zanella, L., Paolis, A. D., Litta, D. D., Cecchetti, V., Falasca, G., Cardarelli, M. (2015). Cadmium-inducible expression of the ABC-type transporter *AtABCC3* increases phytochelatin-mediated cadmium tolerance in *Arabidopsis*. Journal of Experimental Botany, 66(13), 3815-3829. doi:10.1093/jxb/erv185

[53] Burges A, Alkorta I, Epelde L, Garbisu C. (2017). From phytoremediation of soil contaminants to phytomanagement of ecosystem services in metal contaminated sites. International Journal of Phytoremediation. 20(4):384-397. doi:10.1080/15226514.2017.1365340

[54] Buryński M, Klobus G. (2004). Changes of photosynthetic parameters in cucumber leaves under Cu, Cd, and Pb stress. Photosynthetica 42:505–510.

[55] Busch, D., Stark, A., Kammann, C. I., and Glaser, B. (2013). Genotoxic and phytotoxic risk assessment of fresh and treated hydrochar from hydrothermal carbonization compared to biochar from pyrolysis, Ecotox. Environ. Safe., 97, 59–66.

[56] Cabral, A. C. S., Jakovleska, J., Deb, A., Penner-Hahn, J. E., Pecoraro, V. L., & Freisinger, E. (2017). Further insights into the metal ion binding abilities and the metalation pathway of a plant metallothionein from *Musa acuminata*. JBIC Journal of Biological Inorganic Chemistry, 23(1), 91–107. doi:10.1007/s00775-017-1513-9

[57] Cameselle, C., Gouveia, S. (2019). Phytoremediation of mixed contaminated soil enhanced with electric current. J. Hazard. Mater. 361, 95-102.

[58] Canales-Pastrana, R.R., Paredes, M. (2013). Phytoremediation Dynamic Model as an Assessment Tool in the Environmental Management. Open J. Appl. Sci. 03(02), 208-217.

[59] Cano-Reséndiz, O., Rosa, G.D.L., Cruz-Jiménez, G., Gardea-Torresdey, J.L., Robinson, B.H. (2011). Evaluating the role of vegetation on the transport of contaminants associated with a mine tailing using the Phyto-DSS. J. Hazard. Mater. 189(1-2), 472–478.

[60] Cao RS, Fan MY, Hu JW, Ruan WQ, Xiong KN, Wei XH. (2017). Optimizing low-concentration mercury removal from aqueous solutions by reduced graphene oxide-supported Fe_3O_4 composites with the aid of an artificial neural network and genetic algorithm. Materials. 10:2-17.

[61] Caverzan, A., Bonifacio, A., Carvalho, F. E., Andrade, C. M., Passaia, G., Schünemann, M., Margis-Pinheiro, M. (2014). The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice. Plant Sci. 214, 74-87.

[62] Caverzan, A., Passaia, G., Rosa, S.B., Ribeiro, C., Lazzarotto, F., Margis-Pinheiro, M. (2012). Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. Genetics and Molecular Biology 35, 1011–1019.

[63] Çelekli A, Bozkurt H, Geyik F. (2016). Artificial neural network and genetic algorithms for modeling of removal of an azo dye on walnut husk, Desalination and Water Treatment. 57(33):15580-15591.

[64] Cevher-Keskin, B., Selçukcan-Erol, Ç., Yüksel, B., Ertekin, Ö., Yıldızhan, Y., Onarıcı, S., … Memon, A. R. (2018). Comparative transcriptome analysis of *Zea mays* in response to petroleum hydrocarbon stress. Environmental Science and Pollution Research. doi:10.1007/s11356-018-3078-8

[65] Chaâbene, Z., Rorat, A., Rekik Hakim, I., Bernard, F., Douglas, G. C., Elleuch, A., Mejdoub, H. (2018). Insight into the expression variation of metal-responsive genes in the seedling of date palm (*Phoenix dactylifera*). Chemosphere, 197, 123–134. doi:10.1016/j.chemosphere.2017.12.146

[66] Chabukdhara, M., Nema, A.K. (2013). Heavy metals assessment in urban soil around industrial clusters in Ghaziabad, India: Probabilistic health risk approach. Ecotoxicology and Environmental Safety 87, 57-64.

[67] Chandna, R., Augustine, R., Bisht, N. C. (2012). Evaluation of Candidate Reference Genes for Gene Expression Normalization in *Brassica juncea* Using Real Time Quantitative RT-PCR. PLoS ONE. 7 (5). e36918.

[68] Chandra, R., Kang H. (2016). Mixed heavy metal stress on photosynthesis, transpiration rate, and chlorophyll content in poplar hybrids, Forest Science and Technology, 12:2, 55-61.

[69] Chang, CY., Yu, HY., Chen, JJ. (2014). Accumulation of heavy metals in leaf vegetables from agricultural soils and associated potential health risks in the Pearl River Delta, South China Environ Monit Assess 186: 1547.

[70] Chen S, Sun S, Zhao C, Zang M, Wang Q, Liu Q, Li L. (2018). Optimization of biodegradation of polycyclic aromatic sulfur heterocycles in soil using response surface methodology, Petroleum Science and Technology. 36(22):1883-1890. doi:10.1080/10916466.2018.1517165

[71] Chen X, Wang J, Shi Y, Zhao MQ, Chi GY. (2011). Effects of cadmium on growth and photosynthetic activities in pakchoi and mustard. Bot Stud 52:41–46.

[72] Chen, Y., Hu, B., Tan, Z., Liu, J., Yang, Z., Li, Z., & Huang, B. (2015). Selection of reference genes for quantitative real-time PCR normalization in creeping bentgrass involved in four abiotic stresses. Plant Cell Reports, 34(10), 1825–1834. doi:10.1007/s00299-015-1830-9

[73] Chen, Y., Zhi, J., Zhang, H., Li, J., Zhao, Q., & Xu, J. (2017). Transcriptome analysis of *Phytolacca americana* L. in response to cadmium stress. PLOS ONE, 12(9), e0184681. doi:10.1371/journal.pone.0184681

[74] Chen, B.C., Lai, H.Y., Juang, K.W. (2012). Model evaluation of plant metal content and biomass yield for the phytoextraction of heavy metals by switchgrass. Ecotoxicol. Environ. Saf. 80, 393–400.

[75] Cherif, J., Mediouni, C., Ammar, W.B., and Jemal, F. (2011). Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (*Solanum lycopersicum*). J. Environ. Sci. 23: 837–844.

[76] Chibuike, G.U., Obiora, S.C. (2014). Heavy Metal Polluted Soils: Effect on Plants and Bioremediation Methods. Applied and Environmental Soil Science 1, 1-12.

[77] Chirakkara, R.A., Cameselle, C., Reddy, K.R. (2016). Assessing the applicability of phytoremediation of soils with mixed organic and heavy metal contaminants. Rev. Environ. Sci. Biotechnol. 15(2), 299–326.

[78] Chowdhary P., Yadav A., Singh R., Chandra, R., Singh, DP., Raj, A., Bharagava, RN. (2018). Stress response of *Triticum aestivum* L. *and Brassica juncea* L. against heavy metals growing at distillery and tannery wastewater contaminated site, Chemosphere, Volume 206, Pages 122-131.

[79] Choppala, G., Saifullah, Bolan, N., Bibi, S., Iqbal, M., Rengel, Z., Ok, Y. S. (2014). Cellular Mechanisms in Higher Plants Governing Tolerance to Cadmium Toxicity. Critical Reviews in Plant Sciences, 33(5), 374–391. doi:10.1080/07352689.2014.903747

[80] Christensen, J.B., Botma, J.J., Christensen, T.H. (1999). Complexation of Cu and Pb by DOC in polluted groundwater: a comparison of experimental data and predictions by computer speciation models (WHAM and MINTEQA2). Water Res. 33(15), 3231-3238.

[81] Clemens, S. (2006). Evolution and function of phytochelatin synthases. Journal of Plant Physiology, 163(3), 319–332. doi:10.1016/j.jplph.2005.11.010

[82] Cobbett Ch.S. (2000). Phytochelatins and Their Roles in Heavy Metal Detoxification. Plant Physiology, 123 (3) 825-832;

[83] Cortés-Eslava, J., Gómez-Arroyo, S., Risueño, MC., Testillano, PS. (2018). The effects of organophosphorus insecticides and heavy metals on DNA damage and programmed cell death in two plant models, Environmental Pollution, Volume 240, Pages 77-86.

[84] Clemens, S. (2006). Evolution and function of phytochelatin synthases. Journal of Plant Physiology, 163(3), 319–332. doi:10.1016/j.jplph.2005.11.010

[85] Cox, A., Venkatachalam, P., Sahi, S., & Sharma, N. (2016). Silver and titanium dioxide nanoparticle toxicity in plants: A review of current research. Plant Physiology and Biochemistry, 107, 147–163. doi:10.1016/j.plaphy.2016.05.022

[86] DalCorso, G., Farinati, S., Maistri, S., & Furini, A. (2008). How Plants Cope with Cadmium: Staking All on Metabolism and Gene Expression. Journal of

Integrative Plant Biology, 50(10), 1268–1280. doi:10.1111/j.1744-7909.2008.00737.x

[87] Darajeh N, Idris A, Masoumi HRF, Nourani A, Truong P, Rezania S. (2016). Phytoremediation of palm oil mill secondary effluent (POMSE) by *Chrysopogon zizanioides* (L.) using artificial neural networks. International Journal of Phytoremediation. 19(5):413–424.

[88] Darajeh N, Idris A, Reza H, Masoumi F, Nourani A, Truong P, Sairi A. (2016). Modeling BOD and COD removal from Palm Oil Mill Secondary Effluent in floating wetland by *Chrysopogon zizanioides* (L.) using response surface methodology, Journal of Environmental Management. 181:343-352. doi.org/10.1016/j.jenvman.2016.06.060.

[89] Das, N., Bhattacharya, S., & Maiti, M. K. (2016). Enhanced cadmium accumulation and tolerance in transgenic tobacco overexpressing rice metal tolerance protein gene *OsMTP1* is promising for phytoremediation. Plant Physiology and Biochemistry, 105, 297–309. doi:10.1016/j.plaphy.2016.04.049

[90] Dat, J., Vandenabeele, S., Vranov, E., Montagu, M. V., D. I., Breusegem, F. V. (2000). Dual action of the active oxygen species during plant stress responses. Cellular and Molecular Life Sciences (CMLS), 57(5), 779-795. doi:10.1007/s000180050041

[91] Demessie, Z., Woolfson, K. N., Yu, F., Qu, Y., and De Luca, V. (2017). The ATP binding cassette transporter, VmTPT2/VmABCG1, is involved in export of the monoterpenoid indole alkaloid, vincamine in *Vinca minor* leaves. Phytochemistry 140, 118–124, doi:10.1016/j.phytochem.2017.04.019

[92] Dheri, G. S., Singh Brar, M., & Malhi, S. S. (2007). Influence of phosphorus application on growth and cadmium uptake of spinach in two cadmium-contaminated soils. Journal of Plant Nutrition and Soil Science, 170(4), 495–499. doi:10.1002/jpln.200625051

[93] Dias, M. C., Monteiro, C., Moutinho-Pereira, J., Correia, C., Gonçalves, B., & Santos, C. (2012). Cadmium toxicity affects photosynthesis and plant growth at different levels. Acta Physiologiae Plantarum, 35(4), 1281–1289. doi:10.1007/s11738-012-1167-8

[94] Dimkpa, C., Merten, D., Svatoš, A., Büchel, G., Kothe, E. (2009). Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* and sunflower (*Helianthus annuus*), respectively. J. Appl. Microbiol. 107 (5), 1687-1696.

[95] Ding, Y., Chen, Z., and Zhu, C. (2011). Microarray-based analysis of cadmium-responsive microRNAs in rice (*Oryza sativa*). Journal of Experimental Botany, 62, 3563–3573. doi: 10.1093/jxb/err046

[96] Dinh, N., Ent, A., Mulligan, D.R., Nguyen, A.V. (2018). Zinc and lead accumulation characteristics and in vivo distribution of Zn^{2+} in the hyperaccumulator *Noccaea caerulescens* elucidated with fluorescent probes and laser confocal microscopy. Environ. Exp. Bot. 147, 1-12.

[97] Dixit, V., Pandey, V., & Shyam, R. (2001). Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). Journal of Experimental Botany, 52(358), 1101–1109. doi:10.1093/jexbot/52.358.1101

[98] Do, T. H., Martinoia, E., & Lee, Y. (2018). Functions of ABC transporters in plant growth and development. Current Opinion in Plant Biology, 41, 32-38. doi:10.1016/j.pbi.2017.08.003

[99] Dong, C., Wang, Y., Yu, S., Liu, J. (2010). Characterization of a Novel Rice Metallothionein Gene Promoter: Its Tissue Specificity and Heavy Metal Responsiveness. Journal of Integrative Plant Biology, 52(10), 914-924.

[100] Dong, J., Wu, F., & Zhang, G. (2006). Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon esculentum*). Chemosphere, 64(10), 1659–1666. doi:10.1016/j.chemosphere.2006.01.030

[101] Duan, J., He, S., Feng, Y., Yu, Y., Xue, L., Yang, L. (2017). Floating ryegrass mat for the treatment of low-pollution wastewater. Ecol. Eng. 108, 172-178.

[102] Dubey, S., Shri, M., Gupta, A., Rani, V., & Chakrabarty, D. (2018). Toxicity and detoxification of heavy metals during plant growth and metabolism. Environmental Chemistry Letters. doi:10.1007/s10311-018-0741-8

[103] Dubey, S., Shri, M., Gupta, A., Rani, V., & Chakrabarty, D. (2018). Toxicity and detoxification of heavy metals during plant growth and metabolism. Environmental Chemistry Letters, 16(4), 1169-1192. doi:10.1007/s10311-018-0741-8

[104] Duclos, D. V., Björkman, T. (2008). Meristem identity gene expression during curd proliferation and flower initiation in *Brassica oleracea*. J. Exp. Bot. 59 (2), 421-433.

[105] Egnér, H., Riehm, H., Domingo, W.R. (1960). Untersuchungen über die chemische Bodenanalyse als Grundlage für die Beurteilung des Nährstoffzustandes der Böden. II. Chemische Extraktionsmethoden zur Phosphor-und Kaliumbestimmung. Swed J Agri Res 26:199–215.

[106] Eid, E. M., Alrumman, S. A., Farahat, E. A., & El-Bebany, A. F. (2018). Prediction models for evaluating the uptake of heavy metals by cucumbers (*Cucumis sativus* L.) grown in agricultural soils amended with sewage sludge. Environmental Monitoring and Assessment, 190(9). doi:10.1007/s10661-018-6885-y

[107] Ekperusi, A.O., Sikoki, F.D., Nwachukwu. E.O. (2019). Application of common duckweed (*Lemna minor*) in phytoremediation of chemicals in the environment: State and future perspective. Chemosphere. 223, 285-309.

[108] Elloumi, N., Zouari, M., Chaari, L., Jomni, C., Rouina, B., Abdallah, F. (2014). Ecophysiological responses of almond (*Prunus dulcis*) seedlings to cadmium stress. Biologia 69(5):604–609.

[109] Emamverdian, A., Ding, Y., Mokhberdoran, F., & Xie, Y. (2015). Heavy Metal Stress and Some Mechanisms of Plant Defense Response. The Scientific World Journal, 2015, 1–18. doi:10.1155/2015/756120

[110] Ent, A. V., Baker, A. J., Reeves, R. D., Pollard, A. J., Schat, H. (2012). Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. Plant Soil. 362 (1-2), 319-334.

[111] Ergen, S.F., Tunca, E.Ü. (2018). Nanotoxicity modelling and removal efficiencies of ZnONP with consortium. Int. J. Phytoremed. 20, 16-26.

[112] Essaid, H.I., Bekins, B.A., Cozzarelli, I.M. (2015). Organic contaminant transport and fate in the subsurface: evolution of knowledge and understanding. Water Resour. Res. 51(7), 4861–4902.

[113] Fabre, G., Garroum, I., Mazurek, S., Daraspe, J., Mucciolo, A., Sankar, M., Humbel, BM., Nawrath, C. (2016). The ABCG transporter PEC1/ABCG32 is required for the formation of the developing leaf cuticle in *Arabidopsis*. New Phytologist, 209,192-201. doi:10.1111/nph.13608

[114] Fan, M., Hu, J., Cao, R., Ruan, W., & Wei, X. (2018). A review on experimental design for pollutants removal in water treatment with the aid of artificial intelligence. Chemosphere, 200, 330–343. doi:10.1016/j.chemosphere.2018.02.111

[115] Fan, M., Hu, J., Cao, R., Xiong, K., & Wei, X. (2017). Modeling and prediction of copper removal from aqueous solutions by nZVI/rGO magnetic nanocomposites using ANN-GA and ANN-PSO. Scientific Reports, 7(1). doi:10.1038/s41598-017-18223-y

[116] Fang, W., Wei, Y., & Liu, J. (2016). Comparative characterization of sewage sludge compost and soil: Heavy metal leaching characteristics. Journal of Hazardous Materials, 310, 1-10. doi:10.1016/j.jhazmat.2016.02.025

[117] Farinati, S., DalCorso, G., Varotto, S., & Furini, A. (2009). The *Brassica juncea* BjCdR15, an ortholog of *Arabidopsis* TGA3, is a regulator of cadmium uptake, transport and accumulation in shoots and confers cadmium tolerance in transgenic plants. New Phytologist, 185(4), 964–978. doi:10.1111/j.1469-8137.2009.03132.x

[118] Fellet, G., Marchiol, L. (2011). Towards Green Remediation: Metal Phytoextraction and Growth Analysis of Sorghum bicolor under Different Agronomic Management. Low Carbon Econ. 02(03), 144–151.

[119] Fellet, G., Marmiroli, M., and Marchiol, L. (2014). Elements uptake by metal accumulator species grown on mine tailings amended with three types of biochar, Sci. Total Environ., 468–469, 598–608.

[120] Feng, J., Jia, W., Lv, S., Bao, H., Miao, F., Zhang, X., Li, Y. (2017). Comparative transcriptome combined with morpho-physiological analyses revealed key factors for differential cadmium accumulation in two contrasting sweet sorghum genotypes. Plant Biotechnology Journal, 16(2), 558–571. doi:10.1111/pbi.12795

[121] Fernández, A., Sánchez, A., Font, X. (2005). Anaerobic co-digestion of a simulated organic fraction of municipal solid wastes and fats of animal and vegetable origin. Biochemical Engineering Journal 26, 22–28.

[122] Fosso-Kankeu, E., & Mulaba-Bafubiandi, A. F. (2014). Implication of plants and microbial metalloproteins in the bioremediation of polluted waters: A review. Physics and Chemistry of the Earth, Parts A/B/C, 67-69, 242–252. doi:10.1016/j.pce.2013.09.018

[123] Fu, S., Lu, Y., Zhang, X., Yang, G., Chao, D., Wang, Z., Shi, M., Chen, J., Chao, D-Y., Li, R., Ma, JF., Xia, J. (2019). The ABC transporter ABCG36 is required for cadmium tolerance in rice. Journal of Experimental Botany, 70(20), 5909–5918

[124] Fuentes, A., Lorens, M., Saez, J., Aguilar, M.I., Otuno, J.F., Meseguer, V.F. (2004). Phytotoxicity and heavy metals speciation of stabilized sewage sludges. Journal of Hazardous Materials 108, 161-169.

[125] Fusconi, A., Repetto, O., Bona, E., Massa, N., Gallo, C., Dumas-Gaudot, E., & Berta, G. (2006). Effects of cadmium on meristem activity and nucleus ploidy in roots of *Pisum sativum* L. cv. Frisson seedlings. Environmental and Experimental Botany, 58(1-3), 253–260.

[126] Gagetti-Bulgarelli, R., Araujo, P., Tezotto, T., Mazzafera, P., Adrián, S., Andrade. (2016). Expression of metallothionein genes in coffee leaves in response to the absence or excess of Cu and Zn Theoretical and Experimental Plant Physiology Volume 28, Issue 4, pp 371–383.

[127] Gajewska, E., Skłodowska, M. (2005). Antioxidative responses and proline level in leaves and roots of pea plants subjected to nickel stress. Acta. Physiol. Plant. 27 (3), 329-340.
[128] Gajewska, E., Skłodowska, M., Słaba, M., Mazur, J. (2006). Effect of nickel on antioxidative enzyme activities, proline and chlorophyll contents in wheat shoots. Biologia Plantarum 50, 653-659.
[129] Galal, T. M., Shehata, H. S. (2015). Bioaccumulation and translocation of heavy metals by *Plantago major* L. grown in contaminated soils under the effect of traffic pollution. Ecol. Indic. 48, 244-251.
[130] Gantait, S., Panigrahi, J., & Verma, S. K. (2019). Transgenic Ornamentals for Phytoremediation of Metals and Metalloids. Transgenic Plant Technology for Remediation of Toxic Metals and Metalloids, 477-497. doi:10.1016/b978-0-12-814389-6.00022-5
[131] Garroum, I., Bidzinski, P., Daraspe, J., Mucciolo, A., Humbel, B. M., Morel, J. B., Nawrath, C. (2016). Cuticular defects in *Oryza sativa* ATPbinding cassette transporter G31 mutant plants cause dwarfism. Elevated defense responses and pathogen resistance. Plant Cell Physiology, 57(6), 1179-1188. doi:10.1093/pcp/pcw066.
[132] Gautam, M., & Agrawal, M. (2017). Phytoremediation of metals using vetiver (*Chrysopogon zizanioides* (L.) Roberty) grown under different levels of red mud in sludge amended soil. Journal of Geochemical Exploration, 182, 218–227. doi:10.1016/j.gexplo.2017.03.003
[133] Gautam, M., Agrawal, M. (2017). Influence of metals on essential oil content and composition of lemongrass (*Cymbopogon citratus* (DC) Stapf.) grown under different levels of red mud in sewage sludge amended soil. Chemosphere, 175:315–322. doi:10.1016/j.chemosphere.2017.02.065
[134] Ghoshal N, Talapatra S, Moulick A, Chakraborty A, Raychaudhuri SS. (2013). Alterations in transcriptome and proteome on metallothioneins following oxidative stress induced by sublethal doses of cadmium and gamma rays in Plantago ovata. Int J Radiat Biol 89:571–582.
[135] Gichner, T., Patková, Z., Száková, J., Žnidar, I., & Mukherjee, A. (2008). DNA damage in potato plants induced by cadmium, ethyl methanesulphonate and γ-rays. Environmental and Experimental Botany, 62(2), 113–119. doi:10.1016/j.envexpbot.2007.07.013
[136] Gichner, T., Lovecká, P., Kochánková, L., Macková, M., Demnerová, K. (2007). Monitoring toxicity, DNA damage, and somatic mutations in tobacco plants growing in soil heavily polluted with polychlorinated biphenyls. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, Volume 629, Issue 1, Pages 1-6.
[137] Gill, S. S., Khan, N. A., & Tuteja, N. (2011). Differential cadmium stress tolerance in five Indian mustard (*Brassica juncea* L.) cultivars. Plant Signaling & Behavior, 6(2), 293–300. doi:10.4161/psb.6.2.15049
[138] Gill, S. S., Khan, N. A., & Tuteja, N. (2012). Cadmium at high dose perturbs growth, photosynthesis and nitrogen metabolism while at low dose it up regulates sulfur assimilation and antioxidant machinery in garden cress (*Lepidium sativum* L.). Plant Science, 182, 112–120. doi:10.1016/j.plantsci.2011.04.018
[139] Gojkovic, Ž., Vílchez, C., Torronteras, R., Vigara, J., Gómez-Jacinto, V., Janzer, N., … Garbayo, I. (2014). Effect of Selenate on Viability and

Selenomethionine Accumulation of *Chlorella sorokiniana* Grown in Batch Culture. The Scientific World Journal, 2014, 1–13. doi:10.1155/2014/401265

[140] Ghnaya, T., Nouairi, I., Slama, I., Messedi, D., Grignon, C., Abdelly, C., & Ghorbel, M. H. (2005). Cadmium effects on growth and mineral nutrition of two halophytes: *Sesuvium portulacastrum* and *Mesembryanthemum crystallinum.* Journal of Plant Physiology, 162(10), 1133–1140. doi:10.1016/j.jplph.2004.11.011

[141] Gomes, M.A.D.C., Hauser-Davis, R.A., Souza, A.N.D., Vitória, A.P. (2016). Metal phytoremediation: General strategies, genetically modified plants and applications in metal nanoparticle contamination. Ecotoxicol. Environ. Saf. 134, 133–147.

[142] Gong, X., Huang, D., Liu, Y., Zeng, G., Wang, R., Wei, J., Huang, C., Xu, P., Wan, J., Zhang, C. (2018). Pyrolysis and reutilization of plant residues after phytoremediation of heavy metals contaminated sediments: for heavy metals stabilization and dye adsorption. Bioresour. Technol. 253, 64–71.

[143] Gonzalez-Mendoza, D., Moreno, A. Q., & Zapata-Perez, O. (2007). Coordinated responses of phytochelatin synthase and metallothionein genes in black mangrove, *Avicennia germinans*, exposed to cadmium and copper. Aquatic Toxicology, 83(4), 306–314. doi:10.1016/j.aquatox.2007.05.005

[144] Goss, M.J., Tubeileh, A., Goorahoo, D. (2013). A review of the use of organic amendments and the risk to human health. In: Sparks, D.L. (Ed.), Adv. Agron., vol. 120, Academic Press, Elsevier Inc., pp. 275–380.

[145] Goswami, S., Das, S. (2015). A Study on Cadmium Phytoremediation Potential of Indian Mustard, *Brassica juncea*. Int. J. Phytoremediation. 17(6), 583-588.

[146] Gove, L., Cooke, C. M., Nicholson, F. A., & Beck, A. J. (2001). Movement of water and heavy metals (Zn, Cu, Pb and Ni) through sand and sandy loam amended with biosolids under steady-state hydrological conditions. Bioresource Technology, 78(2), 171–179. doi:10.1016/s0960-8524(01)00004-9

[147] Gräfe, K., Shanmugarajah, K., Zobel, T., Weidtkamp-Peters, S., Kleinschrodt, D., Smits, S., & Schmitt, L. (2019). Cloning and expression of selected ABC transporters from the *Arabidopsis thaliana* ABCG family in *Pichia pastoris*. PloS one, 14(1), e0211156. https://doi.org/10.1371/journal.pone.0211156

[148] Grobelak, A., & Napora, A. (2015). The Chemophytostabilisation Process of Heavy Metal Polluted Soil. PLOS ONE, 10(6), e0129538. doi:10.1371/journal.pone.0129538

[149] Grobelak, A., Placek, A., Grosser, A., Singh, B. R., Almås, Å. R., Napora, A., & Kacprzak, M. (2017). Effects of single sewage sludge application on soil phytoremediation. Journal of Cleaner Production, 155, 189–197. doi:10.1016/j.jclepro.2016.10.005

[150] Grobelak, A., Kokot, P., Świątek, J., Jaskulak, M., & Rorat, A. (2018). Bacterial ACC Deaminase Activity in Promoting Plant Growth on Areas Contaminated with Heavy Metals. Journal of Ecological Engineering, 19(5), 150-157. doi:10.12911/22998993/89818

[151] Grobelak, A. (2016). Organic Soil Amendments in the Phytoremediation Process. Phytoremediation 21-39.

[152] Grobelak, A., Kacprzak, M., Grosser, A. (2013). Chemophytostabilisation of Soil Contaminated with Cadmium, Lead and Zinc. Annual Set Environmental Protection 15, 1982-2002.

[153] Grobelak, A., Napora, A., Kacprzak, M. (2015). Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. Ecol. Eng. 84, 22-28.
[154] Grobelak, A., Placek, A., Grosser, A., Singh, B., Almås, Å., Napora, A., Kacprzak, M. (2017). Effects of single sewage sludge application on soil phytoremediation. J Clean Prod 155:189-197.
[155] Grobelak, A., Świątek, J., Murtaś, A,, Jaskulak, M. (2019). Cadmium-induced oxidative stress in plants, cadmium toxicity, and tolerance in plants: from physiology to remediation. Cadmium toxicity and tolerance in plants, 213-231
[156] Guo, J., Dai, X., Xu, W., & Ma, M. (2008). Overexpressing *GSH1* and *AsPCS1* simultaneously increases the tolerance and accumulation of cadmium and arsenic in *Arabidopsis thaliana*. Chemosphere, 72(7), 1020–1026. doi:10.1016/j.chemosphere.2008.04.018
[157] Gupta, B. B., Selter, L. L., Baranwal, V. K., Arora, D., Mishra, S. K., Sirohi, P., Chauhan, H. (2019). Updated inventory, evolutionary and expression analyses of *G (PDR)* type *ABC* transporter genes of rice. Plant Physiology and Biochemistry, 142, 429–439. doi:10.1016/j.plaphy.2019.08.004
[158] Habibul, N., Chen, J., Hu, Y., Hu, Y., Yin, H., Sheng, G., Yu, H. (2019). Uptake, accumulation and metabolization of 1-butyl-3-methylimidazolium bromide by ryegrass from water: Prospects for phytoremediation. Water Research, 156, 82-91.
[159] Hamid, H. A., Jenidi, Y., Thielemans, W., Somerfield, C., & Gomes, R. L. (2016). Predicting the capability of carboxylated cellulose nanowhiskers for the remediation of copper from water using response surface methodology (RSM) and artificial neural network (ANN) models. Industrial Crops and Products, 93, (108-120). doi:10.1016/j.indcrop.2016.05.035. ISSN 0926-6690
[160] Han, X., Yin, H., Song, X., Zhang, Y., Liu, M., Sang, J., Zhuo, R. (2016). Integration of small RNAs, degradome and transcriptome sequencing in hyperaccumulator *Sedum alfredii* uncovers a complex regulatory network and provides insights into cadmium phytoremediation. Plant Biotechnology Journal, 14(6), 1470–1483. doi:10.1111/pbi.12512
[161] Hao, D.-C., Zhang, C.-R., & Xiao, P.-G. (2018). The first Taxus rhizosphere microbiome revealed by shotgun metagenomic sequencing. Journal of Basic Microbiology, 58(6), 501–512. doi:10.1002/jobm.201700663
[162] Hasan, S. A., Hayat, S., & Ahmad, A. (2011). Brassinosteroids protect photosynthetic machinery against the cadmium induced oxidative stress in two tomato cultivars. Chemosphere, 84(10), 1446–1451. doi:10.1016/j.chemosphere.2011.04.047
[163] Hasanuzzaman, M., Nahar, K., Gill, S.S., Alharby, H.F., Razafindrabe, B.H., Fujita, M. (2017). Hydrogen peroxide pretreatment mitigates cadmium-induced oxidative stress in *Brassica napus* L.: an intrinsic study on antioxidant defense and glyoxalase systems. Front. Plant Sci. 8, 1–10.
[164] Hattab, N., Motelica-Heino, M., Faure, O., Bouchardon, J. (2015). Effect of fresh and mature organic amendments on the phytoremediation of technosols contaminated with high concentrations of trace elements. J. Environ. Manage. 159, 37-47.
[165] Hattab, S., Hattab, Flores-Casseres, ML., Boussetta, H., Doumas, P., Hernandez, LE., Banni M. (2016). Characterisation of lead-induced stress molecular biomarkers in *Medicago sativa* plants, Environmental and Experimental Botany, Volume 123, Pages 1-12.

[166] Hayat, S., Hasan, S.A., Ahmad, A. (2011). Growth, nitrate reductase activity and antioxidant system in cadmium stressed tomato (*Lycopersicon esculentum*) cultivars. Biotech. Agron. Soc. Environ. 15:401–414.
[167] Hechmi, N., Aissa, N.B., Abdennaceur, H., Jedidi, N. (2013). Phytoremediation potential of maize (*Zea mays* L.) in co-contaminated soils with pentachlorophenol and cadmium. Int. J. Phytorem. 15(7), 703–713.
[168] Homa, J., Rorat, A., Kruk, J., Cocquerelle, C., Plytycz, B., Vandenbulcke, F. (2015). Dermal exposure of *Eisenia andrei* earthworms: Effects of heavy metals on metallothionein and phytochelatin synthase gene expressions in coelomocytes. Environ Toxicol Chem, 34: 1397-1404.
[169] Hooda, V. (2007). Phytoremediation of toxic metals from soil and waste water. Journal of Environmental Biology 28, 367-376.
[170] Hou, J., Liu, W., Wu, L., Hu, P., Ma, T., Luo, Y., & Christie, P. (2017). Modulation of the efficiency of trace metal phytoremediation by Sedum plumbizincicola by microbial community structure and function. Plant and Soil, 421(1-2), 285–299. doi:10.1007/s11104-017-3466-8
[171] Hou, D., Qi, S., Zhao, B., Rigby, M., Oconnor, D. (2017). Incorporating life cycle assessment with health risk assessment to select the 'greenest' cleanup level for Pb contaminated soil. J. Clean. Prod. 162, 1157–1168.
[172] Hsu, LC., Huang, CY., Chuang, YH., Chen, HW., Chan, YT., Teah, HY., Chen, TY., Chang, CF., Liu, YT., Tzou, YM. (2016). Accumulation of heavy metals and trace elements in fluvial sediments received effluents from traditional and semiconductor industries. Sci Rep-UK 6:34250.
[173] Huang, L., Yan, H., Jiang, X., Yin, G., Zhang, X., Qi, X., Peng, Y. (2014). Identification of Candidate Reference Genes in Perennial Ryegrass for Quantitative RT-PCR under Various Abiotic Stress Conditions. PLoS ONE, 9(4), e93724. doi:10.1371/journal.pone.0093724
[174] Hwang, J., Song, W., Hong, D., Ko, D., Yamaoka, Y., Jang, S., Lee, Y. (2016). Plant ABC Transporters Enable Many Unique Aspects of a Terrestrial Plants Lifestyle. Molecular Plant, 9(3), 338-355. doi:10.1016/j.molp.2016.02.003
[175] Khan, M. I. R., Nazir, F., Asgher, M., Per, T. S., & Khan, N. A. (2015). Selenium and sulfur influence ethylene formation and alleviate cadmium-induced oxidative stress by improving proline and glutathione production in wheat. Journal of Plant Physiology, 173, 9–18. doi:10.1016/j.jplph.2014.09.011
[176] Janani, K., Sivarajasekar, N., Muthusaravanan, S., Ram, K., Prakashman, J., Sivamani, S., Selvaraju, N. (2019). Optimization of EDTA enriched phytoaccumulation of zinc by *Ophiopogon japonicus*: Comparison of Response Surface, Artificial Neural Network and Random Forest models. Bioresource Technology Reports, 100265. doi:10.1016/j.biteb.2019.100265
[177] Jaskulak, M., Grobelak, A., Grosser, A., & Vandenbulcke, F. (2019). Gene expression, DNA damage and other stress markers in *Sinapis alba* L. exposed to heavy metals with special reference to sewage sludge application on contaminated sites. Ecotoxicology and Environmental Safety, 181, 508–517. doi:10.1016/j.ecoenv.2019.06.025
[178] Jaskulak, M., Grobelak, A., & Vandenbulcke, F. (2020). Modelling assisted phytoremediation of soils contaminated with heavy metals – main opportunities, limitations, decision making and future prospects. Chemosphere, 126196. doi:10.1016/j.chemosphere.2020.126196

[179] Jaskulak, M., Rorat, A., Grobelak, A., & Kacprzak, M. (2018). Antioxidative enzymes and expression of *rbcL* gene as tools to monitor heavy metal-related stress in plants. Journal of Environmental Management, 218, 71–78. doi:10.1016/j.jenvman.2018.04.052

[180] Jaskulak, M., Grobelak, A., (2019). Chapter 6 - Cadmium Phytotoxicity — Biomarkers. Cadmium Tolerance in Plants, Academic Press, 177-191, doi.org/10.1016/B978-0-12-815794-7.00006-0.

[181] Jaskulak, M., Rorat, A., Grobelak, A., Chaabene, Z., Kacprzak, M., Vandenbulcke, F. (2019). Bioaccumulation, antioxidative response, and metallothionein expression in *Lupinus luteus* L. exposed to heavy metals and silver nanoparticles. Environ Sci Pollut Res 26: 16040–16052.

[182] Javed, M., Ahmad, I., Usmani, N., Ahmad, M. (2016). Studies on biomarkers of oxidative stress and associated genotoxicity and histopathology in *Channa punctatus* from heavy metal polluted canal, Chemosphere,Volume 151, Pages 210-219.

[183] Hirata, K., Tsuji, N., & Miyamoto, K. (2005). Biosynthetic regulation of phytochelatins, heavy metal-binding peptides. Journal of Bioscience and Bioengineering, 100(6), 593–599. doi:10.1263/jbb.100.593

[184] Kacprzak, M., Grobelak, A., Grosser, A., Napora, A. (2014). The potential of biosolid application for the phytostabilisation of metals. Desalination and Water Treatment 52, 3955-3964.

[185] Kakoei, F., Salehi, H. (2013). Effects of Different Pot Mixtures on spathiphyllum (*Spathiphyllum wallisii*) Growth and Development. J. Cent. Eur. Agric. 14 (2), 140-148.

[186] Nahar, K., Hasanuzzaman, M., Alam, M. M., Rahman, A., Suzuki, T., & Fujita, M. (2016). Polyamine and nitric oxide crosstalk: Antagonistic effects on cadmium toxicity in mung bean plants through upregulating the metal detoxification, antioxidant defense and methylglyoxal detoxification systems. Ecotoxicology and Environmental Safety, 126, 245–255. doi:10.1016/j.ecoenv.2015.12.026

[187] Kang, J., Park, J., Choi, H., Burla, B., Kretzschmar, T., Lee, Y., Martinoia, E. (2011). Plant ABC transporters. Arabidopsis Book 9, e0153, http://dx.doi.org/10.1199/tab. 0153

[188] Kapanen, A., Itavaara, M. (2001). Ecotoxicity tests for compost applications. Ecotoxicology and Environmental Safety 49, 1-16.

[189] Kappen, L., Sommerkorn, M., Schroeter, B. (1995). Carbon acquisition and water relations of lichens in polar regions — potentials and limitations. Lichenol. 27 (6), 531-545.

[190] Karczewska, A., Kabała, C. (2008). Metodyka analiz laboratoryjnych gleb i roślin, Instytut Gleboznawstwa i Ochrony Środowiska Rolniczego, Wrocław.

[191] Kausar, A., Bhatti, H. N., Iqbal, M., & Ashraf, A. (2017). Batch versus column modes for the adsorption of radioactive metal onto rice husk waste: conditions optimization through response surface methodology. Water Science and Technology, 76(5), 1035–1043. doi:10.2166/wst.2017.220

[192] Khan, S., Waqas, M., Ding, F., Shamshad, I., Peter, H. Arp, Li G. (2015). The influence of various biochars on the bioaccessibility and bioaccumulation of PAHs and potentially toxic elements to turnips (*Brassica rapa* L.), Journal of Hazardous Materials, Volume 300,Pages 243-253.

[193] Klein, C., Comero, S., Stahlmecke, B., Romazanov, J., Kuhlbusch, T., Van Doren, E. (2011). NM-Series of Representative Manufactured Nanomaterials:

NM-300 Silver Characterisation, Stability, Homogeneity. Luxembourg; Publications Office of the European Union.

[194] Konlechner, C., Türktas, M., Langer, I., Vaculík, M., Wenzel, W. W., Puschenreiter, M. (2013). Expression of zinc and cadmium responsive genes in leaves of willow (*Salix caprea* L.) genotypes with different accumulation characteristics. Environ. Pollut. 178, 121–127. doi: 10.1016/j.envpol.2013.02.033

[195] Kovacs, H., Szemmelveisz, K. (2017). Disposal options for polluted plants grown on heavy metal contaminated brownfield lands – a review. Chemosphere. 166, 8–20.

[196] Kranner, I., & Colville, L. (2011). Metals and seeds: Biochemical and molecular implications and their significance for seed germination. Environmental and Experimental Botany, 72(1), 93–105. doi:10.1016/j.envexpbot.2010.05.005

[197] Kubátová, P., Hejcman, M., Száková, J., Vondráčková, S., & Tlustoš, P. (2016). Effects of Sewage Sludge Application on Biomass Production and Concentrations of Cd, Pb and Zn in Shoots of Salix and Populus Clones: Improvement of Phytoremediation Efficiency in Contaminated Soils. BioEnergy Research, 9(3), 809-819. doi:10.1007/s12155-016-9727-1 356-019-04972-y

[198] Kumar, D., & Pannu, R. (2018). Perspectives of lindane (γ-hexachlorocyclohexane) biodegradation from the environment: a review. Bioresources and Bioprocessing, 5(1). doi:10.1186/s40643-018-0213-9

[199] Kumar, V., Singh, J., & Kumar, P. (2019). Heavy metal uptake by water lettuce (*Pistia stratiotes* L.) from paper mill effluent (PME): experimental and prediction modeling studies. Environmental Science and Pollution Research. doi:10.1007/s11356-019-04766-2

[200] Kumar, V., Thakur, R.K., Kumar, P. (2019). Assessment of Heavy Metals Uptake by Cauliflower (*Brassica oleracea* var. *botrytis*) Grown in Integrated Industrial Effluent Irrigated Soils: A Prediction Modeling Study. Sci Hortic. 257:108682. doi:10.1016/j.scienta.2019.108682

[201] Kumar, V., Almomin, S., Al-Aqeel, H., Al-Salameen, F., Nair, S., Shajan, A. (2018). Metagenomic analysis of rhizosphere microflora of oil-contaminated soil planted with barley and alfalfa, Plos One, 13(8).

[202] Kumar, D., Kumar, S., Shukla, V., Kumar., N. (2017). Adaptation Strategies of Plants Against Common Inorganic Pollutants and Metals. In: Shukla V., Kumar S., Kumar N. (eds) Plant Adaptation Strategies in Changing Environment. Springer, Singapore.

[203] Kupper, T., Bürge, D., Bachmann, H.J., Güsewell, S., Mayer, J. (2014). Heavy metals in source-separated compost and digestates. Waste Manage. 34, 867–874.

[204] Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y. B., Naidu, R., Megharaj, M. (2017). Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: Technological constraints, emerging trends and future directions. Chemosphere. 168, 944-968.

[205] Kuramshina, Z.M., Smirnova, Y.V., Khairullin, R.M. (2018). Cadmium and Nickel Toxicity for *Sinapis alba* Plants Inoculated with Endophytic Strains of Bacillus subtilis. Russ J Plant Physiol. 65:269–277. doi:10.1134/S1021443718010077

[206] Lambret-Frotté, J., Almeida, L.C., Moura, S.M., Souza, F.L., Linhares, F.S., Alves-Ferreira, M. (2015). Validating Internal Control Genes for the Accurate Normalization of qPCR Expression Analysis of the Novel Model Plant *Setaria viridis*. Plos One 10(8).

[207] Lane, T. S., Rempe, C. S., Davitt, J., Staton, M. E., Peng, Y., Soltis, D. E., Stewart, C. N. (2016). Diversity of ABC transporter genes across the plant kingdom and their potential utility in biotechnology. BMC Biotechnology, 16(1). doi:10.1186/s12896-016-0277-6

[208] Lang, I., and Wernitznig, S. (2011). Sequestration at the cell wall and plasma membrane facilitates zinc tolerance in the moss *Pohlia drummondii*. Environ. Exp. Bot. 74, 186–193. doi: 10.1016/j.envexpbot.2011.05.018

[209] Lanier, C., Bernard, F., Dumez, S., Leclercq, J., Lemière, S., Vandenbulcke, F., Nesslany, F5., Platel, A., Devred, I., Cuny, D., Deram, A. (2016). Combined effect of Cd and Pb spiked field soils on bioaccumulation, DNA damage, and peroxidase activities in *Trifolium repens*. Environ Sci Pollut Res 23: 1755.

[210] Lefèvre, F., Baijot, A., Boutry, M. (2015). Plant ABC transporters: time for biochemistry? Biochemical Society Transactions, 43, 931–936. doi:10.1042/BST20150108

[211] Levin, R. A., Wagner, W. L., Hoch, P. C., Nepokroeff, M., Pires, J. C., Zimmer, E. A., Sytsma, K. J. (2003). Family-level relationships of *Onagraceae* based on chloroplast *rbcL* and *ndhF* data. Am. J. Bot. 90 (1) 107-115.

[212] Li, Q., Zheng, J., Li, S., Huang, G., Skilling, S. J., Wang, L., Li, L., Li, M., Yuan, L., Liu, P. (2017). Transporter-mediated nuclear entry of jasmonoylisoleucine is essential for jasmonate signaling. Molecular Plant, 10, 695-708. doi:10.1016/j.molp.2017.01.010.

[213] Liu, MQ., Yanai, J., Jiang, RF., Zhang, FS., McGrath, SP., Zhao, FJ. (2008). Does cadmium play a physiological role in the hyperaccumulator *Thlaspi caerulescens*? Chemosphere 71:1276–1283.

[214] Liu, T., Liu, B., Zhang, W. (2014). Nutrients and Heavy Metals in Biochar Produced by Sewage Sludge Pyrolysis: Its Application in Soil Amendment. Polish J Environ. Studies 23(1):271-275.

[215] Liu, X., Fu, J., Tang, N., Silva, E.D., Cao, Y., Turner, B.L., Ma, L.Q. (2017). Phytate induced arsenic uptake and plant growth in arsenic-hyperaccumulator *Pteris vittata*, Environmental Pollution, 226, 212-218.

[216] Liu, N., Ding, Y., Fromm, M., Avramova, Z. (2014). Different gene-specific mechanisms determine the revised-response memory transcription patterns of a subset of *A. thaliana* dehydration stress responding genes. Nucleic Acids Res. 42 (9), 5556-5566.

[217] Lone, M.I., He, Z., Stoffella, PJ., Yang, X. (2008). Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. Journal of Zhejiang University SCIENCE B 9, 210–220.

[218] Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

[219] López-Millán, AF., Sagardoy, R., Solanas, M., Abadía, A., Abadía, J. (2009). Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics. Environ Exp Bot 65: 376–385.

[220] Lukacová, Z., Švubová, R., Kohanová, J., and Lux, A. (2013). Silicon mitigates the Cd toxicity in maize in relation to cadmium translocation, cell distribution, antioxidant enzymes stimulation and enhanced endodermal apoplasmic barrier development. Plant Growth Regul. 70, 89–103. doi: 10.1007/s10725-012-9781-4

[221] Luo, Z.B., He, J., Polle, A., Rennenberg, H. (2016). Heavy metal accumulation and signal transduction in herbaceous and woody plants: Paving the way for enhancing phytoremediation efficiency. Biotechnol. Adv. 34(6), 1131–1148.

[222] Ma, B., Lyu, X., Zha, T., Gong, J., He, Y., Xu, J. (2015). Reconstructed metagenomes reveal changes of microbial functional profiling during PAHs degradation along a rice (*Oryza sativa*) rhizosphere gradient, Journal of Applied Microbiology, 118(4), 890-900.

[223] Ma, Y., Rajkumar, M., Zhang, C., Freitas, H. (2016). Inoculation *Brassica oxyrrhina* with plant growth promoting bacteria for the improvement of heavy metal phytoremediation under drought conditions. J. Hazard. Mater. 320, 36–44.

[224] Maco, B., Bardos, P., Coulon, F., Erickson-Mulanax, E., Hansen, L.J., Harclerode, M., Wick, W.D. (2018). Resilient remediation: Addressing extreme weather and climate change, creating community value. Remed. J. 29(1), 7–18.

[225] Madejón, P., Cantos, M., Jiménez-Ramos, M.C. (2015). Effects of soil contamination by trace elements on white poplar progeny: seed germination and seedling vigour Environ Monit Assess 187: 663.

[226] Mahar, A., Wang, P., Ali, A., Awasthi, MK., Lahori, AH., Wang, Q., Li, R., Zhang, Z. (2016). Challenges and opportunities in the phytoremediation of heavy metals contaminated soils: A review. Ecotoxicology and Environmental Safety, 126, 111-121. doi:10.1016/j.ecoenv.2015.12.023

[227] Maksymiec, W., Krupa, Z. (2006). The effects of short-term exposition to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*. Environ Exp Bot 57:187–194

[228] Malinowski, D.P., Belesky, D.P., Lewis, G.C. (2005). Abiotic stresses in endophyte grasses. In: Craig AR, Charles PW, Donald ES (eds) Neotyphodium in cool-season grasses. pp 187–199

[229] Mandal, S., Mahapatra, SS., Patel, R.K. (2015). Enhanced removal of Cr(VI) by cerium oxide polyaniline composite: Optimization and modeling approach using response surface methodology and artificial neural networks, Journal of Environmental Chemical Engineering. 3(2): 870 - 885. doi:/10.1016/j.jece.2015.03.028

[230] Maqbool, A., Ali, S., Rizwan, M., Ishaque, W., Rasool, N., Rehman, M. Z., Wu, L. (2018). Management of tannery wastewater for improving growth attributes and reducing chromium uptake in spinach through citric acid application. Environ. Sci. Pollut. Res. 25(11), 10848-10856.

[231] Marchand, L., Sabaris, C., Desjardins, D., Oustrière, N., Pesme, E., Butin, D., Mench, M. (2015). Plant responses to a phytomanaged urban technosol contaminated by trace elements and polycyclic aromatic hydrocarbons, Environmental Science and Pollution Research, 23(4), 3120-3135.

[232] Marinova, D., Ribarova, Fanny., Atanassova, Maria. (2005). Total phenolics and flavonoids in Bulgarian fruits and vegetables. J. Chem. Technol. Metall. 40, 255-260.

[233] Marques, A.P.G.C., Rangel, A.O.S.S., Castro, P.M.L. (2009). Remediation of Heavy Metal Contaminated Soils: phytoremediation as a potentially promising clean-up technology. Critical Reviews in Environmental Science and Technology 39, 622–654.

[234] Martins, M.Q., Fortunato, A.S., Rodrigues, W.P. (2017). Selection and Validation of Reference Genes for Accurate RT-qPCR Data Normalization in Coffea spp. under a Climate Changes Context of Interacting Elevated [CO_2] and Temperature. Front In Plant Sci 8:307.

[235] Masood, A,. Iqbal, N., Khan, A. (2012). Role of ethylene in alleviation of cadmium-induced photosynthetic capacity inhibition by sulphur in mustard. Plant, Cell & Environment, 35: 524-533.

[236] Matraszek, R., Hawrylak-Nowak, B., Chwil, S. (2016). Interaction Between Cadmium Stress and Sulphur Nutrition Level on Macronutrient Status of Sinapis alba L. Water Air Soil Pollut. 227:355. doi:10.1007/s11270-016-3059-9

[237] Mayo, A.W., Hanai, E.E. (2017). Modeling phytoremediation of nitrogen-polluted water using water hyacinth (*Eichhornia crassipes*), Physics and Chemistry of the Earth, Parts A/B/C. 100:170-180.

[238] Mazari, K., Landa, P., Přerostová, S., Müller, K., Vaňková, R., Soudek, P., Vaněk, T. (2017). Thorium impact on tobacco root transcriptome, Journal of Hazardous Materials, 325, 163-169.

[239] Mendes, L.A., Maria, V.L., Scott-Fordsmand, J.J., Amorim, M.J.B. (2015). Ag Nanoparticles (Ag NM300K) in the Terrestrial Environment: Effects at Population and Cellular Level in *Folsomia candida* (Collembola). Int J Environ Res Public Health 12:12530.

[240] Meng, H., Hua, S., Shamsi, I.H., Jilani, G., Li, Y., Jiang, L. (2012). Cadmium - induced stress on the seed germination and seedling growth of *Brassica napus* L. and its alleviation through exogenous plant growth regulators. Plant Growth Regul. 58:47–59.

[241] Metwally, A., Safronova, V.I., Belimov, A.A., Dietz, K.J. (2005). Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. J. Exp. Bot. 2005;409:167–178.

[242] Mirzahosseini, Z., Shabani, L., Sabzalian, M. R., Sharifi-Tehrani, M. (2014). *Neotyphodium* endophytes may increase tolerance to Ni in tall fescue. Eur. J. Soil. Biol. 63, 33-40.

[243] Mišík, M., Krupitza, G., Mišíková, K., Mičieta, K., Nersesyan, A., Kundi, M., & Knasmueller, S. (2016). The *Tradescantia micronucleus* assay is a highly sensitive tool for the detection of low levels of radioactivity in environmental samples. Environmental Pollution, 219, 1044–1048. doi:10.1016/j.envpol.2016.09.004

[244] Mitton, F. M., Ferreira, J. L., Gonzalez, M., Miglioranza, K. S., Monserrat, J. M. (2016). Antioxidant responses in soybean and alfalfa plants grown in DDTs contaminated soils: Useful variables for selecting plants for soil phytoremediation? Pestic. Biochem. Physiol. 130, 17-21.

[245] Mohamed, B., Mounia, K., Aziz, A., Ahmed, H., Rachid, B., Lotfi, A. (2018). Sewage sludge used as organic manure in Moroccan sunflower culture: effects on certain soil properties, growth and yield components. Science of Total Environment. 627:681–688. doi:10.1016/j.scitotenv.2018.01.258.

[246] Mohammadi, F., Samaei, M. R., Azhdarpoor, A., Teiri, H., Badeenezhad, A., Rostami, S. (2019). Modelling and Optimizing Pyrene Removal from the Soil

by Phytoremediation using Response Surface Methodology, Artificial Neural Networks, and Genetic Algorithm. Chemosphere. 237, 124486.

[247] Mohammadzadeh, A., Tovakoli, M., Motesharezadeh, B., Chaichi, M.R. (2017). Effects of plant growth-promoting bacteria on the phytoremediation of cadmium-contaminated soil by sunflower. Archives of Agronomy and Soil Science 63, 807-816.

[248] Mojiri, A., Aziz, H.A., Tajuddin, R.B.M., Gavanji, S., Gholami, A. (2015). Heavy Metals Phytoremediation from Urban Waste Leachate by the Common Reed (Phragmites australis). Phytoremediation, 75–81.

[249] Mortel, J. E., Schat, H., Moerland, P. D., Themaat, E. V., Ent, S. V., Blankestijn, H., Aarts, M. G. (2008). Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator Thlaspi caerulescens. Plant Cell Environ. 31 (3), 301-324.

[250] Motuzova, G., Minkina, T., Karpova, E., Barsova, N., Mandzhieva, S. (2014). Soil contamination with heavy metals as a potential and real risk to the environment. J. Geochem. Explor. 144, 241-246.

[251] Movafeghi, A., Khataee, A.R., Moradi, Z., Vafaei, F. (2016). Biodegradation of direct blue 129 diazo dye by *Spirodela polyrrhiza*: An artificial neural networks modeling, International Journal of Phytoremediation. 18(4):337-347. doi:/10.1080/15226514.2015.1109588

[252] Murtaza, G., Asghar, R. (2013). Effects of salicylic acid on sucrose synthase activity during seed development and germination in pea (*Pisum sativum* L.). Bangladesh J. Bot. 42 (1), 83-90.

[253] Murtaza, G., Asghar, R., Majid, S.A. (2010). Changes in specific activity of ascorbate peroxidase during seed development of pea (*Pisum sativum* L.) treated with salicylic acid. African Journal of Biotechnology 9, 145-162.

[254] Mutethya, LR., Mwangi, JK., Ndirangu, W. (2017). Heavy metal phytoextraction in sewage sludge using sunflower. Journal of sustainable research in engineering. 4(1): 12–22.

[255] Muthusaravanan, S. (2020). Research Updates on Heavy Metal Phytoremediation: Enhancements, Efficient Post-harvesting Strategies and Economic Opportunities. In: Naushad M., Lichtfouse E. (eds) Green Materials for Wastewater Treatment. Environmental Chemistry for a Sustainable World, vol 38. Springer, Cham

[256] Muthusaravanan, S., Sivarajasekar, N., Vivek, J.S., Paramasivan, T., Naushad, M., Prakashmaran, J., Gayathri, V., Al-Duaij, O.K. (2018). Phytoremediation of heavy metals: mechanisms, methods and enhancements. Environ. Chem. Lett. 16, 1339–1359.

[257] Naguib, A. E., El-Baz, F. K., Salama, Z. A., Hanaa, H. A., Ali, H. F., Gaafar, A. A. (2012). Enhancement of phenolics, flavonoids and glucosinolates of Broccoli (*Brassica olaracea,* var. *Italica*) as antioxidants in response to organic and bio-organic fertilizers. J. Saudi Soc. Agric. Sci. 11 (2), 135-142.

[258] Nahar, N., Rahman, A., Nawani, N.N., Ghosh, S., Mandal, A. (2017). Phytoremediation of arsenic from the contaminated soil using transgenic tobacco plants expressing *ACR2* gene of *Arabidopsis thaliana*, Journal of Plant Physiology, 218, 121-126.

[259] Nahar, K., Hasanuzzaman, M., Alam, M. M., Rahman, A., Suzuki, T., & Fujita, M. (2016). Polyamine and nitric oxide crosstalk: Antagonistic effects on cadmium toxicity in mung bean plants through upregulating the metal

detoxification, antioxidant defense and methylglyoxal detoxification systems. Ecotoxicology and Environmental Safety, 126, 245-255. doi:10.1016/j.ecoenv.2015.12.026

[260] Neag, E., Malschi, D., Maic aneanu, A. (2018). Isotherm and kinetic modelling of Toluidine Blue (TB) removal from aqueous solution using Lemna minor. Int. J. Phytoremed. 20, 1049-1054.

[261] NF ISO 10390, (2005). Soil Quality, Determination of pH. AFNOR, 7 pp.

[262] Nguyen, V. N. T., Lee, S. B., Suh, M. C., An, G., & Jung, K.-H. (2018). OsABCG9 Is an Important ABC Transporter of Cuticular Wax Deposition in Rice. Frontiers in Plant Science, 9. doi:10.3389/fpls.2018.00960

[263] Nguyen, V. N. T., Moon, S., & Jung, K.-H. (2014). Genome-wide expression analysis of rice *ABC* transporter family across spatio-temporal samples and in response to abiotic stresses. Journal of Plant Physiology, 171(14), 1276–1288. doi:10.1016/j.jplph.2014.05.006

[264] Nissim, WG., Cincinelli, A., Martellini, T., Alvisi, L., Palm, E., Mancuso, S., Azzarello, E. (2018). Phytoremediation of sewage sludge contaminated by trace elements and organic compounds. Environmental Research, 164, 356-366, doi:/10.1016/j.envres.2018.03.009.

[265] Niu, K., Shi, Y., Ma, H. (2017). Selection of Candidate Reference Genes for Gene Expression Analysis in Kentucky Bluegrass (*Poa pratensis* L.) under Abiotic Stress. Front in Plant Sci 8:193.

[266] Oconnor, D., Peng, T., Li, G., Wang, S., Duan, L., Mulder, J., Hou, D. (2018). Sulfur-modified rice husk biochar: A green method for the remediation of mercury contaminated soil. Sci. Total Environ. 621, 819–826.

[267] Oconnor, D., Zheng, X., Hou, D., Shen, Z., Li, G., Miao, G., Guo, M. (2019). Phytoremediation: Climate change resilience and sustainability assessment at a coastal brownfield redevelopment. Environ. Int. 130, 104945.

[268] Oh, K., Cao, T., Li, T., Cheng, H. (2014). Study on Application of Phytoremediation Technology in Management and Remediation of Contaminated Soils. J. Clean Energy Technol. 2, 216–220.

[269] Oneto, CD., Otegui, ME., Baroli, I., Beznec, A., Faccio, P., Bossio, E., Lewi, D. (2016). Water deficit stress tolerance in maize conferred by expression of an isopentenyltransferase (IPT) gene driven by a stress- and maturation-induced promoter. Journal of Biotechnology, 220, 66-77.

[270] Oporto, C., Arce, O., Van den Broeck, E., Van der Bruggen, B., Vandecasteele, C. (2006). Experimental study and modelling of Cr (VI) removal from wastewater using *Lemna minor*. Water Res. 40, 1458-1464.

[271] Ouyang, Y. (2002). Phytoremediation: modelling plant uptake and contaminant transport in the soil–plant–atmosphere continuum. J. Hydrol. 266(1-2), 66–82.

[272] Ouyang, Y., Huang, C. H., Huang, D. Y., Lin, D., Cui, L. (2007). Simulating uptake and transport of TNT by plants using STELLA. Chemosphere, 69(8), 1245–1252.

[273] Pal, S., Patra, A. K., Reza, S. K., Wildi, W., Pote, J. (2010). Use of Bio-Resources for Remediation of Soil Pollution. Natural Resources 01 (02), 110-125.

[274] Palacio, S., Maestro, M., Montserrat-Martí, G. (2007). Relationship between Shoot-rooting and Root-sprouting Abilities and the Carbohydrate and Nitrogen Reserves of Mediterranean Dwarf Shrubs. Ann. Bot. 100 (4), 865-874.

[275] Pandey, S.K., Bhattacharya, T., Chakraborty, S. (2015). Metal phytoremediation potential of naturally growing plants on fly ash dumpsite of Patratu thermal power station, Jharkhand, India. Int. J. Phytorem. 18(1), 87-93.
[276] Pant, P. P., Tripathi, A. (2014). Impact Of Heavy Metals On Morphological And Biochemical Parameters Of *Shorea Robusta* Plant. Ekologia. 33 (2), 116-126.
[277] Park, J., Song, W.Y., Ko, D., Eom, Y., Hansen, T.H., Schiller, M., Lee, Y. (2012). The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. The Plant Journal, 69, 278–288.
[278] Paul, A.L., Chaney, R.L. (2017). Effect of soil amendments on Cd accumulation by spinach from a Cd-mineralized soil. J. Environ. Qual. 46, 707–713.
[279] Pedron, F., Grifoni, M., Barbafieri, M., Petruzzelli, G., Rosellini, I., Franchi, E., Vocciante, M. (2017). Applicability of a Freundlich-Like Model for Plant Uptake at an Industrial Contaminated Site with a High Variable Arsenic Concentration. Environments. 4(4), 67.
[280] Peng, C., Cai, Y., Wang, T., Xiao, R., Chen, W. (2016). Regional probabilistic risk assessment of heavy metals in different environmental media and land uses: an urbanization-affected drinking water supply area. Sci Rep-UK 6:37084.
[281] Peters, C.M., Gentry, A.H., Mendelsohn, R.O. (1989). Valuation of an Amazonian rainforest. Nature 339, 655–656.
[282] Phillips, L.A., Greer, C.W., Farrell, R.E., Germida, J.J. (2012). Plant root exudates impact the hydrocarbon degradation potential of a weathered-hydrocarbon contaminated soil, Applied Soil Ecology, 52, 56-64.
[283] Placek, A., Grobelak, A., Kacprzak, M. (2016). Improving the phytoremediation of heavy metals contaminated soil by use of sewage sludge. International Journal of Phytoremediation. 18(6):605-618. doi:10.1080/15226514.2015.1086308.
[284] Placek, A., Grobelak, A., Hiller, J., Stępień, W., Jelonek, P., Jaskulak, M., Kacprzak, M. (2017). The Role of Organic and Inorganic Amendments in Carbon Sequestration and Immobilization of Heavy Metals in Degraded Soils. J. sustain. dev. energy water environ. syst 5(4):509-517.
[285] Plociniczak, T., Kukla, M., Watroba, R., Piotrowska-Seget, Z. (2013). The effect of soil bioaugmentation with strains of Pseudomonas on Cd, Zn and Cu uptake by *Sinapis alba* L. Chemosphere 91, 1332–1337.
[286] Polasky, S., Carpenter, S. R., Folke, C., Keeler, B. (2011). Decision-making under great uncertainty: environmental management in an era of global change. Trends Ecol. Evol. 26(8), 398–404.
[287] Polle, A., Schuetzenduebel, A. (2003). Hirt H, Shinozaki K. Heavy metal signalling in plants: linking cellular and organismic responses. Plant Responses to Abiotic Stress; Berlin-Heidelberg Springer-Verlag 187 - 215
[288] Pulford, I.D., Riddell-Black, D., Stewart, C. (2002). Heavy Metal Uptake by Willow Clones from Sewage Sludge-Treated Soil: The Potential for Phytoremediation, International Journal of Phytoremediation. 4(1):59-72. doi:10.1080/15226510208500073
[289] Qadir, S., Jamshieed, S., Rasool, S., Ashraf, M., Akram, N. A., and Ahmad, P. (2014). "Modulation of plant growth and metabolism in cadmium-enriched environments," in Reviews of Environmental Contamination and Toxicology,

ed. D. M. Whitacre (Cham: Springer International Publishing), 51–88. doi: 10.1007/978-3-319-03777-6_4

[290] Qiao, S., Yamamoto, T., Misaka, M., Isaka, K., Sumino, T., Bhatti, Z., Furukawa. K. (2010). High-rate nitrogen removal from livestock manure digester liquor by combined partial nitritation – anammox process. Biodegradation 21, 11–20.

[291] Qiao, Z., Libault, M. (2013). Unleashing the potential of the root hair cell as a single plant cell type model in root systems biology. Front. Plant. Sci. 4, 484.

[292] Reddy, K.R., Amaya-Santos, G., Yargicoglu, E., Cooper, D.E., Negri, M.C., 2017. Phytoremediation of heavy metals and PAHs at slag fill site: three-year field-scale investigation. Int. J. Geotech. Eng. 13(1), 32–47.

[293] Redondo-Gmez, S., Mateos-Naranjo, E., Andrades-Moreno, L. (2010). Accumulation and tolerance characteristics of cadmium in a halophytic Cd-hyperaccumulator, *Arthrocnemum macrostachyum*. J. Hazard. Mater. 184: 299–307.

[294] Rehman, M., Rizwan, M., Ali, S., Fatima, N., Yousaf, B., Naeem, A., Sabir, M., Ahmad, H.R., Ok, Y.S. (2016). Contrasting effects of biochar, compost and farm manure on alleviation of nickel toxicity in maize (*Zea mays* L.) in relation to plant growth, photosynthesis and metal uptake. Ecotoxicol. Environ. Saf. 133:218-225.

[295] Ren, H., Wan, Y., Zhao, Y. (2018). Phytoremediation of Polychlorinated Biphenyl-Contaminated Soil by Transgenic Alfalfa Associated Bioemulsifier AlnA, Twenty Years of Research and Development on Soil Pollution and Remediation in China, 2018, 645-653.

[296] Ren, Y., Liu, Y., Chen, H., Li, G., Zhang, X., Zhao, J. (2012). Type 4 metallothionein genes are involved in regulating Zn ion accumulation in late embryo and in controlling early seedling growth in *Arabidopsis*. Plant Cell Environ 35:770–789.

[297] Rinaldo, A., Gilbert, B., Boni, R., Krattinger, S. G., Singh, D., Park, R. F., Ayliffe, M. (2017). The Lr34 adult plant rust resistance gene provides seedling resistance in durum wheat without senescence. Plant Biotechnology Journal, 15(7), 894–905. doi:10.1111/pbi.12684

[298] Rizwan, M., Ali, S., Akbar, Z., Shakoor, M.B., Mahmood, A., Hussain, A. (2017). Foliar application of aspartic acid lowers cadmium uptake and Cd-induced oxidative stress in rice under Cd stress. Environ. Sci. Pollut. Res. 24, 21938–21947.

[299] Roohi, R., Jafari, M., Jahantab, E., Aman, M.S., Moameri, M., Zare, S. (2019). Application of artificial neural network model for the identification the effect of municipal waste compost and biochar on phytoremediation of contaminated soils. Journal of Geochemical Exploration. 106399. doi:10.1016/j.gexplo.2019.106399

[300] Rossi, L., Bagheri, M., Zhang, W., Chen, Z., Burken, J.G., Ma, X. (2019). Using artificial neural network to investigate physiological changes and cerium oxide nanoparticles and cadmium uptake by *Brassica napus* plants. Environmental Pollution. 246:381–389. doi:10.1016/j.envpol.2018.12.029

[301] Roy, M., & Mcdonald, L. M. (2015). Metal Uptake in Plants and Health Risk Assessments in Metal-Contaminated Smelter Soils. Land Degradation and Development, 26(8), 785–792. https://doi.org/10.1002/ldr.2237

[302] Sanusi, S.N.A., Halmi, M.I.E., Abdullah, S.R.S., Hassan, H.A., Hamzah, F.M., Idris, M. (2016). Comparative process optimization of pilot-scale total

petroleum hydrocarbon (TPH) degradation by *Paspalum scrobiculatum* L. Hack using response surface methodology (RSM) and artificial neural networks (ANNs). Ecological Engineering. 97:524–534. doi:10.1016/j.ecoleng.2016.10.044

[303] Sarwar, N., Imran, M., Shaheen, M. R., Ishaque, W., Kamran, M. A., Matloob, A., Hussain, S. (2017). Phytoremediation strategies for soils contaminated with heavy metals: Modifications and future perspectives. Chemosphere, 171, 710-721. doi:10.1016/j.chemosphere.2016.12.116

[304] Sasse, J., Schlegel, M., Borghi, L., Ullrich, F., Lee, M., Liu, G. W., Giner, J. L., Kayser, O., Bigler, L., Martinoia, E., and Kretzschmar, T. (2016). *Petunia hybrida* PDR2 is involved in herbivore defense by controlling steroidal contents in trichomes. Plant Cell Environment, 39, 2725–2739, doi:10.1111/pce.12828.

[305] Saveyn, H., Eder, P. (2014). End-of-waste criteria for biodegradable waste subjected to biological treatment (compost & digestate): technical proposals. In: ‘‘JR Scientific and Policy Reports’’. European Comission - Joint Research Centre, Seville. Assessed 20 Mar 2020, at http://www.ftp.jrc.es/EURdoc/JRC87124.pdf

[306] Sawidis, T. (2008). Effect of cadmium on pollen germination and tube growth in *Lilium longiflorum* and *Nicotiana tabacum*. Protoplasma 233, 95–106. doi: 10.1007/s00709-008-0306-y

[307] Selamat, S.N., Halmi, M.I.E.B., Abdullah, S.R.S., Idris, M., Hasan, H.A., Anuar, N. (2017). Optimization of lead (Pb) bioaccumulation in *Melastoma malabathricum* L. by response surface methodology (RSM). Rendiconti Lincei. Scienze Fisiche e Naturali. 29(1):43–51. doi: 10.1007/s12210-017-0656-5

[308] Semane, B., Cuypers, A., Smeets, K., Van Belleghem, F., Horemans, N., Schat, H., Vangronsveld, J. (2007). Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. Physiol. Plant 129:519–528.

[309] Seneviratne, M., Gunaratne, S., Bandara, T., Weerasundara, L., Rajakaruna, N., Seneviratne, G., Vithanage, M. (2016). Plant growth promotion by Bradyrhizobium japonicum under heavy metal stress, South African Journal of Botany 105, 19-24.

[310] Šereš, Z., Maravić, N., Takači, A., Nikolić, I., Šoronja-Simović, D., Jokić, A., Hodur, C. (2016). Treatment of vegetable oil refinery wastewater using alumina ceramic membrane: optimization using response surface methodology, Journal of Cleaner Production. 112(4):3132-3137. doi:10.1016/j.jclepro.2015.10.070.

[311] Shabani, L., Sabzalian, M. R., & Pour, S. M. (2015). Arbuscular mycorrhiza affects nickel translocation and expression of ABC transporter and metallothionein genes in *Festuca arundinacea*. Mycorrhiza, 26(1), 67-76. doi:10.1007/s00572-015-0647-2

[312] Shafaghat, A. 2012. Removal of heavy metals (Pb2 , Cu2 and Cr3) from aqueous solutions using five plants material. Afr. J. Biotechnol. 11(4), 852-855.

[313] Shahid, M., Dumat, C., Khalid, S., Niazi, N.K., Antunes, P.M. (2017). Cadmium bioavailability, uptake, toxicity and detoxification in soil-plant system. Rev. Environ. Contam. Toxicol. 241, 73–137.

[314] Sharavanan, V.J., Sivaramakrishnan, M., Sivarajasekar, N. (2020). Pollutants inducing epigenetic changes and diseases. Environ Chem Lett. 18:325–343. https://doi.org/10.1007/s10311-019-00944-3

[315] Sharkey, T. D. (2005). Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. Plant Cell Environ. 28 (3), 269-277.

[316] Sharma, R., Bhardwaj, R., Handa, N., Gautam, V., Kohli, S.K., Bali, S., Kaur, P., Thukral, A.K., Arora, S., Ohri, P., Vig, A.P. (2016). Chapter 10 - Responses of Phytochelatins and Metallothioneins in Alleviation of Heavy Metal Stress in Plants: An Overview, In Plant Metal Interaction, edited by Parvaiz Ahmad. Elsevier, Pages 263-283.

[317] Sharma, P., Dubey, R. S. (2007). Involvement of oxidative stress and role of antioxidative defense system in growing rice seedlings exposed to toxic concentrations of aluminum. Plant Cell Rep. 26 (11), 2027-2038.

[318] Sharma, R., Bhardwaj, R., Handa, N., Gautam, V., Kaur Kohli, S., Bali, S., Kaur, P., Kumar A., Arora, S., Ohri, P., Pal Vig, A. (2016). Chapter 10 - Responses of Phytochelatins and Metallothioneins in Alleviation of Heavy Metal Stress in Plants: An Overview, Editor(s): Parvaiz Ahmad, Plant Metal Interaction, Elsevier, Pages 263-283.

[319] Sharma, S., Singh, B., Manchanda, V.K. (2014). Phytoremediation: Role of terrestrial plants and aquatic macrophytes in the remediation of radionuclides and heavy metal contaminated soil and water. Environ. Sci. Pollut. Res. 22(2), 946-962.

[320] Shelmerdine, P.A., Black, C.R., Mcgrath, S.P., Young, S.D. (2009). Modelling phytoremediation by the hyperaccumulating fern, *Pteris vittata*, of soils historically contaminated with arsenic. Environ. Pollut. 157(5), 1589–1596.

[321] Shi, Y., Xie, H., Cao, L., Zhang, R., Xu, Z., Wang, Z., Deng, Z. (2017). Effects of Cd- and Pb resistant endophytic fungi on growth and phytoextraction of *Brassica napus* in metal-contaminated soils. Environ. Sci. Pollut. Res. 24, 417–426.

[322] Shibata, Y., Ojika, M., Sugiyama, A., Yazaki, K., Jones, D. A., Kawakita, K., and Takemoto, D. (2016). The full-size ABCG transporters Nb-ABCG1 and Nb-ABCG2 function in pre- and post-invasion defense against *Phytophthora infestans* in *Nicotiana benthamiana*. Plant Cell, 28, 1163–1181. doi: https://doi.org/10.1105/tpc.15.00721

[323] Siliang, L., Wenhao, Y., Tingting, Y., Yan, C. & Wuzhong, Ni. (2013). Effects of Cadmium Stress on Leaf Chlorophyll Fluorescence and Photosynthesis of *Elsholtzia argyi*—A Cadmium Accumulating Plant, International Journal of Phytoremediation, 17:1, 85-92, DOI: 10.1080/15226514.2013.828020

[324] Singh, A. K., Pandey, M. B., Singh, U. P. (2007). Antifungal Activity of an Alkaloid Allosecurinine against Some Fungi. Mycobiology. 35 (2), 62.

[325] Sinha, P., Saxena, R.K., Singh, V.K., Krishnamurthy, L., Varshney, R.K. (2015). Selection and Validation of Housekeeping Genes as Reference for Gene Expression Studies in Pigeonpea (*Cajanus cajan*) under Heat and Salt Stress Conditions. Front Plant Sci 6.

[326] Sivarajasekar, N. (2019). Recent Updates on Heavy Metal Remediation Using Date Stones (*Phoenix dactylifera* L.) – Date Fruit Processing Industry Waste.

In: Naushad M., Lichtfouse E. (eds) Sustainable Agriculture Reviews. 34. Springer, Cham

[327] Smith, S.R. (2009). A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge, Environ. Int. 35, 142–156.

[328] Song, F., Li, J., Fan, X., Zhang, Q., Chang, W., Yang, F., Geng, G. (2016). Transcriptome analysis of *Glomus mosseae*/*Medicago sativa* mycorrhiza on atrazine stress, Scientific Reports, 6(1).

[329] Song, W.-Y., Park, J., Eisenach, C., Maeshima, M., Lee, Y., & Martinoia, E. (2014). ABC Transporters and Heavy Metals. Plant ABC Transporters, 1–17. doi:10.1007/978-3-319-06511-3_1

[330] Soudani, L., Maatoug, M., Heilmeier, H., Kharytonov, M., Wiche, O., Moschner, C., Onyshchenkoc, E., Bouchenafa, N. (2017). Fertilization value of municipal sewage sludge for Eucalyptus camaldulensis plants. Biotechnol Rep 13.

[331] Soudek, P., Petrova, Š., Vankova, R., Song, J., Vanek, T. (2014). Accumulation of heavy metals using Sorghum sp. Chemosphere 104:15–24.

[332] Souza, T.D., Borges, A., Matos, A.T., Veloso, R.W., Braga, A.F. (2018). Optimization of arsenic phytoremediation using *Eichhornia crassipes*, International Journal of Phytoremediation. 20(11):1129-1135, doi:10.1080/15226514.2018.1460308

[333] Stritsis, C., Steingrobe, B., Claassen, N. (2014). Cadmium Dynamics in the Rhizosphere and Cd Uptake of Different Plant Species Evaluated by A Mechanistic Model. Int. J. Phytorem. 16(11), 1104–1118.

[334] Sucher, J., Boni, R., Yang, P., Rogowsky, P., Büchner, H., Kastner, C., Keller, B. (2016). The durable wheat disease resistance gene *Lr34* confers common rust and northern corn leaf blight resistance in maize. Plant Biotechnology Journal, 15(4), 489–496. doi:10.1111/pbi.12647

[335] Sumner, M.E,. and Miller, W.P. (1996). Cation exchange capacity, and exchange coefficients. In: D.L. Sparks (ed.) Methods of soil analysis. Part 2: Chemical properties (3rd ed.). ASA, SSSA, CSSA, Madison, WI

[336] Tang, M., Mao, D., Xu, L., Li, D., Song, S., and Chen, C. (2014). Integrated analysis of miRNA and mRNA expression profiles in response to Cd exposure in rice seedlings. BMC Genomics 15:835. doi: 10.1186/1471-2164-15-835

[337] Thakur, S., Singh, L., Wahid, Z.A. (2016). Plant-driven removal of heavy metals from soil: uptake, translocation, tolerance mechanism, challenges, and future perspectives. Environmental Monitoring Assessment, 188(206). https://doi.org/10.1007/s10661-016-5211-9

[338] Thomaidi, V. S., Stasinakis, A. S., Borova, V. L. & Thomaidis, N. S. (2016). Assessing the risk associated with the presence of emerging organic contaminants in sludge-amended soil: A country-level analysis. Science of Total Environment, 548–549, 280–288. doi:10.1016/j.scitotenv.2016.01.043

[339] Titah, H.S., Halmi, M.I.E.B., Abdullah, S.R.S., Hasan, H.A., Idris, M., Anuar, N. (2018). Statistical optimization of the phytoremediation of arsenic by *Ludwigia octovalvis* - in a pilot reed bed using response surface methodology (RSM) versus an artificial neural network (ANN). International Journal of Phytoremediation. 20(7):721–729.

[340] Toussaint, F., Pierman, B., Bertin, A., Lévy, D., Boutry, M. (2017). Purification and biochemical characterization of NpABCG5/NpPDR5, a plant pleiotropic drug resistance transporter expressed in *Nicotiana tabacum* BY-2

suspension cells. The Biochemical Journal, 474, 1689–1703. doi:10.1042/BCJ20170108

[341] Tóth, G., Hermann, T., Silva, M. D., Montanarella, L. (2016). Heavy metals in agricultural soils of the European Union with implications for food safety. Environ. Int. 88, 299-309.

[342] Trakal, L., Kodešová, R., Komárek, M. (2012). Modelling of Cd, Cu, Pb and Zn transport in metal contaminated soil and their uptake by willow (*Salix* × *smithiana*) using HYDRUS-2D program. Plant and Soil. 366(1-2), 433–451.

[343] Tsakou, A., Roulia, M., Christodoulakis, N.S. (2003). Growth parameters and heavy metal accumulation in Poplar tree cultures (*Populus euramericana*) utilising water and sludge from a sewage treatment plant. Bull. Environ. Contam. Toxicol. 71, 330–337.

[344] Ullah, A., Heng, S., Munis, M. F., Fahad, S., Yang, X. (2015). Phytoremediation of heavy metals assisted by plant growth promoting (PGP) bacteria: A review. Environ. Exp. Bot. 117, 28-40.

[345] Undeman, E., Czub, G., Mclachlan, M.S. (2009). Addressing Temporal Variability When Modelling Bioaccumulation in Plants. Environ. Sci. Technol. 43(10), 3751-3756.

[346] Urbaniak, M., Zieliński, M., Wyrwicka, A. (2016). The influence of the Cucurbitaceae on mitigating the phytotoxicity and PCDD/PCDF content of soil amended with sewage sludge. Int. J. Phytorem. 19(3), 207–213.

[347] US EPA 3051 Method (1994). Microwave-assisted acid digestion of sediments, sludges, soils and oils (pp 1–14). Technical Report, Washington, District of Columbia

[348] US EPA 3051 Method. (1994). Microwave-assisted acid digestion of sediments, sludges, soils and oils (pp 1–14). Technical Report, Washington, District of Columbia

[349] Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Gen Biol 3:0034.

[350] Verma, K., Shekhawat, G. S., Sharma, A., Mehta, S. K., and Sharma, V. (2008). Cadmium induced oxidative stress and changes in soluble and ionically bound cell wall peroxidase activities in roots of seedling and 3-4 leaf stage plants of *Brassica juncea* (L.) czern. Plant Cell Rep. 27, 1261–1269. doi: 10.1007/s00299-008-0552-7

[351] Wahid, A., Perveen, M., Gelani, S., Basra, S.M.A. (2007). Pretreatment of seed with H_2O_2 improves salt tolerance of wheat seedlings by alleviation of oxidative damage and expression of stress proteins. J. Plant. Physiol. 164:283–294

[352] Wang, S., Wei, S., Ji, D., Bai, J. (2015). Co-Planting Cd Contaminated Field Using Hyperaccumulator *Solanum Nigrum* L. Through Interplant with Low Accumulation Welsh Onion, International Journal of Phytoremediation, 17(9), 879-884.

[353] Wang, H., Liu, Y., Peng, Z., Li, J., Huang, W., Liu, Y., Wang, B. (2019). Ectopic Expression of Poplar ABC Transporter PtoABCG36 Confers Cd Tolerance in *Arabidopsis thaliana*. International Journal of Molecular Sciences, 20(13), 3293. doi: 10.3390/ijms20133293

[354] Wang, H., Tang, X., Wang, H., & Shao, H. (2015). Proline accumulation and metabolism-related genes expression profiles in Kosteletzkya virginica seedlings under salt stress. Frontiers in Plant Science, 6.

[355] Wang, Q., Gu, M., Ma, X., Zhang, H., Wang, Y., Cui, J., Gui, J. (2015). Model optimization of cadmium and accumulation in switchgrass (*Panicum virgatum* L.): potential use for ecological phytoremediation in Cd-contaminated soils. Environ. Sci. Pollut. Res. 22(21), 16758–16771.

[356] Wang, V., Liu, Y., Li, SS., Han, GZ. (2015). Insights into the origin and evolution of the plant hormone signaling machinery. Plant Physiology, 167, 872-886. doi:10.1104/pp.114.247403

[357] Wang, X., Wang, C., Sheng, H., Wang, Y., Zeng, J., Kang, H., Zhou, Y. (2016). Transcriptome-wide identification and expression analyses of ABC transporters in dwarf polish wheat under metal stresses. Biologia Plantarum, 61(2), 293-304. doi:10.1007/s10535-016-0697-0

[358] Wang, Z., Bai, M., Oh, E., Zhu, J. (2012). Brassinosteroid Signaling Network and Regulation of Photomorphogenesis. Annu. Rev. Genet. 46 (1), 701-724.

[359] Williams, BP., Aubry, S., Hibberd, JM. (2012). Molecular evolution of genes recruited into C4 photosynthesis. Trends in Plant Science, 17(4), 213-220.

[360] Włóka, D., Placek, A., Rorat, A., Smol, M., Kacprzak, M. (2017). The evaluation of polycyclic aromatic hydrocarbons (PAHs) biodegradation kinetics in soil amended with organic fertilizers and bulking agents. Ecotoxicol. Environ. Saf. 145, 161-168.

[361] Wójcik, M., Tukiendorf, A. (2014). Accumulation and tolerance of lead in two contrasting ecotypes of *Dianthus carthusianorum*, Phytochemistry, Volume 100, Pages 60-65.

[362] Xie, X., Wang, G., Yang, L., Cheng, T., Gao, J., Wu, Y., Xia, Q. (2015). Cloning and characterization of a novel *Nicotiana tabacum* ABC transporter involved in shoot branching. Physiologia Plantarum, 153, 299-306. doi:10.1111/ppl.12267

[363] Xin, Y., Huang, B., Dai, H., Mu, Y. (2017). Characterization of root morphology and root-derived low molecular weight organic acids in two sweet potato cultivars exposed to cadmium. Archives of Agronomy and Soil Science 63, 723-734.

[364] Xu, Y., Plaza, F.J., Liang, X., Davis, T.W., Nichols, J., Fu, J.K., Koranchie-Boah, P. (2020). A hydro-thermal-geochemical modeling framework to simulate reactive transport in a waste coal area under amended and non-amended conditions. Heliyon. 6(1), e02803.

[365] Yaashikaa, P.R., Kumar, P.S., Saravanan, A. (2019). Modelling and Cr(VI) ion uptake kinetics of Sorghum bicolor plant assisted by plant growth–promoting *Pannonibacter phragmetitus*: an ecofriendly approach. Environ. Sci. Pollut. Res. 1-12.

[366] Yadav, B., Molina, I., Ranathunge, K., Castillo, I. Q., Rothstein, S. J., Reed, J. W. (2014). ABCG transporters are required for suberin and pollen wall extracellular barriers in *Arabidopsis*. Plant Physiology. 26, 3569–3588. doi:10.1105/tpc.114.129049

[367] Yadav, S.K. (2010). Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants, South African Journal of Botany, Volume 76, Issue 2, Pages 167-179, ISSN 0254-6299,

[368] Yadava, K.K., Gupta, N., Kumar, A., Reece, L.M., Singh, N., Rezania, S., Khan, S.A. (2018). Mechanistic understanding and holistic approach of phytoremediation: a review on application and future prospects. Ecol. Eng. 120, 274–298.

[369] Yang, Y., Chen, Q., Wall, J.D., Hu, Z. (2012). Potential nanosilver impact on anaerobic digestion at moderate silver concentrations. Water Res 46:1176–1184.

[370] Yang, Y., Chen, W., Wang, M., Li, Y., Peng, C. (2017). Evaluating the potential health risk of toxic trace elements in vegetables: accounting for variations in soil factors. Sci. Total Environ. 584: 942–949

[371] Yıldırım, K., Uylaş, S. (2016). Genome-wide transcriptome profiling of black poplar (*Populus nigra* L.) under boron toxicity revealed candidate genes responsible in boron uptake, transport and detoxification, Plant Physiology and Biochemistry, 2016, 109, 146-155.

[372] Zha, Q., Xiao, Z., Zhang, X., Han, Z., Wang, Y. (2016). Cloning and functional analysis of *MxNRAMP1* and *MxNRAMP3*, two genes related to high metal tolerance of *Malus xiaojinensis*, South African Journal of Botany 102, 75-80,

[373] Zhang, F.Q., Zhang, H.X., Wang, G.P., Xu, L.L., Shen, Z.G. (2009). Cadmiuminduced accumulation of hydrogen peroxide in the leaf apoplast of *Phaseolus aureus* and *Vicia sativa* and the roles of different antioxidant enzymes. J Hazard Mater. doi:10.1016/j.jhazmat.2009.02.002

[374] Zhang, L., Rylott, E.L., Bruce, N. C., Strand, S.E. (2018). Genetic modification of western wheatgrass (*Pascopyrum smithii*) for the phytoremediation of RDX and TNT, Planta, 1-9.

[375] Zhang, M.K., Liu, Z.Y., Wang, H. (2010). Use of Single Extraction Methods to Predict Bioavailability of Heavy Metals in Polluted Soils to Rice. Commun. Soil. Sci. Plan. 41(7), 820–831.

[376] Zhang, S., Song, J., Cheng, Y., Christie, P., Long, J., Liu, L. (2018). Derivation of reliable empirical models describing lead transfer from metal-polluted soils to radish (*Raphanus sativa* L.): Determining factors and soil criteria. Sci. Total Environ. 613-614, 72–80.

[377] Zhang, S., Song, J., Gao, H., Zhang, Q., Lv, M.C., Wang, S., Sun, W. (2016). Improving prediction of metal uptake by Chinese cabbage (*Brassica pekinensis* L.) based on a soil-plant stepwise analysis. Sci. Total Environ. 569-570, 1595–1605.

[378] Zhao, X., Joo, J.C., Lee, J.K., Kim, J.Y. (2019). Mathematical estimation of heavy metal accumulations in *Helianthus annuus* L. with a sigmoid heavy metal uptake model. Chemosphere. 220, 965–973.

[379] Zhou, Q., Guo, J., He, C., Shen, C., Huang, Y., Chen, J., Yang, Z. (2016). Comparative Transcriptome Analysis between Low- and High-Cadmium-Accumulating Genotypes of Pakchoi (*Brassica chinensis* L.) in Response to Cadmium Stress, Environmental Science & Technology, 2016, 50(12), 6485-6494.

[380] Zhu, H., Ai, H., Cao, L., Sui, R., Ye, H., Du, D., Chen, L. (2018). Transcriptome analysis providing novel insights for Cd-resistant tall fescue responses to Cd stress, Ecotoxicology and Environmental Safety, 2018, 160, 349-356.

[381] Zimeri, A.M., Dhankher, O.P., McCaig, B., Meagher, R.B. (2005). The plant MT1 metallothioneins are stabilized by binding cadmium and are required for cadmium tolerance and accumulation. Plant Molec Biol 58:839–855.