A global approach for recovery of arable land through improved phytoremediation coupled with advanced liquid biofuel production and climate friendly copper smelting process

Deliverable D2.2: Report on plant growth and phytoremediation capacity optimization

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presented by Phy2Climate project consortium

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2	Fraunhofer	Fraunhofer Gesellschaft	BEN
3	AUR	Aurubis AG	BEN
4	LEITAT	LEITAT Technological Center	BEN
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13	LTC	Litoclean SL	BEN
14	CLH	Compania Logistica de Hidrocarburos S.A.	BEN
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MoM	Minutes of Meeting	
MAN	Procedures and user manuals	
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1. EXECUTIVE SUMMARY

Pot test experiments have been carried out by each Pilot Site Leader with the global aim of determining the best phytoremediation strategy to be applied in the demo contaminated site.
For this purpose, the pilot site leaders have defined a harmonized pot tests experimental plan for the sake of comparability and reproducibility of results.
The harmonized pot tests experimental plan includes both a sampling and a monitoring plan and defines a common framework in which pot tests have been conducted and is described in section 3.
Accordingly, although each pilot site has its own characteristics (type of soil, type of contaminant, plant species, amendments, climatic conditions, etc.), the 5 pilot sites of Spain, Serbia, Lithuania, Argentina, and India have assessed the progress of the phytoremediation strategy throughout pot tests, employing a common set of soil remediation and plant growth indicators.
Apart from stating the main parameters of the experimental design such as controls, number of replicates and the duration of the experiments, the most relevant parameters for the soil and energy crop characterisation have been agreed upon.
Moreover, to give answer to the specific characteristics of each pilot site, a specific phytoremediation strategy has been defined for each of them. In sections 4-8, the phytoremediation strategies applied to each Pilot Sites are described. They include a brief description of the contaminated site to recollect and summarize the information about the specific contamination of each Pilot Site (described in detail in deliverable D2.1, sections 4-8), the description of the experimental design followed to perform the pot tests and the specific sampling and monitoring campaign carried out to evaluate phytoremediation capacity of the tested treatments as well as biomass production potential. Apart from phytoremediation efficiency of the tested treatments, the estimation of biomass production is also presented since it is an important factor to be considered to meet the Specific Objective 1.1 (production of >40 kg energy crop per site and growing season must be reached) of Phy2Climate Project.
Finally, it is worth noticing that pot tests results will allow to define the final harmonized strategy to follow along the site phytoremediation period (3.5 years). Therefore, deliverable D 2.1 will be updated in M15 accordingly.

2. INTRODUCTION

Phytoremediation can be described as “the use of plants and associated soil microbes to reduce the concentrations or toxic effects of contaminants in the environments” (Greipsson, 2011[1]).
Phytoremediation has been largely studied in the last three decades for the removal of a large set of contaminants, such as heavy metals, radionuclides, and organic pollutants (total petroleum hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAH), and pesticides (Ali et al., 2013)[2].
It is a well socially accepted technique being an eco-friendly, cost-effective, and very efficient way of restoring contaminated areas which can be then available for different uses (food, feed, and feedstock for biofuels/biodiesels). Especially at urban sites it is an efficient tool to remove contaminants without the need of digging activities or other works with heavy machinery.

[1] Greipsson, S., 2011. Phytoremediation. Nat. Educ. Knowl. 2, 7.
[2] https://doi.org/10.1016/j.chemosphere.2013.01.075

Phytoremediation can occur by means of different mechanisms depending on the pollution sources, and are well summarized by Ali et al., 2013[3]:

- **Phytoextraction:** uptake of contaminants from soil or water by plant roots and their translocation/accumulation to the aboveground biomass (e.g., metals translocate to shoots).
- **Phytofiltration:** adsorption or absorption of contaminants from waters by plants. Phytofiltration may be rhizofiltration (use of plant roots), blastofiltration (use of seedlings) or caulofiltration (use of excised plant shoots).
- **Phytostabilization or phytoimmobilization:** use of specific plants that can stabilize the contaminants in contaminated soils so to avoid/minimize their mobility and bioavailability. It is efficiently applied to immobilize metals. However, this technique does not allow the degradation/removal of the contaminants but only limits their migration by inactivating/immobilizing them.
- **Phytovolatilization:** plants can uptake the pollutants from soil and convert them into volatile forms that are released into the atmosphere. This technique can be efficiently used for organic pollutants and some heavy metals, but it must be noticed that it does not eliminate the problem but only transfers it from one state to another (from soil to atmosphere).
- **Phytodegradation:** degradation of organic pollutants by plants with the help of enzymes, and it is not dependent on rhizospheric microorganisms. Phytodegradation is limited to the removal of organic pollutants because heavy metals are non-biodegradable.
- **Rhizodegradation:** degradation of organic pollutants in the soil by microorganisms in the rhizosphere (a layer of about 1 mm around the root). Plants can increase the microbial activity by the secretion of organic substances (such as carbohydrates, amino acids, flavonoids) that favour the growth and activity of rhizospheric microorganisms but also by the secretion of enzymes than can degrade the pollutants in soil. Rhizodegradation is also limited to the removal of organic pollutants because heavy metals are non-biodegradable.
- **Phytodesalination:** removal of salts from salt-affected soils using halophytic plants.

The 5 Pilot Sites of Argentina, India, Lithuania, Serbia, and Spain have different sources of contamination such as heavy metals and metalloids, petroleum hydrocarbons and polycyclic aromatic hydrocarbons (PAH), and Fe, Na, K in excess concentration. Hence different phytoremediation strategies, developing different phytoremediation mechanisms, must be applied.

Before applying the phytoremediation on the contaminated sites, pot trials have been carried out by each Pilot Sites Leader with the global aim of determining the best phytoremediation strategy to be applied in the specific contaminated site.

Despite the specific characteristics of each site (type of soil, type of contaminant, plant species, amendments, climatic conditions, etc.), pot trials have been performed following a harmonized pot tests experimental plan defined by all Pilot Site Leaders to ensure comparability and reproductivity of results. The harmonized pot tests experimental plan defines a common framework in which pot tests must be performed. Specifically, it provides sampling (including

[3] https://doi.org/10.1016/j.chemosphere.2013.01.075

type and frequency of sampling and storing procedure for both soil and energy crops) and monitoring (including soil and energy crop characterisation) common procedures and defines the main parameters of the experimental design such as controls, number of replicates and the duration of the experiments.

Specific targets have been pursued, depending on the site-specific characteristics such as:

- Optimization of the soil – plant species – amendments – fertilizers – biostimulants matrix
- Biomass production including seeds, for its valorisation as feedstock for biofuels/biodiesel production
- Seed germination under hostile conditions (contaminated soils) for later transplantation to the pilot parcels
- Assessment of the phytoremediation mechanism (rhizosphere effect or translocation to roots/stems/leaves/seeds) to, additionally, determine the possible environmental impact of the loss of the contaminated aboveground biomass

To meet the main and specific objectives, each Pilot Site Leader has defined its own experimental plan to perform pot trials, based on the agreed common framework in which to conduct phytoremediation actions.
Accordingly, each Pilot Site Leader, after characterising the contaminated site and defining the main contamination sources and contamination level, has defined the set of vegetative species and amendments/fertilizers/biostimulants to be investigated as well as the specific experimental conditions (experimental design and experimental set-up).
Specific parameters for soil and energy crop characterisation have been measured together with the common ones, with a minimum frequency of 1 sampling event for season. In particular, translocation and bioaccumulation factors have varied among pilot sites since they are strictly connected to the specific contaminants. Additionally, the common parameters for soil characterisation included physical parameters (water content, texture), chemical parameters (pH, electrical conductivity, N, C, S, Total C (CT), Total N (NT), organic matter, Mg, Ca, B, Fe, Mn, Na, K, Cd, Cr, Cu, Pb, As, P (available), K (available), P (total), K (total), TPH, PAH), and biological parameters (microbial biomass). Common parameters for energy crop characterisation included yield of production of biomass that is an important factor to estimate the future available feedstock for biofuel/biodiesel production.
Finally, visual inspections have also been agreed as important indicators of the plants' response to the hostile conditions.

It is worth noticing that, unfortunately, due to the COVID situation in India, their phytoremediation strategy could not be defined and performed when this deliverable was submitted and, therefore, it is not included in this document.

3. HARMONIZED POT TRIALS EXPERIMENTAL PLAN

A harmonized experimental plan establishing a common framework for the design of experiments, as well as for sampling and monitoring program, has been followed by each Pilot Site Leader to carry out the preliminary pot tests that will help to establish the most appropriate phytoremediation strategy. This will allow to guarantee the comparability and reproducibility of results among Pilot Sites, as it is established in the proposal.

3.1 Experimental design

The main parameters of the experimental design that have been agreed upon are the type of controls, number of replicates and the duration of the experiments and are detailed in the following sections.

3.1.1 Controls

A minimum of 2 controls has been stated to be the necessary for proper interpretation of results, and specifically:

- Contaminated soil without vegetation: to discard other factors contributing to the decrease of the concentration of the target contaminants in soil, for example, lixiviation processes.
- Non-contaminated soil with vegetation: to control the optimal vegetative growing.

3.1.2 Replicates

A minimum of 3 replicates per investigated treatment has been stated to be necessary to ensure feasibility of the results.

3.1.3 Experimental duration

To assess the effect of the phytoremediation strategy it has been stated that pot tests had to last a minimum of 3 months.

3.2 Sampling program

The sampling program that has been agreed upon in the pot tests framework, include sampling of both soil and energy crops.
The sampling and analysis of soils will be used to monitor the soil quality improvement and the reduction of the target contaminants cause by the remediation action implemented. Furthermore, the sampling and analysis of the planted energy crops will serve to determine the bioaccumulation factors of the existing contaminants (both in above and belowground biomass) and to quantify the biomass generated, to have a preliminary estimation of the biomass that will be produced in field and that will be used for biofuel feedstock.

3.2.1 Soil sampling

The harmonized strategy for soil sampling that has been agreed upon, provides the following instructions:

Table 3.1 Soil sampling program for pot tests

Number of samples	1
Type of sample	Composite samples/soil bulk
Sample conservation	Air-dried (except for the analysis of specific target contaminants, as will be defined in the sections 4-8 describing the site-specific experimental design)
Sample frequency	Minimum of 1 sampling campaign must be performed (or rather after harvesting)

3.2.2 Energy crops sampling

Concerning energy crop sampling, the following common strategy has been defined:

Table 3.2 Energy crop sampling program for pot tests

Number of samples	1
Type of sample	Composite samples: one sample of all aboveground biomass (including stems, leaves, seeds) and of one sample of all belowground biomass (roots) per pot. In case seeds are needed for biodiesel production, they will be harvested separately, and stored in a different bag.
Sample conservation	Air-dried (except for the analysis of specific target contaminants, as will be defined in the sections 4-8 describing the site-specific experimental design)
Sample frequency	Minimum of 1 sampling campaign must be performed (or rather after harvesting)

3.3 Monitoring program

A harmonized monitoring plan has been agreed among the Pilot Sites Leaders for the sake of comparison, establishing the most important parameters that need to be monitored in case of performing a phytoremediation action including both soil and energy crops characterization and distinguishing between general and site-specific parameters.

3.3.1 Soil characterization

The harmonized strategy for soil characterization during pot tests include a minimum of one analysis (after harvesting) of a set of physical, chemical, and biological parameters both general and site-specific, detailed in the following **Fehler! Verweisquelle konnte nicht gefunden werden.**:
aracterisation

Table 3.3 Soil Characterisation

SOIL CHARACTERISTICS	Units
Physical parameters	
Texture	granulometric composition
Water content	%
Chemical parameters	
MEASURED IN ALL PILOT SITES	
pH	-
Electrical conductivity	mS/cm
Chemical parameters	
MEASURED IN ALL PILOT SITES	
K (available)	mg/kg dry matter
P (available)	mg/kg dry matter
K (Total)	mg/kg dry matter
S	mg/kg dry matter
B	mg/kg dry matter
Cu	mg/kg dry matter
Zn	mg/kg dry matter
Organic matter	mg/kg dry matter
Total C	mg/kg dry matter
Total N	mg/kg dry matter
SITE-SPECIFIC PARAMETERS*	
Total Petroleum Hydrocarbons (TPH)	mg/kg dry matter
Polycyclic Aromatic Hydrocarbons (PAH)	mg/kg dry matter
Cd	mg/kg dry matter
Cr	mg/kg dry matter
Pb	mg/kg dry matter
As	mg/kg dry matter
Na	mg/kg dry matter
Biological parameters	
Microbial biomass	CPU/ml

***To be noticed that dry matter will be referred as DM from now on.**

3.3.2 Energy crops characterization

A harmonized strategy for energy crops characterization during pot tests has also been stated, being crucial for the Phy2Climate project to analyse the accumulation of contaminants in plants and to evaluate biomass production. Accordingly, a minimum of one analysis (after harvesting) of the following parameters has been stated to be performed:

Table 3.4 Energy crops characterization

ENERGY CROP CHARACTERISTICS	Units
ABOVEGROUND BIOMASS	
Translocation Factor (from root to leaves)	**%**
Biomass (leaves, stems and seeds)	g DM /m^2

Table 3.4 Energy crops characterization

ENERGY CROP CHARACTERISTICS	Units
Yield (compared to control)	%
Stress/damage of plants	-
Bioaccumulation Factors (BAF)	mg/kg DM
BELOWGROUND BIOMASS	
Bioaccumulation Factors (BAF)	mg/kg DM
Biomass (roots)	g DM. /m^2
Yield (compared to control)	%

It must be considered that bioaccumulation and translocation factors are site specific because each pilot site will determine their specific bioaccumulation and translocation factors according to the contaminants they have in soil.

Translocation factors, biomass and yield have been measured to give response to the Phy2Climate specific objectives (**Fehler! Verweisquelle konnte nicht gefunden werden.**) and to be able to quantify the defined KPIs (Table 3.6).

Table 3.5 Specific objectives of the Phy2Climate project to be considered when defining the monitoring program of pot test

Specific Objectives	
1.1	Production of >40 kg energy crop per site and growing season
1.3	Soil remediation rate equivalent to <20 years of total decontamination and transition to arable land
3.1	Cost reduction of land remediation in factor >5 compared to excavation and disposal threshold
3.2	Cost reduction of feedstock for biofuels >50%

Table 3.6. KPIs of the Phy2Climate project established in the proposal to evaluate phytoremediation of Pilot Sites

KPIs to evaluate phytoremediation pilots	
KPI 1	Contaminant uptake rate of energy crops equivalent to a < 20 years full remediation cycle
KPI 2	Yield of the energy crops >85% in comparison with the crop yield in clean soil conditions
KPI 3	Plant translocation factor of the contaminants from the roots to the aerial biomass > 1
KPI 4	Cost reduction of factor >5 compared with the excavation and disposal threshold

Finally, visual inspections have been also performed during and at the end of pot tests. Parameters such as stress and damage of plants, pests, seeds germination level, soil surface cover percentage, height of plants, plants density, and plants exuberance, have been considered.

4. SPANISH SITE POT TRIALS EXPERIMENTAL PLAN

4.1 Objectives

An experimental design testing several phytoremediation treatments (several vegetative species and several amendments) has been proposed and followed to meet the following objectives:

- Determine the most effective species for the phytoremediation of soils contaminated by petroleum hydrocarbons (TPH/PAH) but also considering the potential for biofuel production.
- Determine the amendment/s that can positively influence phytoremediation of soils contaminated by petroleum hydrocarbons (TPH and PAH) and ensure the proper growth of the plants.
- Determine nutrients deficit in the plants and the possible pests that could occur in the field so to establish remedial measures to be applied in the following field activities.

4.2 Materials and methods

4.2.1 Description of the set-up

4.2.1.1 Site and soil description

The contaminated soil was collected from an industrial site belonging to the company Exolum, formerly known as Compañía Logística de Hidrocarburos S.A. (CLH), and a partner in the present project. The site is in the north-eastern part of Spain, within the autonomous community of Catalonia and specifically at the west of the city of Tarragona, next to the Francolí river and near to the Mediterranean Sea, where the Port of Tarragona is located (see Figure 4.1). Therefore, the soil is highly influenced by human activity and the land use in the study area is industrial, surrounded by other industrial facilities, roads, highways, and the railway.

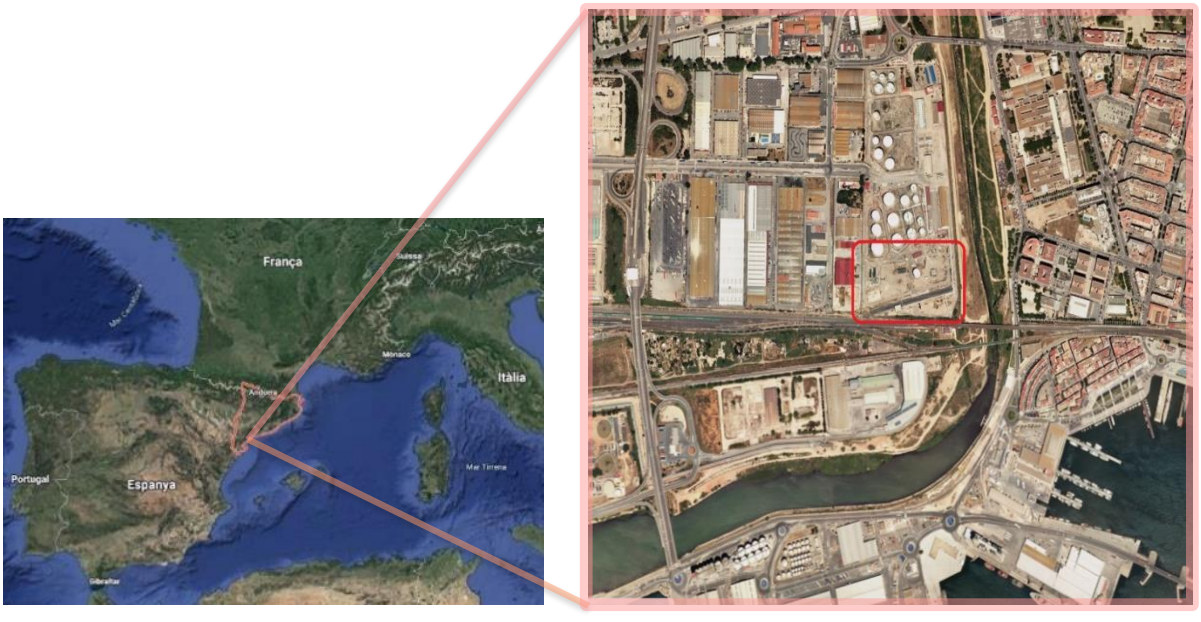


Figure 4.1 Location of the contaminated site in Tarragona, Spain

The detailed description of the site can be found in deliverable D 2.1, section 4.1.

The phytoremediation pilot test will be implemented at the southern part of the site (see Figure 4.2). In this unpaved area, soil contamination by TPH presented an average concentration of 2400 mg/kg back in 2014, when the last soil monitoring event took place. In this case, most of the hydrocarbons detected were in the diesel-range organics (C10-C28). Additionally, the light non-aqueous phase liquid (LNAPL), originally present, was no more detected thanks to an on-going remediation treatment kicked off in January of 2016 and described in detail in deliverable D 2.1, section 4.2.2.

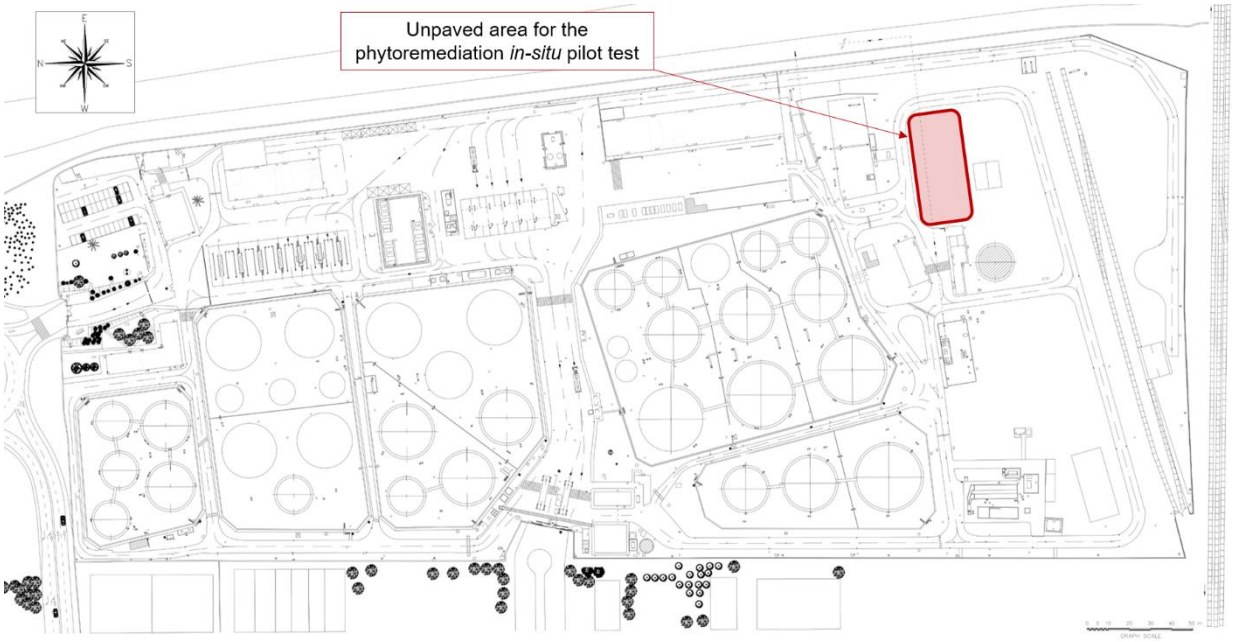


Figure 4.2 Facilities map and location of the phytoremediation pilot area at the Spanish site

The initial characterization of the phytoremediation pilot area, prior to carrying out the pot tests, is described in deliverable D 2.1 section 4.2. It was performed by sampling 4 points (P1, P2, P3, P4) distributed in a way that guarantees the characterization of the contamination variability throughout the site. The following parameters were determined during the initial characterisation campaign:

- Physical parameters (water content, texture)
- Chemical parameters (pH, electrical conductivity, N, C, S, Total C (CT), Total N (NT), organic matter, Mg, Ca, B, Fe, Mn, Na, K, Cd, Cr, Cu, Pb, As, P (available), K (available), P (total), K (total), TPH, PAH)
- Biological parameters (microbial biomass)

The analytical methods used to determine the physico-chemical parameters, are described in the Annex I of deliverable D 2.1 and are summarized in the following section 4.4.1.

Summarizing the most interesting results of the initial characterisation sampling campaign, the soil contamination in this area resulted to be mainly located at a depth of 2 – 4 m. The highest

concentration of TPH was detected at the greatest depth of sampling point 4-P4, where 5486 mg TPH/kg of soil were detected.
Moreover, Pb was also found to be present, regardless of the SP and depth. On the other side, S and B were rarely found. Finally, regarding water content, it increased with depth, getting in SP4 to values around 20% at -4 m, which was near to the water table.

Hence, to perform the pot tests, a 1-ton sample of contaminated soil was taken by means of a digger (due to the high compaction grade of the site) at the greatest depth (4 m) of P4, where the highest concentration of TPH was detected. The 1-ton sample was transferred by LITOCLEAN to LEITAT facilities where it was characterized before to proceeding with the experimental pot tests and the most interesting and important characteristics are summarized in the following table (average value of 6 replicates):

Table 4.1 Main parameters of 1-ton soil sample used to perform pot tests

Parameter	Unit	Value
Water content	%	17.60
pH	1:2,5 H_2O	8.37
EC	µS/cm	117.52
Organic matter (OM)	mg/kg DM	2.58
Clay	%	9.6
Silt	%	29.7
Sand	%	24.1
Textural class	-	Silt loam
TPH	mg/kg DM	4,042.10
PAH	mg/kg DM	12.10
P available	mg/kg DM	<LQ
K available	mg/kg DM	66.95
Mg	mg/kg DM	23,523.41
Ca	mg/kg DM	15,5312.17
S	mg/kg DM	<LQ
B	mg/kg DM	6.62
Cu	mg/kg DM	12.90
Fe	mg/kg DM	11,656.56
Mn	mg/kg DM	290.72
Mo	mg/kg DM	<LQ
Zn	mg/kg DM	<LQ
Total C	mg/kg DM	7.61
Total N	mg/kg DM	0.03
Cd	mg/kg DM	<LQ
Total Cr	mg/kg DM	10.18
Pb	mg/kg DM	13.94
As	mg/kg DM	<LQ
Na	mg/kg DM	112.18

The values in Table 4.1 have been used as a reference point to determine the effect of the investigated phytoremediation strategies.

The contaminated soil was well homogenized and mixed with the selected amendments in the established percentages according to each treatment specification, before to be transferred to the pots.

Finally, it must be highlighted that a commercial substrate (universal substrate, Flower premium) has been also used to have a reference of biomass production under ideal conditions (porosity, permeability, etc.) and good balance of nutrients for plant growth.

4.2.1.2 Soil amendments

The literature review showed the importance of soil amendments in the efficiency improvement of phytoremediation strategies in the case of soils contaminated by petroleum hydrocarbons (TPH/PAH) (Hussain et al., 2018)[4].
Four amendments were selected to be tested (biochar, compost, PGPR and common fertilizer) and are described below:

- **Biochar:** is produced by pyrolysis and it is carbon (C) rich and contains macronutrients and between 70 and 90% of stable C.
- **Compost:** is made by decomposing organic materials into simpler organic and inorganic compounds in a process called composting. Composting produces a soil-like material that contains organic matter, C, available macronutrients, and between 2 and 14% stable C.
- **PGPR (plant growth promoting rhizobacteria):** as described by Bhattacharyya et al., 2012[5], PGPR are "rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, rhizosphere engineering, production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC), quorum sensing (QS), signal interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOC), induction of systemic resistance, promoting beneficial plant-microbe symbioses, interference with pathogen toxin production, etc.".
- **Common fertilizer (NPK: nitrogen (N), phosphorus (P) and potassium (K)).**

Biochar and compost were selected as innovative ways to recycle macronutrients within agricultural systems, while minimising environmental impacts, in accordance with the circular economy principle of 'closing the loop' by returning organic residue/waste to agricultural soils (Oldfield et al. 2018)[6].
PGPR were selected because they represent an attractive alternative to the use of chemical fertilizers, pesticides, and other supplements.

Specifically, the contaminated soil was amended with compost (5% v/v), biochar (5% v/v), PGPR (0.05 gr/pot at several applications), common fertilizer (5 gr/pot), and with the mix of three of them with the same proportion (5% v/v for compost and biochar, and 0.05 gr/pot of PGPR).
Commercial amendments were used for this study and supplied by local providers: Compost was provided by Compost Segrià, biochar was provided by Carbón Vivo, common fertilizer was

[4] https://doi.org/10.1016/j.envexpbot.2018.05.012
[5] https://doi.org/10.1007/s11274-011-0979-9
[6] https://doi.org/10.1016/j.jenvman.2018.04.061

provided by Agroquímics Fabregat-Duran S.A., PGPR (Bacillus subtilis SEIB23, Radisei) was provided by Seipasa Natural Technology.
The concentrations of biochar and compost amendments were selected based on literature review (Hussain et al., 2018[7]; Bielská et al., 2017 [8]; Zhang et al., 2016 [9].).
For PGPR application, the provider instructions were followed. Specifically, the first application of 0.05 gr/pot was done between 1 and 2 weeks after sowing, depending on the crop. The first application is more effective once the seeds have started their germination because the existence of growing plant tissues (radicle) is key for the interaction of the microorganisms with the crop.
Subsequently, after the first application, at least 2 more applications at the same dose (0.05 gr/pot) were done at an interval of 2 to 4 weeks, depending on the crop, in order to re-inoculate and maintain populations of active microorganisms.
The fertilizer was applied following the provider instructions and 20 gr/pot were added.

4.2.1.3 Vegetative species

A detailed and careful bibliographic review was performed with the aim of selecting the most appropriate plant species taking into consideration their TPH/PAH removal efficiency as well as their biofuel potential production.
Finally, it was decided to select 4 plant species, among the ones suggested in the Grant Agreement, to be investigated during the preliminary pot tests.
Specifically, the following plant species were selected:

- ***Sorghum sp.*:** plant that phytoremediates petroleum hydrocarbons according to Germida et al. 2002[10]
- ***Brassica Napus*:** Diesel-tolerant species with potential for phytoremediation according to La Calle et al. 2018[11]
- ***Panicum Virgatum*:** plant that phytoremediates petroleum hydrocarbons, according to Germida et al. 2002[12]
- ***Heliantus Annuus*:** plant that tolerates petroleum hydrocarbons according to Germida et al. 2002[13]

All the selected species have shown a good potential for biofuel production and extensive literature has been found regarding their use for TPH removal investigated at the lab scale or through preliminary studies based on pot tests and is detailed in section 4.4.1 of deliverable D 2.1.

Commercial seeds of the four selected plant species, supplied by a local provider (Can Torra), were used to perform pot tests. Specifically, according to the recommended dosage of plant species, twenty seeds of each specie were sown per pot.

[7] https://doi.org/10.1016/j.envexpbot.2018.05.012
[8] https://doi.org/10.1016/j.scitotenv.2017.03.230
[9] https://doi.org/10.1016/j.jes.2015.12.023
[10] https://doi.org/10.1016/S0166-2481(02)80015-0
[11] https://doi.org/10.1016/j.scitotenv.2017.10.334
[12] https://doi.org/10.1016/S0166-2481(02)80015-0
[13] https://doi.org/10.1016/S0166-2481(02)80015-0

Before seeding, the seeds were disinfected with 5% of sodium hypochlorite for 30 minutes and then rinsed with distilled water.
The characterisation of the seeds together with a germination test were also conducted and are described in the following section 4.2.1.5.

4.2.1.4 Experimental design to perform pot tests

An experimental design has been followed to perform the pot tests and is schematized in Table 4.2Table 4.2.
Twenty-four different phytoremediation treatments have been analysed. For each of the 4 selected vegetative species (*Sorghum sp., Brassica napus, Panicum virgatum, Helianthus annuus*) 5 amendments have been tested (contaminated soil amended with 1) compost 2) biochar 3) PGPR 4) common fertilizer and 5) mix of compost/biochar/PGPR namely "All" from now on) and the phytoremediation capacity of each vegetative specie has also been tested in case of using contaminated soil without amendments. Each treatment had 5 replicates, for a total number of 120 experiments (pots).
Moreover, a control with a commercial substrate has been also performed for each vegetative specie, to have a reference of biomass production under ideal conditions (pH, EC, porosity, permeability, etc) and good balance of nutrients for plant growth. In this case 5 replicates have been carried out for a total number of 20 control experiments (pots).
Finally unplanted controls consisting of unplanted contaminated soil without amendments and unplanted contaminated soil with amendments have also been tested with 5 replicates for a total number of 30 more control experiments (pots):

- Compost + contaminated soil (5 replicates).
- Biochar + contaminated soil (5 replicates).
- PGPR + contaminated soil (5 replicates).
- Common fertilizer+ contaminated soil (5 replicates).
- Mix of compost/biochar/PGPR + contaminated soil (5 replicates).
- Contaminated soil (5 replicates).

Controls of unplanted contaminated soil without amendments have been selected to determine the effect of leaching on TPH decrease, because these controls have been irrigated in the same way as the other treatments.
On the other hand, controls with unplanted contaminated soil with amendments have been used to determine the effect of the amendment (and not also the vegetative specie), if there were any, on the TPH removal.

Table 4.2 Experimental design to test different phytoremediation treatments and controls

AMENDMENTS	VEGETATIVE SPECIES				
	Sorghum sp.	*Brassica Napus*	*Panicum Virgatum*	*Helianthus Annuus*	Without species
Compost	X	X	X	X	X
Biochar	X	X	X	X	X
PGPR	X	X	X	X	X
Common Fertilizer	X	X	X	X	X
Mix = Compost, biochar and PGPR	X	X	X	X	X
Contaminated soil	X	X	X	X	X
Non-contaminated soil	X	X	X	X	

For the sake of simplicity, a nomenclature has been adopted to refer to the investigated treatments and controls and is reported in the following table:

Table 4.3 Nomenclature of the investigated treatments and controls

NOMENCLATURE	DESCRIPTION
INVESTIGATED PHYTOREMEDIATION TREATMENTS	
TR1	HELIANTHUS ANNUUS +CONTAMINATED SOIL
TR2	HELIANTHUS ANNUUS+COMPOST+CONTAMINATED SOIL
TR3	HELIANTHUS ANNUUS+PGPR+CONTAMINATED SOIL
TR4	HELIANTHUS ANNUUS+BIOCHAR+CONTAMINATED SOIL
TR5	HELIANTHUS ANNUUS+COMMON FERTILIZER+CONTAMINATED SOIL
TR6	HELIANTHUS ANNUUS+ALL+CONTAMINATED SOIL
TR7	SORGHUM SP.+CONTAMINATED SOIL
TR8	SORGHUM SP.+COMPOST+CONTAMINATED SOIL
TR9	SORGHUM SP.+PGPR+CONTAMINATED SOIL
TR10	SORGHUM SP.+BIOCHAR+CONTAMINATED SOIL
TR11	SORGHUM SP.+COMMON FERTILIZER+CONTAMINATED SOIL
TR12	SORGHUM SP.+ALL+CONTAMINATED SOIL
TR13	BRASSICA NAPUS+CONTAMINATED SOIL
TR14	BRASSICA NAPUS+COMPOST+CONTAMINATED SOIL
TR15	BRASSICA NAPUS+PGPR+CONTAMINATED SOIL
TR16	BRASSICA NAPUS+BIOCHAR+CONTAMINATED SOIL
TR17	BRASSICA NAPUS+COMMON FERTILIZER+CONTAMINATED SOIL
TR18	BRASSICA NAPUS+ALL+CONTAMINATED SOIL
TR19	PANICUM VIRGATUM+CONTAMINATED SOIL
TR20	PANICUM VIRGATUM+COMPOST+CONTAMINATED SOIL
TR21	PANICUM VIRGATUM+PGPR+CONTAMINATED SOIL
TR22	PANICUM VIRGATUM+BIOCHAR+CONTAMINATED SOIL
TR23	PANICUM VIRGATUM+COMMON FERTILIZER+CONTAMINATED SOIL
TR24	PANICUM VIRGATUM+ALL+CONTAMINATED SOIL

Table 4.3 Nomenclature of the investigated treatments and controls

NOMENCLATURE	DESCRIPTION
CONTROLS	
TR25	CONTAMINATED SOIL+COMPOST
TR26	CONTAMINATED SOIL+PGPR
TR27	CONTAMINATED SOIL+BIOCHAR
TR28	CONTAMINATED SOIL+COMMON FERTILIZER
TR29	CONTAMINATED SOIL+ALL
TR30	HELIANTHUS ANNUUS+NON-CONTAMINATED SOIL
TR31	SORGHUM SP.+NON-CONTAMINATED SOIL
TR32	BRASSICA NAPUS+NON-CONTAMINATED SOIL
TR33	PANICUM VIRGATUM+NON-CONTAMINATED SOIL
TR34	CONTAMINATED SOIL

Pot tests were performed using square plastic pots of 15x15x20 cm with a total volume of 3.4 L that were placed on plastic tray to contain leaching. An amount of 3 kg of soil was added to each pot. In case of using amendments, the soil was well mixed with the specific amendment and in the specific concentrations (according to each treatment specification) before being transferred to the pot. In each case a layer of 2-3 cm of gravels has been added to the bottom of the pot to help drainage.

Twenty seeds have been sown for pot (equidistant furrows were made in the soil and 3-4 seeds were deposited into each hole). Then, after germination, the seedlings of each plant species were thinned to three plants per pot to avoid ineffective competition dynamics.

The pot tests were performed in an outdoor experimental zone at LEITAT, from June 2021 to September 2021 (see Figure 4,3). The growing periods of plants were 75 to 120 days.

After harvesting, the physico-chemical characterization of the soil has been performed with the aim of determining the phytoremediation effect of each treatment as well as the effect of the selected amendments. The analytical methods used to perform the necessary measurements are described in the Annex I of deliverable D 2.1 and are summarized in the following section 4.4.1.

The analysis of the morphology of roots and of biomass and seeds production has also been performed and are described in the following section 4.4.2. As already mentioned, the potential biomass production in the field is essential in this Project because the produced biomass will be used as feedstock for biofuels production. On the other hand, morphology of the roots has been analysed because field experience has shown that plants developing well-branched roots in the soil generally show greater phytoremediation efficiency.

Moreover, while pot tests were running, visual inspections have been carried out to monitor i) presence/absence of pest ii) presence/absence of nutritional deficiencies iii) height iv) number of true leaves, v) phenological stages and vi) presence/absence of phytopathologies and are described in the following section 4.4.2.

Figure 4.3 Pot tests performed outdoor, at LEITAT facilities

4.2.1.5 Germination test

First, the characterisation of seeds was performed. The moisture content of the seeds was determined using oven method at 103ºC ± 2ºC for 24h, following the Canadian methods and procedures for testing seed (available at: https://inspection.canada.ca/).
After selecting the seeds as much uniform in size and shape as possible (to reduce impurity probability), a purity test was also performed for each plant species. Subsequently, 100 seeds of each plant species were weighted, and the extrapolation of 1000 seeds weight was calculated. The purity test was used to determine which proportion of sample was pure crop seed and which proportion was seeds of weeds, seeds of other crops, and inert matter.

Then, a preliminary and qualitative germination test was performed prior to the pot tests to test the germination response of the 4 selected plant species to different moisture conditions, and substrate and in case of applying or not a disinfection procedure of the seeds. The effect of the different amendments was not tested.

For each plant species, 15 seeds were sown in a 9-cm-diamenter Petri dish at different moisture conditions (low, medium, and high) into four different substrates (5 g of liquid nutritious medium, 15 g of commercial substrate, 15 g of wet contaminated soil (stored refrigerated) and 15 g of air-dried contaminated soil). In all cases the use of non-disinfected and disinfected seeds was

investigated, except for the case of the air-dried contaminated soil where only disinfected seeds were tested. Three replicates of each condition were performed.
Seeds were disinfected using 5% of sodium hypochlorite for 30 minutes, and then rinsed with distilled water.
The tested moisture conditions (low, medium, and high) were obtained by adding the following amount of distilled water to the substrates:

Table 4.4 Tested moisture conditions for the germination test

	High moisture	Medium moisture	Low moisture
Liquid nutritious medium	2 mL	1 mL	0.5 mL
Commercial substrate	6 mL	4 mL	1.5 mL
Dry contaminated soil	5 mL	3 mL	1 mL
Wet contaminated soil	2 mL	1 mL	0 mL

The germination experiment lasted 5 days and was carried out in temperature-controlled chamber (MP control, MP-1200-STAB) under constant temperature (25ºC), humidity (50%) and light conditions (14h light/10 h dark, 250 lux). The temperature fluctuation was controlled within ± 2ºC.

Seeds were examined daily, and seedlings were considered germinated when the radicle had extended more than 2 mm beyond the seed coat (Schopfer et al., 1984 [14]; Zhang et al., 2015[15]).

It is worth noticing that purity test and a precise germination test will be performed for each stock of seed that will be applied in field for each sowing season, to adjust the seed dosage and to ensure to meet the specific objective 1.1 of the Project (see Table 3.5).

4.2.2 Sampling campaign

A unique sampling campaign was performed at the end of the pot tests, after 4 months from the sowing, to take into consideration the different vegetative cycles of the selected species. *Helianthus annuus* and *Sorghum sp*. species reached the mature stage in only 3 months from sowing. *Brassica napus* stopped growing after the same period (about 3 months) because it was sown a bit over the best sowing season.
The whole sampling campaign was performed in one day; however, the ideal time of collection differed among the species because the plants were not at the same growth stage.
First, the above ground biomass (stem and leaves as a bulk sample stored in a bag and seeds stored in a different bag) was collected from each treatment/pot and frozen for future analysis on nutritional deficit of the plants. Literature review revealed that the phytoremediation of soils contaminated by TPH is not due to the translocation factor in leaves/stems/seeds but mainly is due to the degradation caused by the growth of microorganisms in the soil that can be promoted by the plants (rhizospheric effect).
Then the pots were gently crushed and shaken in a vat to carefully collect the roots.

[14] https://doi.org/10.1104/PP.76.1.155
[15] https://doi.org/10.1071/CP14269

An amount of 250 g of the soil was collected, including the one that was firmly attached to the roots, and was frozen for the analysis of TPH and PAH concentrations, so to avoid decrease of TPH and PAH trough volatilization.
The rest of soil was air-dried and sieved through a 2 mm sieved-mesh and was stored for the other analytical measurements (analysis of physical (water content) and chemical parameters (pH, electrical conductivity, N, S, Total C, Total N, P (available), K (total), K (available), Mg, Ca, B, Cu, Fe, Mn, Cd, Cr, Pb, As, Na, organic matter, TPH, PAH).

Before being analysed, soil was well mixed to ensure a homogeneous bulk sample.

4.3 Irrigation regime

Hand watering was provided as needed, depending on the climatic conditions and on the specie, to maintain 80% of the pots' water holding capacity and prevent run-out from the bottoms of the pots.

4.4 Monitoring program

The monitoring program was conducted according to the common framework during the pot tests duration (4 months, from May 2021 to September 2021). The monitoring results will not only provide important information relating to the efficiency of the investigated phytoremediation treatments and the relative biomass production but also, they will be a useful register to prevent adverse events in field, e.g., presence of pest or nutritional deficiencies in plants.

4.4.1 Soil characterization

The collected soil samples were initially (before starting the pot tests, see Table 4.1) and finally (end of the pot tests, after about 4 months from sowing) analysed to determine physicochemical parameters.

The following parameters were determined before and after the pot tests:

- Physical parameters (water content).
- Chemical parameters (pH, electrical conductivity, N, C, S, Total C (CT), Total N (NT), organic matter, Mg, Ca, B, Fe, Mn, Na, K, Cd, Cr, Cu, Pb, As, P (available), K (available), P (total), K (total), TPH, PAH).

The analytical methods used to determine the physicochemical parameters, are described in the Annex I of deliverable D 2.1 and are detailed here:

- **Water content (moisture)** was determined by gravimetric method with oven (Memmert, Germany), based on the sample mass loss when submitted to 105ºC.
- **The soil reaction (pH)** was measured potentiometrically in a 1:5 soil:water suspension using a digital pH meter (HQ40d, HACH).
- **Electrical conductivity (EC)** was measured in a 1:5 soil:water suspension according to the UNE 77308 (2001).
- **N, C, S, Total C (CT) and Total N (NT)** were measured with total combustion of the sample using an Elemental Analysis (Eurovector, model EuroEA3000, EA system, Italy).

- **Organic matter** was measured by using loss on ignition method (Schulte and Hopkins, 1996). For the spectrometric element analysis, the dried sample (0.5 g) was extracted with 9 mL of HNO_3 (65%) and 3 mL of HCl (32%). The digestion was performed using a microwave (Anton Paar, model Multiwave 7000, Austria) based on EPA 3051A method ("Microwave assisted acid digestion of sediments, sludges and oils").
- **Concentrations of magnesium (Mg), calcium (Ca), Boron (B), iron (Fe), manganese (Mn), sodium (Na), potassium (K), cadmium (Cd), Chromium (Cr), copper (Cu), lead (Pb) and arsenic (As)** were measured with inductively coupled plasma mass spectrometry (Agilent technologies, Model 7500, ICP-MS system, USA).
- **Available phosphorous P (available) and potassium K (available)** were extracted with ammonium lactate solution.
- **Total P and K** were analysed using ICP-MS (Agilent technologies, Model 7500, ICP-MS system, USA). Metals concentrations were adjusted to the dry matter content of the soil (105ºC, 24h).
- **TPH concentration** of the soil samples (preliminary frozen at -20ºC to avoid losses for volatilization) was measured according to the methods UNE EN ISO 16558-1, ISO 16703 and EPA 8015b. **PAH** concentration was determined according to NEN ISO 18287.

  The percentage of TPH removal was determined as follows:

$$\% \, TPH \, Removal = \frac{TPH_0 - TPH_f}{TPH_0} \, x \, 100$$

  Where TPH_0 is the initial concentration of total petroleum hydrocarbons (before starting pot tests,see Table 4.1) and TPH_f is final concentration of total petroleum hydrocarbon (at the end of the pot test) for each pot test.

It is worth noticing that all analysis had a quality control procedure including the analysis of certified reference material (ISE 850, WEPAL), laboratory control samples and solvent blanks. All analytical results (physical, chemical assays) are reported as the average of three replicates.

4.4.2 Energy crop characterization

The energy crop characterisation was performed at the end of the pot tests (after 4 months from sowing). Plants were gently harvested, and the above ground biomass (leaves/stems/seeds) and roots were gently separated and stored into plastic bags, according to the specifications detailed in the sampling campaign section 4.2.2.
After harvesting, biomass samples were rinsed with deionized water, dried with a tissue to remove the excess of water and then air-dried for a week.
After that, the aboveground biomass (leaves/stems/seeds) was measured gravimetrically using both wet and dry weights (Ogbo et al., 2010[16]). To determine dry weight, the above ground biomass was dried in an oven (Memmert, Germany) at 70 °C for 72 h until constant mass was reached (Peng et al., 2009[17]).

[16] Ogbo EM, Tabuanu A, Ubebe R (2010) Phytotoxicity assay of diesel fuel-spiked substrates remediated with Pleurotus tuberregium using Zea mays. Int J Appl Res Nat Prod 3:12–16
[17] http://dx.doi.org/10.1016/j.jhazmat.2009.03.036

The morphology of the roots of each specie was also inspected.

Moreover, while pot tests were running, visual inspections have been carried out every 15 days to monitor six parameters selected based on the adaptation of climatic and edaphic conditions, and evolution of energy crops with time, and are defined as follows:

- **Presence/absence of pest:** a pest record is documented evidence that indicates the presence or absence of specific pest at a plant species in a certain time. Some organisms (caterpillars, aphids, etc.) can cause damage in leaves, stems, flowers and fruits. For this reason, visual inspections involve a regular checking of pot test to register an early detection of pest, helping to prevent or minimize a pest outbreak.

- **Presence/absence of phytopathologies:** refers to organisms that cause infectious diseases including fungi, oomycetes, bacteria, viruses, viroids, protozoa, nematodes, etc.
- **Presence/absence of nutritional deficiencies:** nutrient deficiency occurs when a plant lacks enough of an essential nutrient required for growth. Without sufficient essential nutrients, plants will not grow well and will show various symptoms to express the deficiency.
- **Height:** the distance from the base of the plant at ground level to the top of the highest leaf (for *Brassica napus and Panicum virgatum*) or inflorescence/panicle (for *Helianthus annuus and Sorghum sp.*) of mature plant, measured in centimetres using a meter stick.
- **Number of true leaves:** these are determined by counting the number of true green leaves (excluding cotyledons) in all plant species. Also, leaf senescence, characterized by loss of chlorophyll and leaf yellowing-browning, were counted, and noted in observations.
- **Phenological stages:** an observable stage or phase in the annual life cycle of a plant that can be defined by a start (germination) and end point (harvest). For *Helianthus annuus*[18], *Brassica napus*[19] and *Sorghum sp.*[20] a phenological stage handbook was adopted to validate the phenological stages. This concept does not apply to *Panicum virgatum* since it a perennial species.

4.5 Results

4.5.1 Germination test

A qualitative germination test has been performed to evaluate the effect of moisture conditions and substrate on germination of seeds of the four selected vegetative species (*Helianthus annuus, Brassica napus, Panicum virgatum and Sorghum sp.*). The preliminary germination test response shows that, regardless of the plant species and the conditions studied (moisture and substrate), germination started 24 hours after the experiment was initiated. Under controlled conditions (climatic chamber: 25ºC, 50% humidity, 14h light/10h dark) the successful completion of germination of all plant species occurred in a maximum of 5 days (see Figure 4.4). No water

[18] Stages of Sunflower Development. NDSU extension. Available at: Stages of Sunflower Development — Publications (ndsu.edu)
[19] PALOL, MIQUEL (2008). "Les plantes cultivades. La colza." ISBN: 8460945901
[20] Sorghum growth and development. K-STATE Research and Extension. Available: Sorghum growth and development

stress symptoms, inhibition, and delay of the progress of seed germination was observed for none of the four plant species and none of the investigated conditions (moistures and substrates).

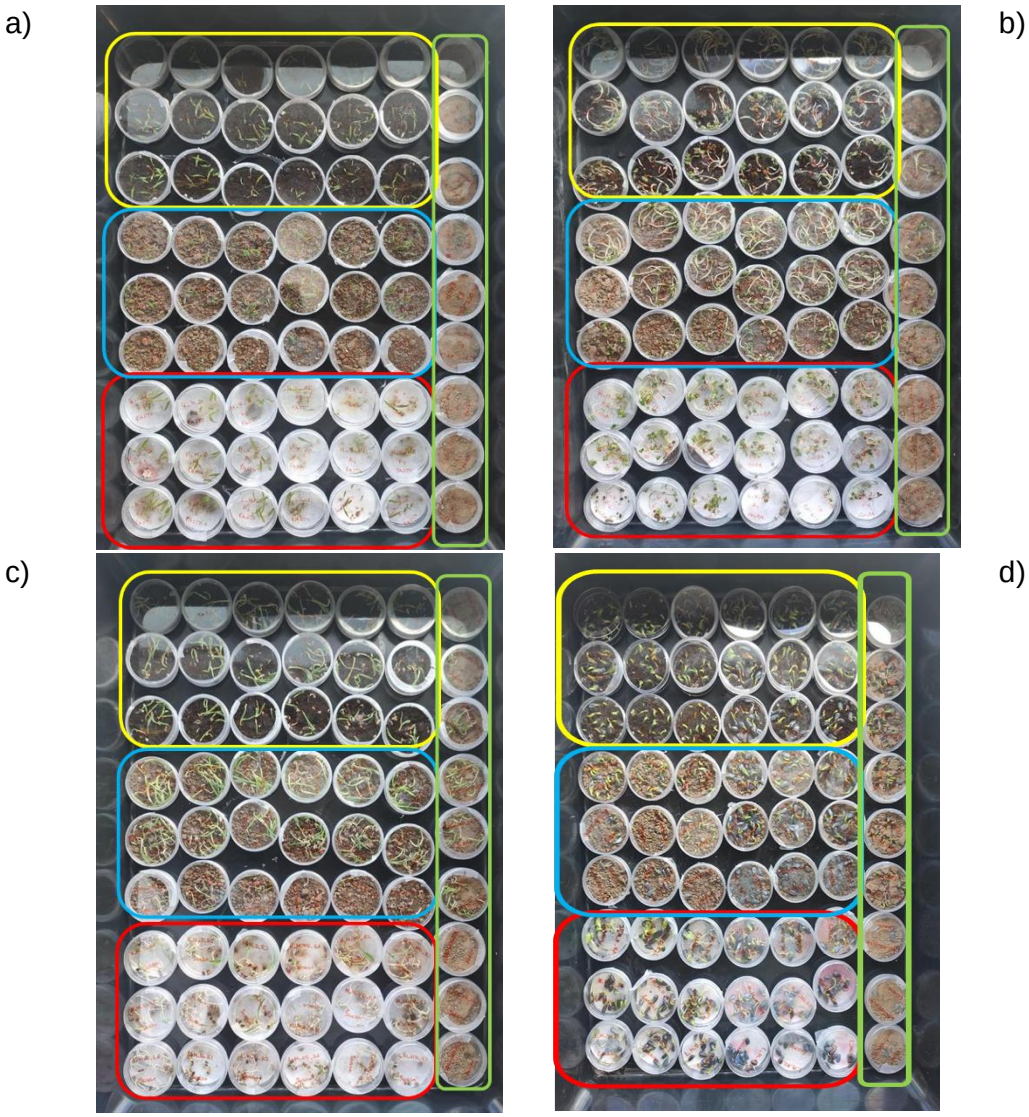


Figure 4.4 Preliminary germination test results for a*) Panicum virgatum b) Brassica napus c) Sorghum sp. d) Helianthus annuus* and for different substrate conditions highlighted with different colours (yellow box: commercial substrate, blue box: wet contaminated soil (from P4) stored in a refrigerator; red box: liquid nutritive medium; green box: contaminated soil (from P4), dried and then rewet using the moisture specifications reported in Table 4.4).

These pictures recollect all the investigated moisture conditions. The first three plates of the first line for each substrate condition (highlighted with the coloured boxes) are 3 replicates of the low moisture condition and disinfected seeds while the following three plates are 3 replicates of the low moisture condition and non-disinfected seeds. The first three plates of the second line for each substrate condition are 3 replicates of the medium moisture condition and disinfected seeds while the following three plates are 3 replicates of the medium moisture condition and non-disinfected seeds. The first three plates of the third line for each substrate condition are 3 replicates of the high moisture condition and disinfected seeds while the following three plates are 3 replicates of the high moisture condition and non-disinfected seeds. In the case of the

green box, representing the dried and rewet contaminated soil, only disinfected seeds have been used hence the first three plates (in column) are the low moisture conditions, the other three the medium moisture condition, and the last three the high moisture condition.

The seedlings evaluation revealed a normal grow of all plant species according to *Seedling Evaluation Handbook* (1992 Edition), published by the Association of Official Seed Analysts (AOSA).

It must be observed that for this preliminary, qualitative germination test the different amendments selected for this study (nor different concentrations) were not tested. This aspect will be considered in the further detailed germination tests that are planned to be performed before starting field activities and for each stock of seeds that will be applied in field for each sowing season, with the aim of adjusting the seed dosage and to ensure to meet the specific objective 1.1 of the Project (see table 3.5).

Moreover, the specific characteristics of the four plant species seeds utilized in pot tests are presented in Table 4.5 (all the analytical results are reported as the average of three replicates). The results showed a high proportion of pure crop seeds with mean values ranging between 98.2% and 100%, for all the species.

Table 4.5 Characterisation of the four plant species seeds used for pot tests

Species	Moisture (%)	Purity (%)	Weight of 100 seeds	Extrapolated Test Weight (1000 seeds)
Helianthus annuus	3.2 (±1.1)	99.7 (±0.2)	6.07 (±0.1)	60.7
Brassica napus	5.9 (±0.6)	100 (±0.0)	0.53 (±0.1)	5.3
Panicum virgatum	2.4 (±1.2)	98.8 (±0.3)	0.27 (±0.1)	2.7
Sorghum sp.	8.4 (±1.9)	98.2 (±0.4)	1.90 (±0.1)	19

4.5.2 Visual inspections

From the morphological data collected by visual inspections every 15 days, a graph has been elaborated with the average height reached by each plant species in each treatment and is shown in Figure 4.5

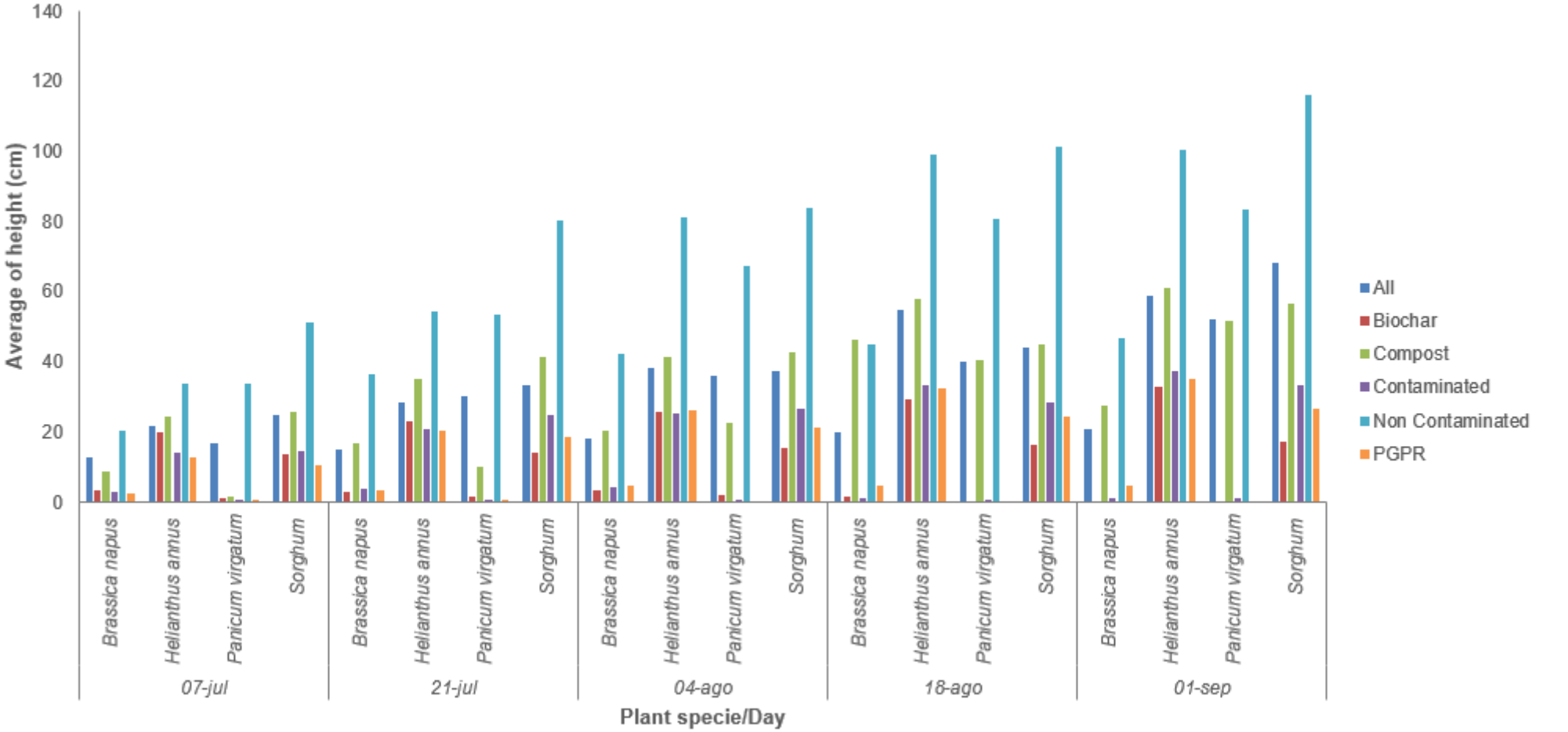


Figure 4.5 Average height reached during the growing season by each plant species in each investigated phytoremediation treatment

It must be noticed that results for the common fertilizer are not reported because there was no germination in pots probably due to the addition of a non-optimal concentration of fertilizer.
To establish the concentration of the fertilizer, three preliminary experiments were performed by comparing, for all the selected vegetative species, the results in terms of germination after adding 5, 10 and 20 gr/pot of common fertilizer to the contaminated soil. It was concluded that for concentration greater than 5 gr/pot, germination was inhibited. Hence, a concentration of 5 gr/pot was fixed for the pot tests. Hence, further pot tests will be performed for the optimal solutions, or rather for the selected vegetative species, in parallel with further field activities to investigate the effect of common fertilizer.
Also, PGPR and biochar did not allow the proper germination of *Brassica napus* and *Panicum virgatum* species in pots. As above mentioned, these conditions (e.g., different amendments and in different concentrations) were not tested in the preliminary, qualitative germination test and will be taken into account in the planning of the further germination test that will be performed preliminary to the field activities.
Focusing on soil additives, the best results, at least for the parameters that can be detected through visual inspection, have been obtained for compost and for the mixture of compost, biochar and PGPR (Figure 4.5).
On the other hand, focusing on the vegetative species, the plants that have shown the best results are *Sorghum sp.* and *Helianthus annuus*.
These preliminary results must be validated with the information concerning the efficiency of the tested phytoremediation strategies in terms of TPH/PAH removal.
Concerning the number of leaves it was counted only for *Helianthus annuus, Brassica napus* and *Sorghum sp.*, because it was strictly related to determine the phenological stages, especially, vegetative stages.
Regarding pest analysis, caterpillars and aphids were detected in *Brassica napus* during the pot trials (see Figure 4.6 a) and b), respectively) and were treated with natural products. These pests caused several damages to the plants such as holes or eating leaves (produced by caterpillars) and curled leaves (produced by aphids). Nevertheless, some syrphids species, a natural enemy of aphids, were seen flying over infested plants. In addition, some pollinators like honeybees (*Apis mellifera*) and bumblebee (*Bombus terrestris*) have been detected during flowering stage of sunflower (*Helianthus annuus*) (see Figure 4.6c)). On the other hand, regarding the phytopatologies, mildew was detected in some of the Brassica napus pot tests.

a) b) c)

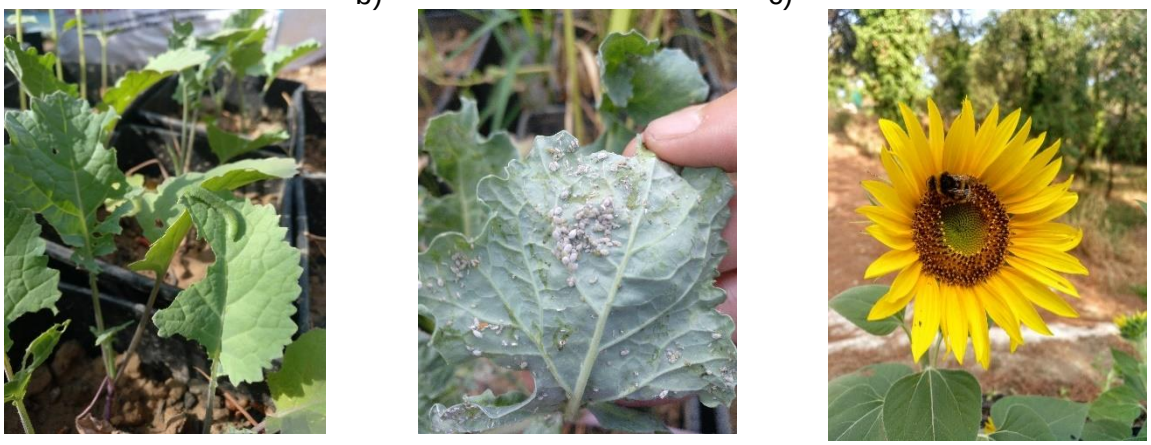


Figure 4.6 Visual inspections: Pests and pollinators detected in the case of *Brassica napus* and *Helianthus annuus*

Finally, nutritional deficiencies were detected in all plant species, especially, in the phytoremediation treatments using biochar and PGPR.
However, other treatments also showed nutritional deficiencies, at specific periods, depending on their phenological stages' necessities. For this reason, it can be concluded that fertilization will be essential during field activities for two reasons: first, i) contaminated soil may not have the necessary balance of minerals and other nutrients to allow the desired vegetation to grow adequately, and second ii) the success of phytoremediation is often attributed to microbial degradation, and the microbial populations will also exert demand on nutrients in the soil [21].

4.5.3 Soil characterization

The physical and chemical parameters have been analysed and reported in Table 4.6 for all the investigated phytoremediation treatments with the exception of the treatments with *Brassica Napus* and *Panicum Virgatum* alone and combined with PGPR and biochar (TR13, TR15, TR16, TR19, TR21 and TR22), and all the treatments with commercial fertilizer (TR5, TR11, TR17, TR23,TR28) because as already mentioned, in these cases the germination did not take place and hence soil analysis was not performed because it was assumed that no phytoremediation effect could be detected. Moreover, the aim of this study was also to produce biomass for biofuel/biodiesel production.

[21] https://doi.org/10.1002/047127304X

Table 4.6 Physico-chemical parameters analysed to determine the effectiveness of the investigated phytoremediation strategies (investigated phytoremediation treatments are highlighted in green while control experiments in orange)

	WC	pH	EC	P available	K available	Mg	Ca	S	B	Cu	Fe	Mn	Mo	Zn	Total C	Total N	Cd	Cr Total	Pb	As	Na	TPH	PAH
	%	-	*µS/cm*	*mg/kg DM*																			
TR1	2.4 ±0.3	8.8 ±0.1	388.0 ±123.1	<LQ	118.5 ±9.2	26,846.0 ±2,201.2	169,324.0 ±10,790.4	<LQ	<LQ	<LQ	13,924.5 ±1,248	341 ±22.6	1 ±0.01	50 ±1.4	6.2 ±0.1	<LQ	<LQ	21.5 ±7.8	13 ±0.01	7 ±1.4	<LQ	123 ±51.2	<LQ
TR2	2.1 ±0.5	8.2 ±0.1	319.3 ±40.9	58.5 ±6.4	128.5 ±10.6	28,507.0 ±3,403.3	179,688.5±20,546.4	<LQ	<LQ	13.5 ±0.7	12,862.0 ±1,492	294.5 ±27.6	<LQ	70.5 ±3.5	7.8 ±0.3	<LQ	<LQ	29.5 ±20.5	12 ±1.4	6.5 ±0.7	<LQ	142.98 ±4.5	0.13 ±0.01
TR3	2.4 ±0.8	8.5 ±0.01	128.3 ±8.5	<LQ	51 ±5.7	29,036.0 ±245.4	190,157.0 ±459.6	<LQ	<LQ	<LQ	12,978.0 ±271.5	293 ±7.1	<LQ	48 ±1.4	7.1 ±0.3	<LQ	<LQ	12.0 ±0.01	10.5 ±0.7	7 ±0.01	<LQ	267.65 ±68.9	<LQ
TR4	4.3 ±1-3	8.9 ±0.1	163.3 ±7.8	<LQ	190.5 ±7.8	24,836.0 ±72.1	152,264.0 ±6,619.9	<LQ	<LQ	<LQ	12,175.0 ±116	283.5 ±7.8	<LQ	46.5 ±3.5	5.5 ±0.4	<LQ	<LQ	15.0 ±1.4	8.5 ±2.1	6 ±0.01	<LQ	212.34 ±90.2	0.885± 0.2
TR6	6.0 ±1.6	8.5 ±0.1	411.0 ±39.7	58.5 ±16.3	279 ±12.7	22,917 ±1,579	147,065.5±1,368.3	<LQ	<LQ	16 ±7.1	11,989.5 ±1,147.6	268 ±41	<LQ	69.5 ±14.8	7.3 ±0.5	<LQ	<LQ	21.0 ±2.8	10.5 ±0.7	6.5 ±0.7	<LQ	147.98 ±8.5	0.68 ±0.1
TR7	3.0 ±0.9	9.0 ±0.1	176.6 ±37.3	<LQ	94.5 ±9.2	27,467.5 ±17.7	177,575.5±3,376.4	<LQ	<LQ	<LQ	13,452.5 ±244	301.5 ±4.9	<LQ	47 ±0.01	5.5 ±1.0	<LQ	<LQ	18.0 ±2.8	11 ±	8 ±0.6	<LQ	270.75 ±68.2	<LQ
TR8	1.8 ±0.4	8.2 ±0.01	235.8 ±16	61 ±14.1	75.5 ±41.7	26,571 ±1,069.9	175,150.5±6,695.6	<LQ	<LQ	17.5 ±0.7	12,693.0 ±362	279.5 ±4.9	<LQ	79.5 ±0.7	7.5 ±0.3	<LQ	<LQ	15.5 ±0.7	9.5 ±0.7	6.5 ±07	<LQ	145.84 ±10.2	<LQ

Table 4.6 Physico-chemical parameters analysed to determine the effectiveness of the investigated phytoremediation strategies (investigated phytoremediation treatments are highlighted in green while control experiments in orange)

	WC	pH	EC	P available	K available	Mg	Ca	S	B	Cu	Fe	Mn	Mo	Zn	Total C	Total N	Cd	Cr Total	Pb	As	Na	TPH	PAH
	%	-	*µS/cm*	*mg/kg DM*																			
TR9	0.5 ±0.01	8.3 ±0.1	144.0 ±7.1	<LQ	87.5 ±24.7	27,997.0 ±786.3	180,188.5±2,311.5	<LQ	<LQ	<LQ	14,212.0 ±940.5	336 ±4.2	<LQ	85.5 ±53.0	7.6 ±0.1	<LQ	<LQ	14 ±1.4	11 ±1.4	7.5 ±0.7	195 ±90.5	322.29 ±265.7	<LQ
TR10	5.1 ±0.1	8.7 ±0.01	172.9 ±13.7	<LQ	199.5 ±13.4	24,119.0 ±649.1	157,976.5±1,639.8	<LQ	<LQ	<LQ	11,426.0 ±1,033.8	266.5 ±27.6	<LQ	42 ±4.2	7.8 ±1.1	<LQ	<LQ	10.5 ±0.7	10.5 ±0.7	6.5 ±0.7	<LQ	522.88 ±4.6	0.88 ±0.4
TR12	1.5 ±0.1	8.3 ±0.1	419.3 ±28.5	64.5 ±3.5	210.5 ±24.7	24,160.0 ±1230.4	153,925.0 ±10,257.3	<LQ	<LQ	12.5 ±2.1	12,250.0 ±388.9	279 ±4.2	1 ±0.01	67 ±2.8	6.6 ±0.6	<LQ	<LQ	16.5 ±2.1	11 ±0.01	6.5 ±0.7	<LQ	126.3 ±12	0.5 ±0.01
TR14	1.8 ±0.8	8.2 ±0.1	294.3 ±37.2	61 ±19.8	159.5 ±29	28,490.0 ±1000.6	186,375.5±146.5	<LQ	<LQ	18.5 ±2.1	13,731.5 ±427.8	321 ±25.5	<LQ	81.5 ±12	7.3 ±0.2	<LQ	<LQ	17 ±1.4	10.5 ±0.7	7.5 ±0.7	<LQ	134.44 ±16.5	<LQ
TR18	4.7 ±0.9	8.7 ±0.1	479.0 ±78.6	53±7.1	311.5 ±40.3	24,006.0 ±57.3	150,101.5±6,854	<LQ	<LQ	15 ±1.4	12,238.5 ±1,000.6	279.5 ±30.4	5 ±0.01	70.5 ±2.1	7. 6±1.0	<LQ	<LQ	17.5 ±2.1	10.5 ±0.7	6.5 ±0.7	<LQ	221.47 ±25.1	0.67 ±01
TR20	4.4 ±0.4	8.2 ±0.1	270.3 ±16.4	63 ±19.8	163.5 ±24.7	26,166.0±1171	172,913.5±8,398.3	<LQ	<LQ	17 ±1.4	12,868.5 ±47.4	311.5 ±27.6	<LQ	76 ±1.4	7.1 ±1.5	<LQ	<LQ	16 ±1.4	10 ±0.01	6.5 ±0.7	<LQ	167.76 ±49.1	<LQ
TR24	4.7 ±2.3	8.6 ±0.01	366.0 ±59.1	62.5 ±16.3	274.5 ±16.3	22,871.0 ±1488.5	143,434.5±8,845.2	<LQ	<LQ	17.5 ±0.7	12,319.0 ±446.9	272.5 ±10.6	<LQ	70.5 ±3.5	6.9 ±1.4	<LQ	<LQ	16.5 ±0.7	11 ±0.01	6 ±0.01	<LQ	154.99 ±16.2	0.585 ±0.1

Table 4.6 Physico-chemical parameters analysed to determine the effectiveness of the investigated phytoremediation strategies (investigated phytoremediation treatments are highlighted in green while control experiments in orange)

	WC	pH	EC	P available	K available	Mg	Ca	S	B	Cu	Fe	Mn	Mo	Zn	Total C	Total N	Cd	Cr Total	Pb	As	Na	TPH	PAH
	%	-	*µS/cm*	*mg/kg DM*																			
TR25	3.6 ±0.4	8.3 ±0.1	181.4 ±21.9	45 ±7.1	179 ±12.7	24,117.0 ±253.1	161,793.5±7,733.6	<LQ	<LQ	16 ±1.4	11,349.5 ±561.9	264.5 ±14.8	<LQ	71 ±0.01	7.0 ±0.2	<LQ	<LQ	16.5 ±2.1	12 ±2.8	6 ±0.01	<LQ	290.45 ±15.4	<LQ
TR26	1.7 ±0.8	8.5 ±0.01	121.5 ±18.5	<LQ	59.5 ±6.4	29,239.0 ±1,609.4	190,079.5±11,006.1	<LQ	<LQ	<LQ	13,062.5 ±1,320.2	314.5 ±37.5	<LQ	49.5 ±4.9	6.7 ±0.3	<LQ	<LQ	13.5 ±0.7	10 ±0.01	7 ±1.4	<LQ	221.55 ±47.4	<LQ
TR27	3.9 ±0.6	8.9 ±0.01	246.7 ±18	<LQ	183 ±17	24,313.0±297.7	158,807.5±1,451.7	<LQ	<LQ	<LQ	11,602.0 ±465.3	254.5 ±16.3	<LQ	44.5±3.5	7.0 ±1	<LQ	<LQ	13.5 ±2.1	9 ±1.4	6 ±0.01	<LQ	338.65 ±101.3	0.895 ±0.4
TR29	5.6 ±0.7	8.6 ±0.01	381.0 ±77.5	72.5 ±16.3	366 ±9.9	23,264.0 ±586.2	151,425.5±8,334.7	<LQ	<LQ	18 ±1.4	11,8410 ±499.2	271 ±21.2	6 ±0.01	74 ±9.9	7.4 ±2.7	<LQ	<LQ	18 ±0.01	11 ±1.4	6 ± 0.01	<LQ	335.74 ±31.6	0.485 ±0.01
TR30	69.0 ±16.6	8.0 ±0.1	1,170.7 ±546.9	802 ± 96.2	27,22.5 ±811.1	3,501 .0 ±2,969.8	44,398.0 ±33,062.9	<LQ	<LQ	42 ±32.5	4,813.5 ±2,885.7	104.5 ±75.7	<LQ	139.5 ±94.0	17.8 ±1.3	<LQ	<LQ	26 ±0.01	7 ±5.7	2 ±0.01	628.5± 569.2	/	/
TR31	56.2 ±16.7	8.2 ±0.1	1,254.7 ±414.6	887 ±5.7	2,524 ±285.7	3,211.0 ±2,742.2	45,110.0 ±34,226.8	<LQ	12 ±0.01	44.5 ±36.1	5,,903 ±4575	112 ±86.3	<LQ	147 ±111.7	15.1 ±8.4	<LQ	<LQ	36 ±0.01	8.5 ±7.8	3 ±0.01	1,171.0 ± 1,368	/	/
TR32	36.9 ±3.5	8.3 ±0.01	1,055.3 ±321.4	1,052.5 ±132.2	2,503.5 ±350	1,626.5 ±157.7	25,094.0 ±1,236	<LQ	<LQ	24.5 ±0.7	3,141.5 ±38.9	65 ±2.8	<LQ	92.5 ±2.1	21.7 ±1.2	<LQ	<LQ	<LQ	5 ±1.4	1 ±0.01	205 ±83.4	/	/

Table 4.6 Physico-chemical parameters analysed to determine the effectiveness of the investigated phytoremediation strategies (investigated phytoremediation treatments are highlighted in green while control experiments in orange)

	WC	pH	EC	P available	K available	Mg	Ca	S	B	Cu	Fe	Mn	Mo	Zn	Total C	Total N	Cd	Cr Total	Pb	As	Na	TPH	PAH
	%	-	*µS/cm*	*mg/kg DM*																			
TR33	60.7 ±6.6	8.2 ±0.01	735.0 ±30.4	755 ±1.4	1,825.0 ±183.8	3,916.5 ±364.2	58,304.5 ±9,253.9	<LQ	<LQ	53.5 ±24.7	6,084.5 ±975.1	131 ±26.9	<LQ	194.5 ±30.4	16.3 ±3.0	<LQ	<LQ	14.5 ±6.4	10.5 ±4.9	5 ±4.2	463 ±289.9	/	/
TR34	3.9 ±0.6	8.8 ±0.2	281.2 ±77.7	<LQ	117 ±4.2	25,667 ±357.1	165,341.5±3,324.1	<LQ	16 ±1.4	<LQ	13,451.5 ±402.3	307.5 ±12	1.5 ±0.7	48 ±0.01	6.4 ±0.4	<LQ	<LQ	17.5 ±3.5	12 ±0.01	7 ±0.01	<LQ	186.91 ±70.6	<LQ

The results shown in Table 4.6 have been compared with the initial characterisation of the 1-ton sample of contaminated soil, used for pot tests and reported in Table 4.1 and the discussion is reported below.

Concerning physical parameters, the moisture content of soil, also referred to as water content, at the end of pot tests show mean values below 6% in all treatments, while it increased to 69% (the highest value obtained) in the case of control experiments with commercial garden substrate (TR30, TR31, TR32, TR33). This may be due to the presence of some material in the commercial substrate with high water retention capacities.

Concerning chemical parameters, the results show that pH average values remained almost constant and basic conditions have been likely observed (in accordance with the high values of Mg and Ca detected).
The average values of pH at the end of pot tests range between 8.0 and 9.0 for the planted pot tests and between 8.3 and 8.9 for the unplanted pot tests (controls).
Concerning the electrical conductivity (EC), the investigated treatments that have been analysed have shown an increase (from slightly to more significant) of mean values of EC.

All of the analysed treatments have shown optimum to high values of available K (the interpretation of the results was performed taking as reference the *Guia d'interpretació d'anàlisis de sòls i plantes available at:* http://agricultura.gencat.cat). However, regarding available P, it must be considered that the contaminated soil (or rather the starting point, see Table 4.1Table 4.1) showed a value < limit of quantification (LQ). The values below LQ have been maintained and no improvement has been detected in the case of using the vegetative species alone (TR1, TR7) and in case of using the combination with biochar (TR4, TR10) and PGPR (TR3, TR9). Whereas, in the case of using compost (TR2, TR8, TR14, TR20), and the mix of biochar/compost/PGPR (TR6, TR12, TR18, TR24), the available P content has been increased. These results have been validated by the controls with contaminated soil + PGPR (TR26) and contaminated soil + biochar (TR27) that have also shown P available values below LQ while controls with compost (TR25) and the mix of compost/biochar/PGPR (TR29) have shown an increase of the available P. Hence, as expected, it is possible to conclude that compost is essential for the proper supply of P to the plants.
However, it is important to stress that total N content mean values were found to be under the limit of quantification (<LQ) in all the analysed treatments and nitrogen deficiency was also observed by visual inspections. Meanwhile, regarding total C, a very similar value among the whole set of investigated and analysed treatment was detected at the end of pot tests period and was about 7 mg/kg DM. In comparison with the optimal commercial substrate (non-contaminated soil: TR30, TR31, TR32, TR33) the value of total C is lower.

By comparing the values obtained at the end of the pot experiments for the investigated treatments with the initial values presented in Table 4.1, observations regarding the metal content showed that in all cases:

- Mean values of Mo, S, and Cd remained below LQ.
- Mean values of As slightly increased (but this was probably related to the analytical error).
- Mean values of Fe significantly decreased.
- Mn, Pb, Total Cr did not significantly change their mean value.

- Zn significantly increased its mean value almost reaching the values detected for the commercial substrate (TR30-TR33).
- Mean values of Na and B decreased reaching values below the detection quantification limit.

Based on these results and comparing them to the Catalonian legislation of contaminated soils, which establishes Generic Reference Levels for each metal (https://www.boe.es/buscar/pdf/2009/BOE-A-2009-17181-consolidado.pdf), it can be concluded that metals and metalloids at the end of the pot tests were below the reference levels.

Finally, to better visualize the results in terms of percentage removal of TPH (the target contaminant of the Spanish Site) a graph has been elaborated and is presented in Figure 4.7.

The graph depicts the removal efficiency expressed as

$$\frac{\mathrm{TPHf} - \mathrm{TPH0}}{\mathrm{TPH0}} \, x \, 100$$

where TPH_0=4042.10 mg/kg DM (average value of 6 replicates) and TPH_f is the final average value (3 replicates), for each of the investigated phytoremediation treatments (with the exception of the treatments with *Brassica Napus and Panicum Virgatum* alone and combined with PGPR and biochar and all the treatments with commercial fertilizer because as already mentioned, in these cases the germination was negligible, and it was assumed that no phytoremediation effect could be detected).

The evaluation of the TPH removal efficiency has also been performed for the unplanted controls of contaminated soil with and without amendments and is presented in Figure 4.8.

As can be observed, in all cases, including the control experiments of contaminated soil (with and without amendments), a clear and similar decrease of TPH was observed, ranging between a minimum of 87% and a maximum of 97%.
The mean values of TPH detected at the end of the pot tests, despite having significantly decreased (if compared to the initial value: see Table 4.1) are still higher than the limits established by "Real Decreto, 9/2005, de 14 de enero «BOE» núm. 15, de 18 de enero de 2005 Referencia: BOE-A-2005-895, Anexo IV (particularly the limits established for "other uses" category). However, considering the sharp decrease in just only 4 months of pot tests, these are very promising results suggesting the decontamination of the site below established limits, in few years.
A sharp decrease of PAH was also observed for all the treatments and including the control experiments. In most of the cases, PAH values below the quantification limit (<LQ) have been recorded.

These observations would suggest that the phytoremediation of the TPH-contaminated soil is mainly due to the degradation driven by microorganisms in soils rather than the plant species, and that the microbial activity is not influenced by neither the plants nor their combination with the amendments.
Literature review has shown that the most abundant biota in the soil is microbes that can perform several functions including recycling of organic matter that improve fertility of the soil and plant health.

The soil microorganisms that can be considered beneficial are those that develop a symbiotic association with roots of plants and that helps in nutrients mineralization and availability. However, another important role of the microorganisms is the capacity of developing plant growth hormones and antagonists against plant pest, parasites or diseases.

Soil beneficial microbes are:

- PGPR (Plant growth-promoting rhizobacteria) that colonize the plants' roots directly or indirectly
- PGFP (Plant growth-promoting fungi) which affect plants at their different growing stages

- BSMs (Beneficial soil microbes): "According to an estimate one gram of soil may contain 10^{10}-10^{11} bacteria, 6,000-50,000 bacterial species and up to 200m fungal hyphae (Blackwell. 2011[22]) and most of them are considered as beneficial for the for soil and also for the growth of plants, decomposition of organic matter nutrients uptake and also help in the growth of plants (Nihorimbere *et al.*, 2011[23]).

The decrease of TPH/PAH in the control experiments performed with contaminated soil but without plants and amendments can be also due to the additional lixiviation caused by the absence of plants but also volatilization and photolysis (Peng et al., 2009[24]). However, the action of BSMs is suspected to be one of the main contributors.
In this framework, it is important to stress that according to literature review longer chain TPH are degraded by microorganisms while shorter chain TPH or volatile substances are generally lost through leaching and also volatilization if exposed to the natural elements.
Hence, further pot tests will be performed with the selected vegetative species and amendments, under controlled conditions (climatic chamber), in parallel with field activities, to determine the effect of lixiviation on TPH/PAH decrease. Specifically, pots will be irrigated until the field capacity is reached and leachates will be collected to be analysed (the pots will be placed in trays to collect the leachates) to estimate the losses due to leaching. Moreover, the effect of irrigation on TPH/PAH decrease will be tested too, by performing control tests with contaminated soil and no plants nor amendments with irrigation and without irrigation, under controlled conditions (climatic chamber: 25ªC, 14h light/10 h dark, 50% humidity).
In addition, TPH/PAH decrease in the control tests with contaminated soil and no plants nor amendments will be tested under anoxic conditions. This should help validate that the decrease in TPH/PAH concentration is effectively due to the degradation of microorganisms in the rhizosphere (in this case, we don't expect a decrease in TPH/PAH concentration, since microbial activities should decrease or stop in absence of oxygen).

These results led to the conclusion that all selected species showed a good tolerance to the petroleum hydrocarbons concentration in the contaminated soil and that phytoremediation efficiency won't be a key parameter for the selection of the vegetative species to be applied in field. Thus, they will be determined depending on the biomass production needs to meet the Specific Objective 1.1 (see Table 3.5) and will be explained in the following section 4.5.4.

[22] http://dx.doi.org/10.3732/ajb.1000298

[23] Venant Nihorimbere, Marc Ongena, Maïté Smargiassi, Philippe Thonart. Beneficial effect of the rhizosphere microbial community for plant growth and health . Biotechnol. Agron. Soc. Environ. 2011 15(2), 327-337

[24] http://dx.doi.org/10.1016/j.jhazmat.2009.03.036

Moreover, tendency to develop pests (recorded by visual inspections) will be also considered based on the pot tests' results as detailed in the following section 4.5.4.
Another variable that will be considered for the selection of the vegetative specie to be applied in field will be the shape of the roots. Field experience has shown that plants developing well-branched roots in the soil generally show greater phytoremediation efficiency. Details about this particular aspect will be further elaborated in the section 4.5.4.
In the same way, it seems that the amendments do not influence phytoremediation effect, hence, they will be selected depending on the nutritional needs of the selected vegetative species and highlighted by primary (NPK) and secondary (S, Ca, Mg) macronutrients and micronutrients values recorded during the pot trials and reported in Table 4.6.

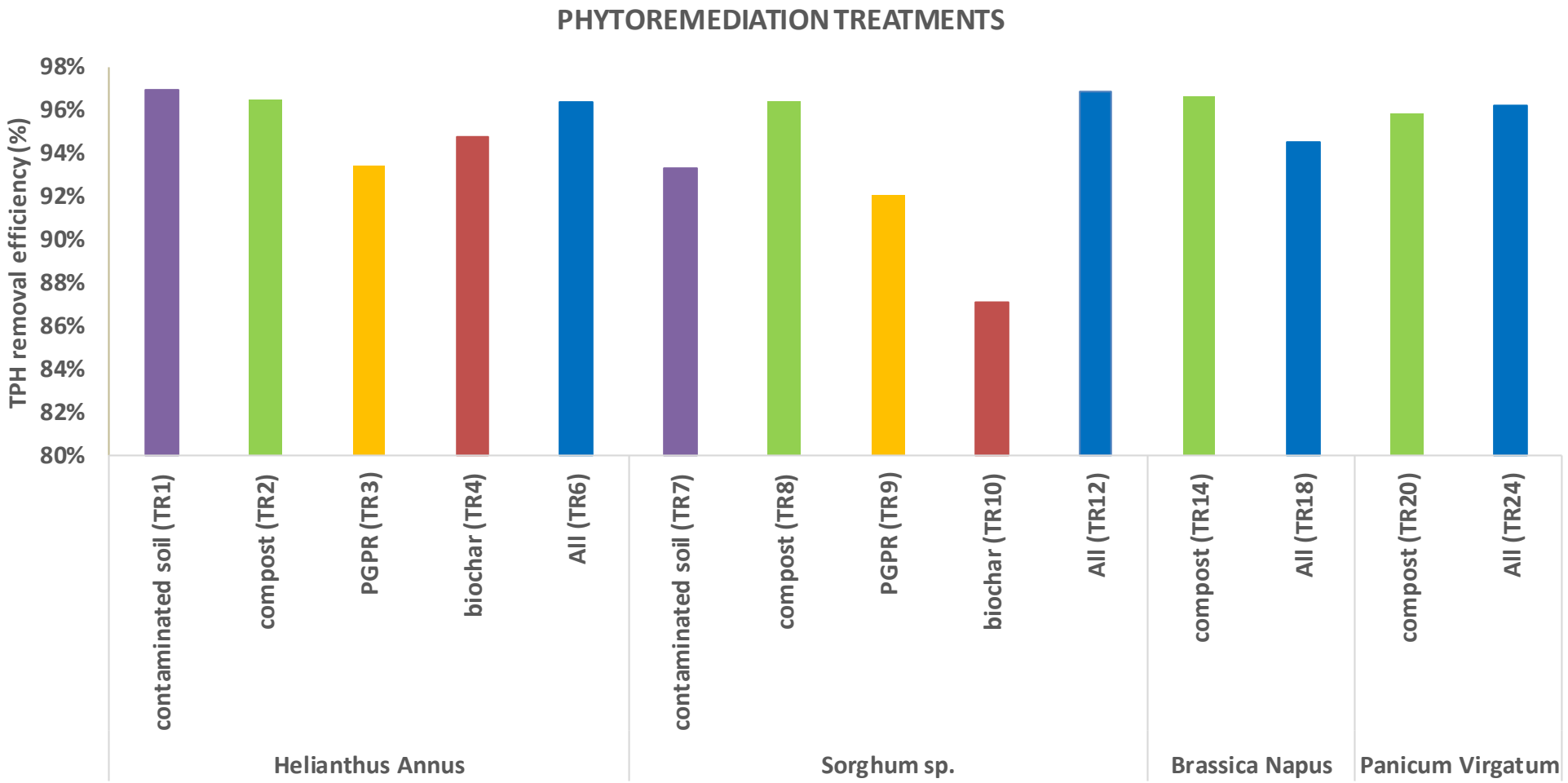


Figure 4.7 TPH removal efficiency (%) of the investigated phytoremediation treatments

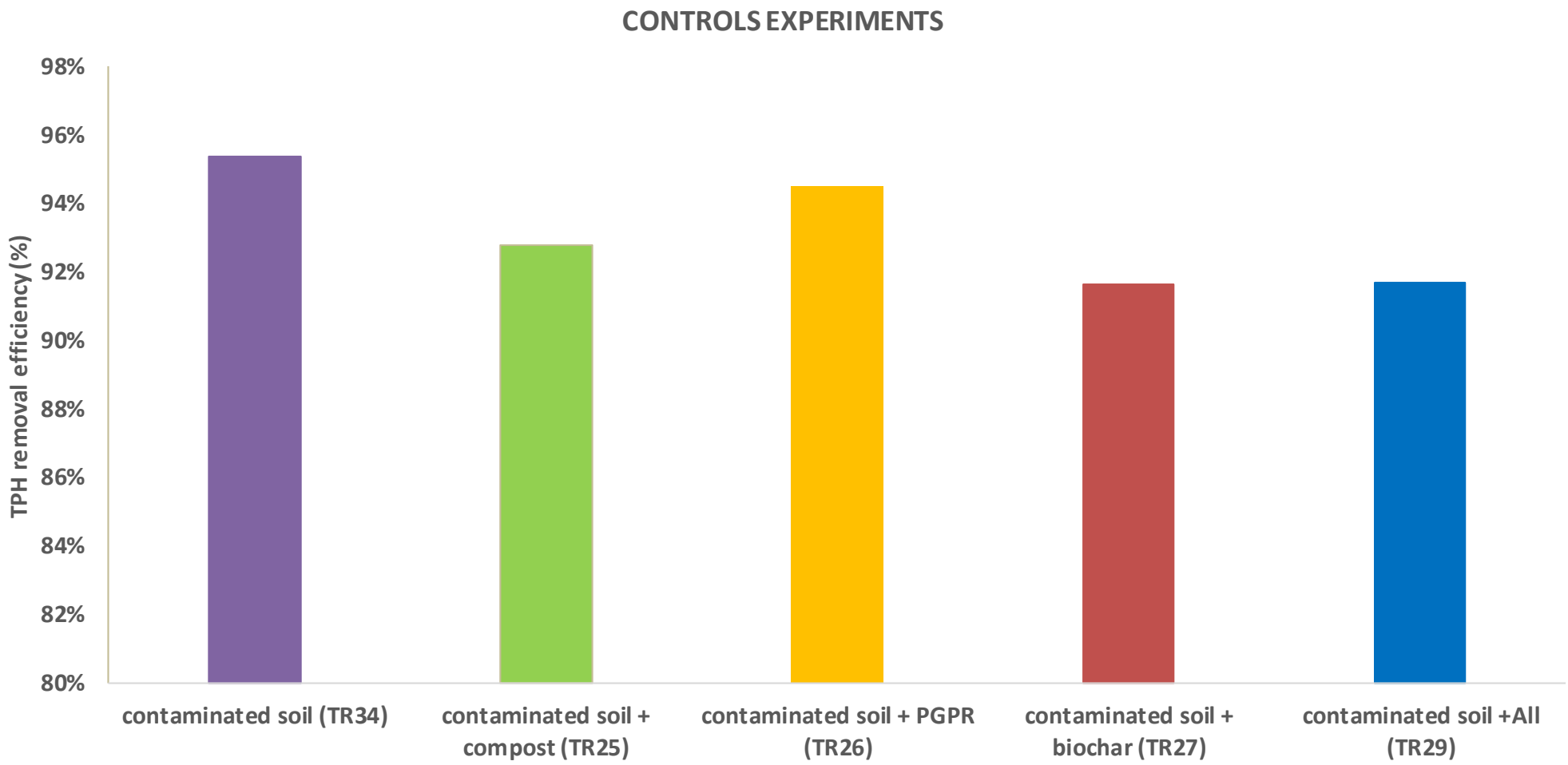


Figure 4.8 TPH removal efficiency (%) recorded for control experiments

4.5.4 Energy crop characterization

The dry biomass production has been calculated for each investigated treatment, with the exception of the treatments with *Brassica Napus* and *Panicum Virgatum* alone and combined with PGPR and biochar (TR13, TR15, TR16, TR19, TR21 and TR22), and all the treatments with commercial fertilizer (TR5, TR11, TR17, TR23, TR28) because as already mentioned, in these cases the germination was negligible, and it was assumed that no phytoremediation effect could be detected. Dry biomass production is an important parameter for the future field activities because it will be valorised for biofuel/biodiesel production. Hence, plants should not only be capable of removing high concentrations of TPH and PAH, but they should also produce high aerial biomass per year/season.
As described in section 4.4.2, aboveground biomass (leaves/stems/seeds) was measured gravimetrically using both wet and dry weights. To determine dry weight, the aboveground biomass was dried in an oven at 70 °C for 72 h until constant mass was reached. Results of wet and dry weight of stems and leaves (collected as a bulk sample) and seeds are presented in Table 4.7. The main contribution to the biomass production is given by stems and leaves and it is represented in Figure 4.9.

Focusing on dry biomass production given by stems and leaves, *Sorghum sp.* has shown the best results in case of using compost (TR8) and the mixture of compost, biochar and PGPR (TR12) with a production of maximum 37.4 g and 43.4 g, respectively, against an optimal value of 73.5 g recorded in the control experiment conducted with commercial substrate (TR31). Also, *Panicum virgatum* has shown a good performance in terms of dry biomass production from stems and leaves. Particularly in this case, the best results were given by the combination with compost (TR20) and the mix of compost, biochar and PGPR (TR24) that provided a maximum of 27.7 g and 31.5 g, respectively, against an optimal value of 55.5 g recorded in the control experiment conducted with commercial substrate (TR33).
Whereas, the other 2 species, *Helianthus annuus* and *Brassica napus*, produced dry biomass values lower than 5 g, with a maximum biomass production of 4.8 g (TR6, combination with the mix of amendments) and 2.5 g (TR18, combination with the mix of amendments), respectively.

Regarding seed production, only *Helianthus annuus* and *Sorghum sp.* generated seeds. In this case *Helianthus annuus* combined with the mixture of compost, biochar and PGPR (TR6) showed the best results followed by *Sorghum sp.* also in the case of its combination with the mixture of compost, biochar and PGPR (TR12) and with a value of 8.6 and 3 g of dry seeds, respectively.
The other treatments, in both cases (*Helianthus annuus* and *Sorghum*) generated less than 1 g. However, it must be considered that the dry weights of seeds include the inflorescences or the stem, where the seeds are deposited.

Finally, the morphology of the roots of each specie was also inspected resulting in taproots in the case of *Helianthus annuus* and *Panicum virgatum* and in well branched roots in the case of *Sorghum sp*. and *Brassica napus.*

In Figure 4.10 it is possible to observe the different morphologies of the roots that the 4 vegetative species have developed in case of being applied in combination with compost and with the mixture of amendments (compost/biochar/PGPR), or rather the treatments that have shown the best results in terms of both TPH/PAH removal and biomass production.

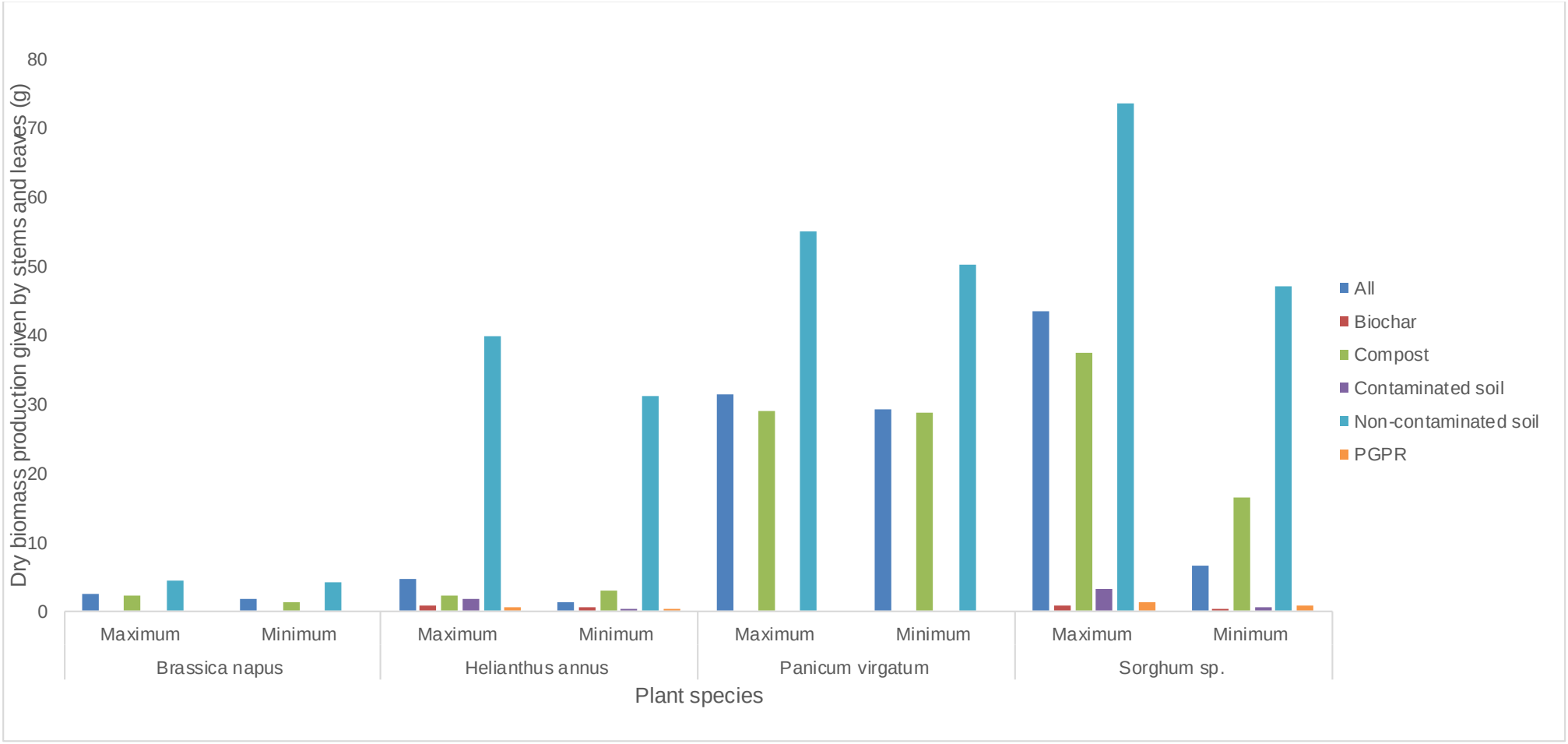


Figure 4.9 Minimum and maximum value of wet and dry biomass given by stems and leaves treated as a bulk sample for all the investigated treatments

Table 4.7 Minimum and maximum value of wet and dry biomass given by stems and leaves treated as a bulk sample and seeds for all the investigated treatments (to be noticed that the standard deviation was not reported because only one sample was used for this analysis)

		Wet stems and leaves (g)	Dried stems and leaves (g)	Wet seeds (g)	Dried seeds (g)
TR1	MAX	4	1.7	2.6	1.2
	MIN	1.7	0.1	0.8	0.8
TR2	MAX	11.5	3.1	9.3	3.7
	MIN	10.2	2.3	9.1	3.5
TR3	MAX	2.7	0.6	1.5	0.8
	MIN	1.3	0.3	0.7	0.7
TR4	MAX	2.7	0.8	1.4	0.7
	MIN	2.1	0.5	0.8	0.6
TR6	MAX	21.7	4.8	28.1	8.6
	MIN	2.9	1.2	7.8	2
TR7	MAX	19.4	3.2	-	-
	MIN	1.5	0.5	-	-
TR8	MAX	48	37.4	2.8	1.6
	MIN	47.1	16.4	2.6	1.4
TR9	MAX	2.7	1.2	-	-
	MIN	2.3	0.8	-	-
TR10	MAX	1.7	0.9	-	-
	MIN	0.3	0.1	-	-
TR12	MAX	70.5	43.4	4.5	3
	MIN	40	26.6	1.4	1
TR14	MAX	16.1	2.2	-	-
	MIN	13.2	1.3	-	-
TR18	MAX	17.9	2.5	-	-
	MIN	12	1.8	-	-

Table 4.7 Minimum and maximum value of wet and dry biomass given by stems and leaves treated as a bulk sample and seeds for all the investigated treatments (to be noticed that the standard deviation was not reported because only one sample was used for this analysis)

		Wet stems and leaves (g)	Dried stems and leaves (g)	Wet seeds (g)	Dried seeds (g)
TR20	MAX	29.1	27.7	-	-
	MIN	28.7	27.6	-	-
TR24	MAX	33.3	31.5	-	-
	MIN	29.2	29.2	-	-
TR30	MAX	40.6	39.9	23.2	15.8
	MIN	31.2	11.1	10.4	9.8
TR31	MAX	144.9	73.5	18.4	12.9
	MIN	85	47.2	11.2	5.5
TR32	MAX	33.7	4.4	-	-
	MIN	35.8	4.2	-	-
TR33	MAX	115.8	55.1	-	-
	MIN	106.2	50.3	-	-

a)

b)

Figure 4.10. Morphology of the roots of *Helianthus annuus, Brassica napus, Sorghum sp. and Panicum virgatum* developed in case of being combined with a) the mixture of compost, biochar, PGPR and b) only compost

4.6 Conclusions

Based on the pot tests results, a phytoremediation strategy to be applied in the Spain Pilot Site has been defined.

Specifically, pot tests have highlighted that:

i) The remediation of the petroleum hydrocarbons contaminated soil was most likely induced by the action of BSMs.
ii) The selection of a specific plant nor of a specific amendment seem to have an effect on the microbial activity
iii) The highest biomass production has been recorded in the case of the phytoremediation treatments using *Sorghum sp.* combined with compost and with the mix of amendments (compost/biochar/PGPR).
iv) All the plants have proved to be TPH tolerant, however, *Sorghum sp.* and *Helianthus annuus* have shown to be more resistant to the detected concentrations.
v) *Sorghum sp.* and *Panicum virgatum* have shown a branching of the roots all over the soil while *Helianthus annuus* and *Brassica napus* have shown a taproot.

According to points i) and ii), it has been concluded that the selection of the vegetative species to be applied in field won't be performed according to the TPH removal efficiency (because all the treatments and controls have shown almost the same decrease) but on biomass production needs to meet the Specific Objective 1.1 (Table 3.5).
For this reason, the *Sorghum sp.,* and the mix of amendments (compost/biochar/PGPR) have been selected to be applied in field because according to point iii) this phytoremediation strategy has shown the highest biomass production. At the same time, the addition of PGPR will provide the microorganisms that are mainly responsible of the TPH degradation, while compost and biochar will ensure the supply of both macro and micronutrients to the soil.

Moreover, since field experience has shown that plants developing well-branched roots in the soil generally show greater phytoremediation efficiency, according to point v) *Sorghum sp.* showed *to be a good choice also because it develops well* branching roots.
Once the vegetative species is selected, the amendments will be determined accordingly, depending on the nutritional needs of the selected vegetative species.
Moreover, it has been decided to apply a crop rotation to avoid a season with bare soil and to ensure that biomass production needs will be met. Specifically, *Sorghum sp.* will be rotated with *Brassica napus* that, despite showing less biomass production (but it must be taken into account that it was not sowed in the optimal sowing season), will help to limit runoff and leaching of NPK. Moreover, the use of different crops with different root morphology will contribute to improve soil structure.
Hence a pot field of ≥800 m^2 will be sowed first with *Sorghum sp. (Sowing:* April/May; Harvesting: September/October*) and the Brassica Napus* (Sowing: September/October; Harvesting: May/June). The pot field size will depend on the final ongoing germination and purity test results that will determine the final need in terms of m^2 to fulfill the specific objective 1.1 (see Table 3.5).
After harvesting, the mix of stems, leaves and seeds will be dried and sent to Fraunhofer Institute (WP3) for biofuel production.
However, the conclusion that phytoremediation of the petroleum hydrocarbons contaminated soil is mainly due to the degradation operated by microorganisms in soil will be validated by

performing another set of pot tests in parallel with field activities. This because according to literature review, longer chain TPH are degraded by microorganisms while shorter chain TPH or volatile substances are generally lost through leaching and also volatilization if exposed to the natural elements, hence the following further analysis will be performed:

- The TPH decrease in the control tests with contaminated soil and no plants nor amendments will be tested under anoxic conditions. This should help validate that the decrease in TPH concentration is effectively due to the degradation of microorganisms in the rhizosphere (in this case, we don't expect a decrease in TPH concentration, since microbial activities should decrease or stop in absence of oxygen).
- The effect of irrigation on TPH decrease in the control tests with contaminated soil and no plants nor amendments will be investigated by testing a set of control pot tests with irrigation and another set without irrigation, under controlled conditions (climatic chamber: 25ªC, 14h light/10 h dark, 50% humidity). These pot tests will also allow to determine the effect of lixiviation in the TPH decrease because leachates will be collected once the field capacity is reached and will be analysed (the pots will be placed in trays to collect the leachates) to estimate the losses due to leaching.

5. SERBIAN SITE POT TRIALS EXPERIMENTAL PLAN

5.1 Objectives

It is well known that non-hyperaccumulating *Brassica* species have potential for heavy metal accumulation and can tolerate high concentrations of heavy metals in their shoots. The objective of pot trials was to assess if rapeseed (*Brassica napus*) as energy crop, has potential to be used for phytoextraction of heavy metals from soil from Serbian pilot site. The effect of different PGPR commercial products in promoting rapeseed growth was also validated and rapeseed potential to uptake heavy metals was also compared to other energy crops such as sunflower, hemp and white mustard.

5.2 Materials and methods

5.2.1 Description of the set-up

Soil/sediment used in pot tests was collected at the Serbian pilot site on May 12th, 2021. Approximately 500 kg of polluted soil/sediment was collected at the site, then transported to IFVCNS facilities, and manually mixed and homogenized before placed in pots.

Unpolluted soil used as control was collected at the agricultural field which is used by IFVCNS for rapeseed growing. Unpolluted sediment used as control was collected at Special Nature Reserve “Zasavica”. According to previous research the sediment on this location has low concentrations of heavy metals and basic physical and chemical properties are similar to sediment at the pilot site.

Pot experiments were performed on open air under natural weather conditions. In order to assess the full plants potential to grow in polluted soil, large plastic pots (height 30 cm/diameter 36cm) were selected, and each was filled with 20 kg of sediment or soil.

Following energy crops were selected for pot trials: rapeseed *(Brassica napus)*, white mustard *(Brassica alba)*, sunflower *(Helianthus annuus)*, hemp *(Cannabis sativa)* and sorghum (*Sorghum bicolor)*. Sorghum showed very low germination (only one plant emerged) so it was discarded from the experiment. Plants sown in unpolluted sediment showed also low germination and slow development. 3 weeks after sowing, plants sown in unpolluted sediment dried up and were discarded from the experiment.

Following commercial PGPR products were applied during growth of rapeseed in order to assess if they have effect on plant growth and metals uptake:

- Commercial product based on *Trichoderma* strains (Trichoderma asperellum T34 strain) **Trifender Pro**, produced by Kwizda Agro (available at: https://www.kwizda-agro.at/bioprodukte/trifender-pro~p3354)
- Commercial product based on rizosphere bacteria, Trichoderma sp. And Mycorrihzae, **Panorama Bio Plus** produed by Baktersol (available at: https://www.baktersol.com/en/proizvod/)
- Commercial product based on auxins and gibberellins producing bacteria will be applied foliar **Bio eho,** produced by Biofor System (available at: http://biofor.rs/proizvodi/bioeho/)

A pot experiment with polluted sediment started on May 17th and lasted to July 28th, 2021. A total of 21 pots were filled with 20 kg of polluted sediment each. On May 18th sowing was performed. All energy crops were sown in 3 replicates in polluted sediment but the number of seeds per pot varied depending on plant variety. Number of pots per energy crop and sediment type is presented in Table 5.1.

Table 5.1. Number of pots per energy crop and sediment type

Energy crop	Treatment with PGPR product	Lebel	No of pots with Polluted sediment	No of pots Unpolluted sediment	No of pots Unpolluted soil (CS)
Rapeseed	No treatment	OR_0	3	3	3
Rapeseed	Trifender Pro	OR_T	3	/	/
Rapeseed	Panorama Bio Plus	OR_P	3	/	/
Rapeseed	Bio Eho	OR_B	3	/	/
Hemp	No	HE	3	/	/
Sunflower	No	SF	3	/	/
White mustard	No	WM	3	/	/

Figure 5.1 Pot experiment set up

Seeding density for hemp and sunflower was 5 seeds per pot and for rapeseed and white mustard was 10 seeds per pot. After emerging, plants were timed to one plant per pot for hemp and sunflower and two plants per pot for rapeseed and white mustard.

Three days after sowing, pots with rapeseed were treated with commercial PGPR products: Trifender Pro and Panorama Bio Plus. In accordance with producers' instructions, both products were diluted with tap water to concentration of 0.05%. 20 ml per pot of diluted PGPR product was spread evenly over a soil surface using hand sprayer. Immediately after application, soil was gently watered in order to promote product penetration in deeper soil layers.

Three weeks after rapeseed plants emerged, Bio Echo product was applied foliar. In accordance with producers' instructions Bio Eho was diluted 1000 times (0,2 ml in 200ml of water) and each plant in a pot was treated with 10 ml of solution using hand sprayer.

Six weeks after sowing each pot was fertilized with ammonium nitrate fertilizer. An amount of 5.97 g of fertilizer which corresponds to 2 g of pure nitrogen) was added in each pot in form of water solution.

Ten weeks after sowing, on 28.07. 2021 harvest was performed.

5.2.2 Sampling campaign

Soil samples were collected from each pot at the beginning of pot experiment and after the harvest. A small probe was used to take four subsamples form each pot. Subsamples were then mixed thoroughly and transferred in plastic bags.

Energy crops were sampled only at the end of the pot experiment. Plants high was carefully measured before cutting aboveground parts. Roots were carefully removed from pots and the excess of soil was gently removed by hand. Aboveground parts of plants were transferred in paper bags and left in dark and dry place to dry. Roots were transferred in plastic bags and stored in refrigerator until washing. In order to remove soil residues, roots were thoroughly washed in tap water and after that rinsed in distilled water. After washing, ro ots were placed in a dry and dark place for drying.
After plan material was dried, it was measured and grind in laboratory mill.

5.3 Irrigation regime

Pots were irrigated manually by adding 2.5 L of water every second or third day after plants emerging. During July, pots were watered every morning.

5.4 Monitoring program

Analytical methods used for soil characterization and energy crops characterization is given in the Table 5.2.

Table 5.2. Analytical methods for soil/sediment and energy crop characterization

Parameters	Method	Short description	Method detection limit
Texture - granulometric composition	ISO 11277:2009	Method by sieving and sedimentation - ISO 11277:2009. Sieving soil samples through series of sieves in the range of 2 mm to 0.063 mm. Sedimentation method for fraction 0.063 m to <0.002 mm, by withdrawing the 25 ml of suspension at defined time and depth in the cylinder.	0.1%
Water content	EN 12880:2000	Oven drying at 105°C to the constant weight.	0.1%
pH	ISO 10390:2005	5 g/50 g H_2O mixing for specified time	0.02
Electrical conductivity	ISO 11265:1994	20 g/100 g H_2O mixing for 30 min.	0.0005 mS/cm

Table 5.2. Analytical methods for soil/sediment and energy crop characterization

Parameters	Method	Short description	Method detection limit
P	Internal laboratory method	Phosphorus is extracted with ammonium lactate solution, then colored with ammonium molybdate and analyzed on UV/VIS spectrometer	0,1 mg/100g
Total N	ISO 11261:1995	Kjeldahl method - Digestion of soil sample with ccH_2SO_4, distillation in H_3BO_3, titration with HCl	43 mg/kg
Organic matter	CEN - EN 12879	Loss of ignition at 550°C	/
Total C	TOC analyser	Acidification of soil sample with HCl to remove inorganic C. Analysis on TOC analyser with IR detector	10 mg/kg
Microbial biomass	Internal method	Petri cultivation	NA
K (Total)	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO3:H2O2) Analysis on ICP-MS.	0.5 mg/kg
K (available)	Internal laboratory method	Potassium is extracted with ammonium lactate solution, then analyzed using atomic flame emission spectroscopy.	0.4 mg/100g
Mg	EPA3051a EPA6020B	MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Analysis on ICP-MS.	0.5 mg/kg
Ca	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.5 mg/kg
S (as sulphate)	Internal method	Water extraction, analysis IC	0.5 mg/kg
B	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	ICP-MS 0.1 mg/kg
Cu	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.028 mg/kg
Fe	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.24 mg/kg
Mn	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.014 mg/kg
Mo	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.025 mg/kg
Zn	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C.	0.18 mg/kg

Table 5.2. Analytical methods for soil/sediment and energy crop characterization

Parameters	Method	Short description	Method detection limit
		Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	
Cd	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.028 mg/kg
Cr	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.014 mg/kg
Pb	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.014 mg/kg
As	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.021 mg/kg
Na	EPA3051a EPA6020B	Soil-MW digestion with ccHNO_3:ccHCl=3:1, at 170°C. Energy plant - MW extraction (HNO_3:H_2O_2) Analysis on ICP-MS.	0.5 mg/kg
Hydrocarbons (TPH)	ISO 9377-2:2000(E) and EPA8000B)	Ultrasound hexane extraction (EPA3550b), cleanup: sulphur removal with activated copper powder (EPA3660b), cleanup with silica gel (EPA3630C). Extract evaporation with nitrogen gass (EPA3630C). Analysis GC-FID (ISO 9377-2:2000(E) and EPA8000B)	25 mg/kg
Polyaromatic Hydrocarbons (PAH)	GC-MS (series of EPA method)	Ultrasound hexane extraction (EPA3550b), cleanup: sulphur removal with activated copper powder (EPA3660b), cleanup with silica gel (EPA3630C). Extract evaporation with nitrogen gass (EPA3630C). Analysis GC-MS (EPA8270C)	0.003 mg/kg Each PAH
Organochlorine pesticide and polychlorinated biphenyl's	GC-ECD (series of EPA method)	Ultrasound hexane extraction (EPA3550b), cleanup: sulphur removal with activated copper powder (EPA3660b), cleanup with silica gel (EPA3630C). Extract evaporation with nitrogen gass (EPA3630C). Analysis GC-ECD	0.003 mg/kg Each compound
BCR	Arin et al., 2008[25]	Exchangeable fraction – MW extraction with acetic acid Reducible fraction – MW extraction with NH_2OH_HCl Oxidizable fraction – MW extraction H_2O_2 and CH_3COOH. Residual fraction - MW extraction with HNO_3 and HCl	same as for soil

[25] Arin M., Kazi T., Jamali M., Jalbani N., Afridi H., Baig J. (2008), Journal of Hazardous Materials 154 (2008) 998–1006.

Table 5.2. Analytical methods for soil/sediment and energy crop characterization

Parameters	Method	Short description	Method detection limit
		Analysis on ICP-MS.	
Bioavailable fraction of organic pollutants	Spasojevic et al., 2015[26]	Bioavailability was assessed by desorption experiments with a non-exhaustive extraction procedure with Tenax resin. After extraction, procedure is the same as for PAH, OCP, PCB.	0.003 mg/kg Each compound

5.5 Results

5.5.1 Soil characterization

The general chemical and physical parameters of soil/sediment characterization have been presented in the Tables 5.3 and 5.4. Based on the TOC content contaminated sediment can be considered as rich in organic carbon. TOC in agricultural soil used as control was not measured but the average TOC content on field from which the sample was collected is 1.23% which classifies this soil as low to medium rich in carbon. According to the CEC value contaminated sediment can be classified as light-colored loams and silt loams. The control soil and contaminated sediment have similar texture. There is slightly higher clay content, and lower send content in the contaminated sediment compared to the control soil samples.

Table 5.3 Basic physical and chemical characterization pf soil and sediment (na- not analysed)

Sample	TOC	CEC (meq/100 g sample)	Texture		
			Sand (50 µm - 2000 µm)	Silt (2 µm - 50 µm)	Clay (< 2 µm)
Agricultural soil	na	na	39.4±9.1	37.1±7.2	23.5±5.2
Contaminated sediment	2.87±0.7	1.19±0.1	33.4±7.0	36.4±3.9	30.5±3.2

Both, control soil and contaminated sediment can be considered as slightly alkaline. pH in all treatment marginally decreased at the end of the pot experiment. Soils are rich in organic matter and nutrients (N, P, K). But even so, due the observed plant stress because of nitrogen deficiency, all pot has been fertilized with ammonium nitrate fertilizer (Table 5.4 and figure 5.2). According to domestic soil classification available P and K are in optimum to high range. Soil samples were similar in texture, except for sample 3 that is rich in sand fraction (>72%). Investigated soil is rich in organic matter and available potassium and phosphorous. The contaminated sediment has significantly higher organic matter content (loss of ignition), compared to the control soil. The control soil and contaminated sediment has relatively high

[26] Spasojevic J., Maletic S., Roncevic S., Radnovic D., Cucak D., Trickovic J., Dalmacija B. (2015), Journal of Hazardous Materials. 283, 60-69.

content of nitrogen and phosphorous. No significant changes of the organic matter, nitrogen phosphorous and sulphur content has been observed after the harvesting.

The content of the selected metal(oid)s at the beginning and end of the experiment is presented on the Figure 5.2. According to national sediment legislation[27] sediment is considered as highly contaminated, since heavy metals, such as Cu, Cr, exceeded remediation values. While concentrations of Zn, Cd, Cr and Pb exceed target value. Other heavy metal(oid)s content is below the target value. The control soil and contaminated sediment have relatively high content of the Fe and Mn, and its concentration has not been changed after the harvesting. Both elements belong to the nutrients, and it is not considered as a toxic.

According to the literature hyperaccumulators are those plants which are able to accumulate and tolerate unusually large amounts of metals in comparison with other plants[28]. Baker and Brooks (1989) defined hyperaccumulator for different metals based on their dry weight shoot metal concentrations such as 0.01% for Cd and 0.1% for Cr, Cu, Pb and Ni, and 1% for Mn and Zn. Based on this statement in the best case for a given experimental conditions (20 kg of soil/sediment and maximum 100 g of obtained plant material) expected change in the soil/sediment are as follow 0.0005 mg/kg for Cd and 0.05 mg/kg for Cr, Cu, Pb and Ni, and 0.5 mg/kg for Mn and Zn. This level of concentration changes, less than 1%, is in the range of the measurement uncertainty of the analytical methods for determination these analytes. Therefore, the changes in concentration in control soil and contaminated sediment is not expected to be notified.

However, during the experiment the marginal reduction of the Ni, Zn, Cd, As, Cr (for all OR and WM variants) and Pb for about 10% was observed in all variants. And there is no significant difference between grown plants for these metals. In the case of copper significantly higher reduction (25%) was observed for the treatment OR_0 and OR_B. On contrary, the reduction up to 25% for chromium was observed for the HE and SF variants. This reduction can be attributed to the leaching of the bioavailable metal content (Figure 5.3), since the experiment was performed in the open air, with no control of rainfall, and the leachate water from the pot was not monitored.

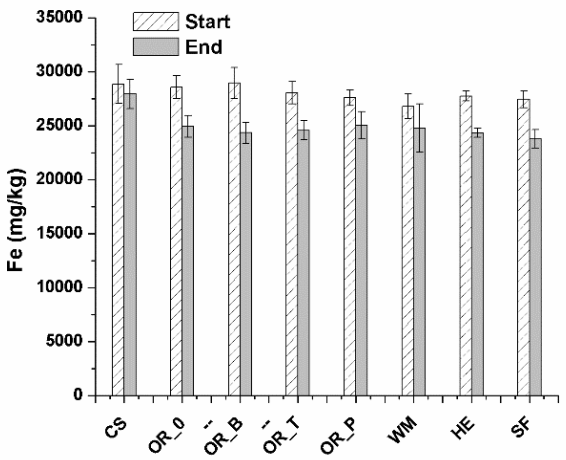
Table 5.4 Basic physical and chemical characterization of soil and sediment in each pot

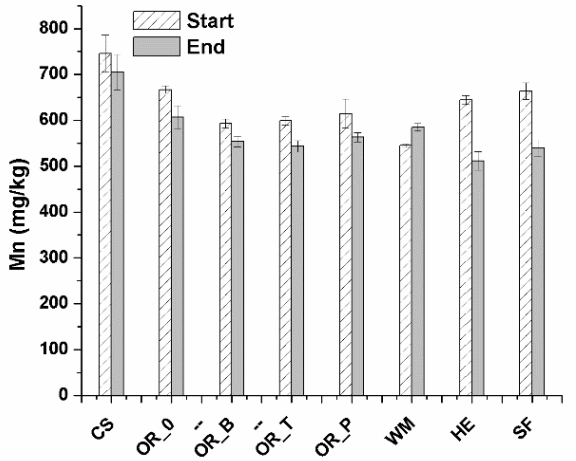
Pot	Time	pH	Eh	OM*	Total N	Total P	Availa-ble P	S**	Na	K	Availa-ble K	Mg	Ca
			µS/cm	%	mg/kg	mg/kg	P_2O_5/100g)	mg/kg	g/kg	g/kg	K_2O/100g	g/kg	g/kg
CS	Before sowing	7.85	142.5	6.6	1,870	919	29.5	45.7	0.597	17.2	37.8	83.4	191
	After harvest	8.07	102	7.1	2,170	950	/	41.6	/	/	/	/	/
OR_0	Before sowing	7.67	388	11.7	2,510	1,710	102.8	26.4	0.753	16.6	38.2	31.3	78.4
	After harvest	7.95	232	12.0	2,310	1,310	/	28.1	/	/	/	/	/

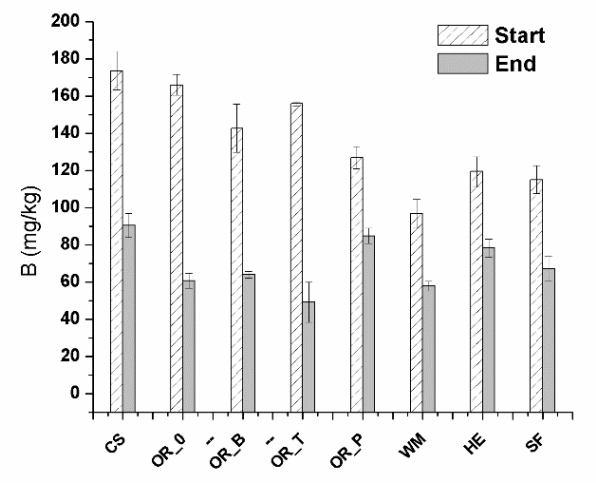
[27] Regulation on limit values of pollutants in surface waters, groundwater and sediment and timelines for reaching of the values ("Official Gazette RS" no. 50/12)
[28] Baker AJM, Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metal elements—a review of their distribution, ecology and phytochemistry. Biorecovery 1:81–126

Table 5.4 Basic physical and chemical characterization of soil and sediment in each pot

Pot	Time	pH	Eh	OM*	Total N	Total P	Availa-ble P	S**	Na	K	Availa-ble K	Mg	Ca
			µS/cm	%	mg/kg	mg/kg	P_2O_5/100g)	mg/kg	g/kg	g/kg	K_2O/100g	g/kg	g/kg
OR_B	Before sowing	7.63	389	12.1	2,640	1,630	99.3	38.9	0.704	13.2	38.0	30.7	81.4
	After harvest	7.99	231	12.0	2,630	1,470	/	31.4	/	/	/	/	/
OR_T	Before sowing	7.68	357	11.1	3,140	1,631	105.2	26.2	0.626	15.7	38.4	32.5	84.8
	After harvest	7.90	211	11.5	2,720	1,470	/	26.5	/	/	/	/	/
OR_P	Before sowing	7.63	378	10.3	2,530	1,475	95.9	50.4	0.664	9.98	36.1	25.4	67.5
	After harvest	7.91	311	11.3	2,850	1,360	/	43.1	/	/	/	/	/
WM	Before sowing	7.64	440	10.9	2,730	1,440	100.7	54.2	0.693	16.5	37.6	30.5	73.0
	After harvest	7.76	213	10.1	2,540	1,200	/	45.6	/	/	/	/	/
HE	Before sowing	7.66	396	11.9	2,600	1,530	93.3	38.4	0.686	15.9	36.7	22.7	70.0
	After harvest	7.88	143	11.3	2,540	1,330	/	33.2	/	/	/	/	/
SF	Before sowing	7.69	360	10.5	2,930	1,530	93.6	63.6	0.63	14.5	37.8	27.6	67.6
	After harvest	7.93	256	12.0	2,417	1,340	/	43.5	/	/	/	/	/



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End
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20
40
60
80
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120
140
160
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200
CS
OR_0
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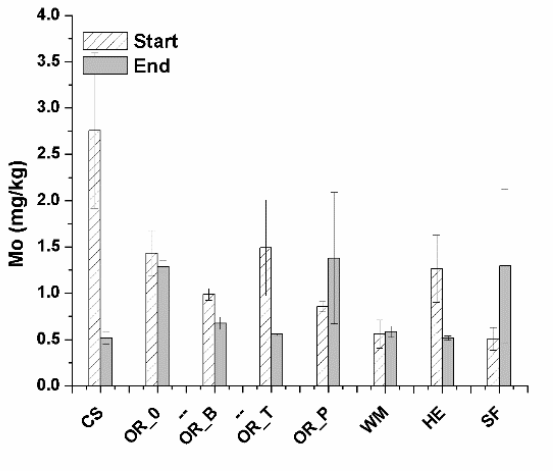
WM
HE
SF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1.0
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2.0
2.5
3.0
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OR_0
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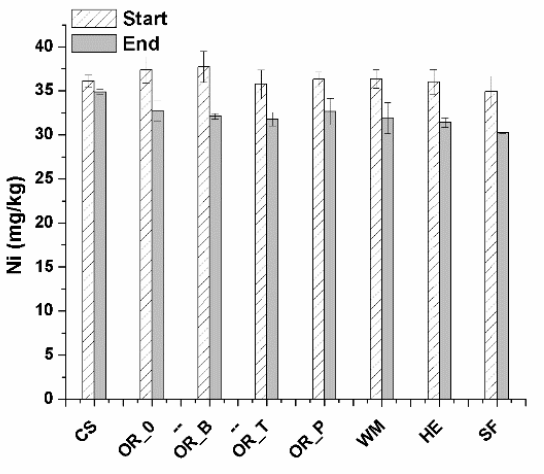
OR_T
OR_P
WM
HE
SF


Start
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Ni (mg/kg)
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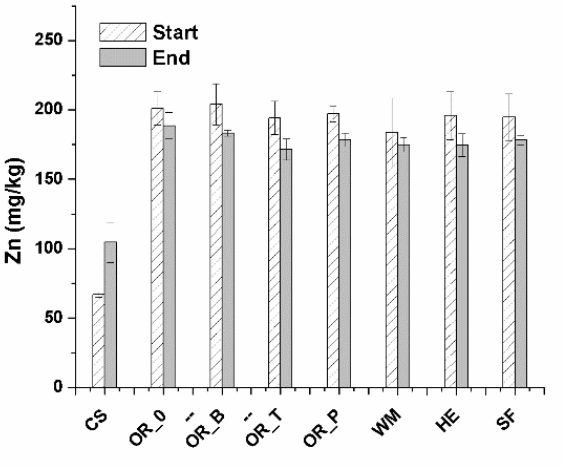
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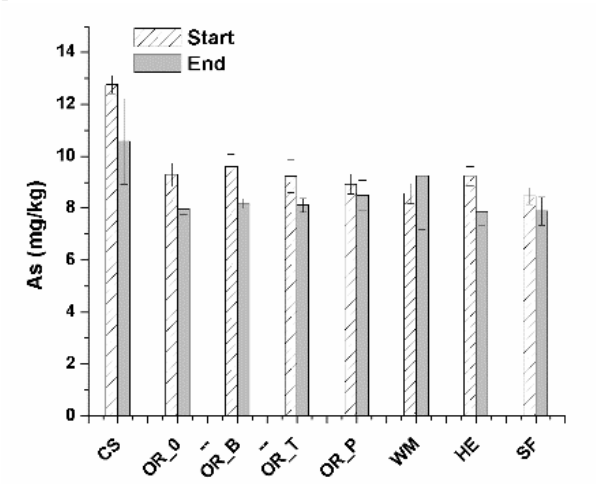
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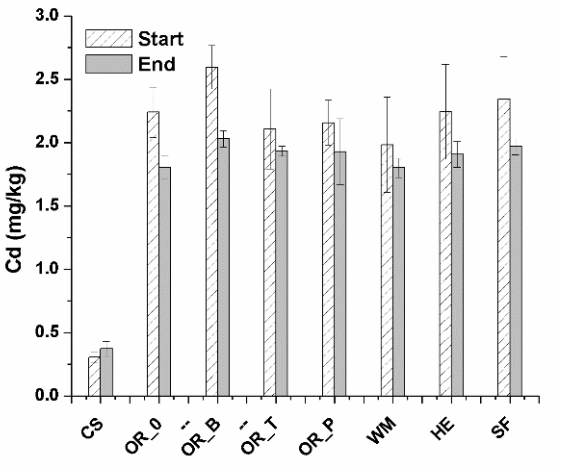
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End
Cd (mg/kg)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
CS
OR_0
OR_B
OR_T
OR_P
WM
HE
SF

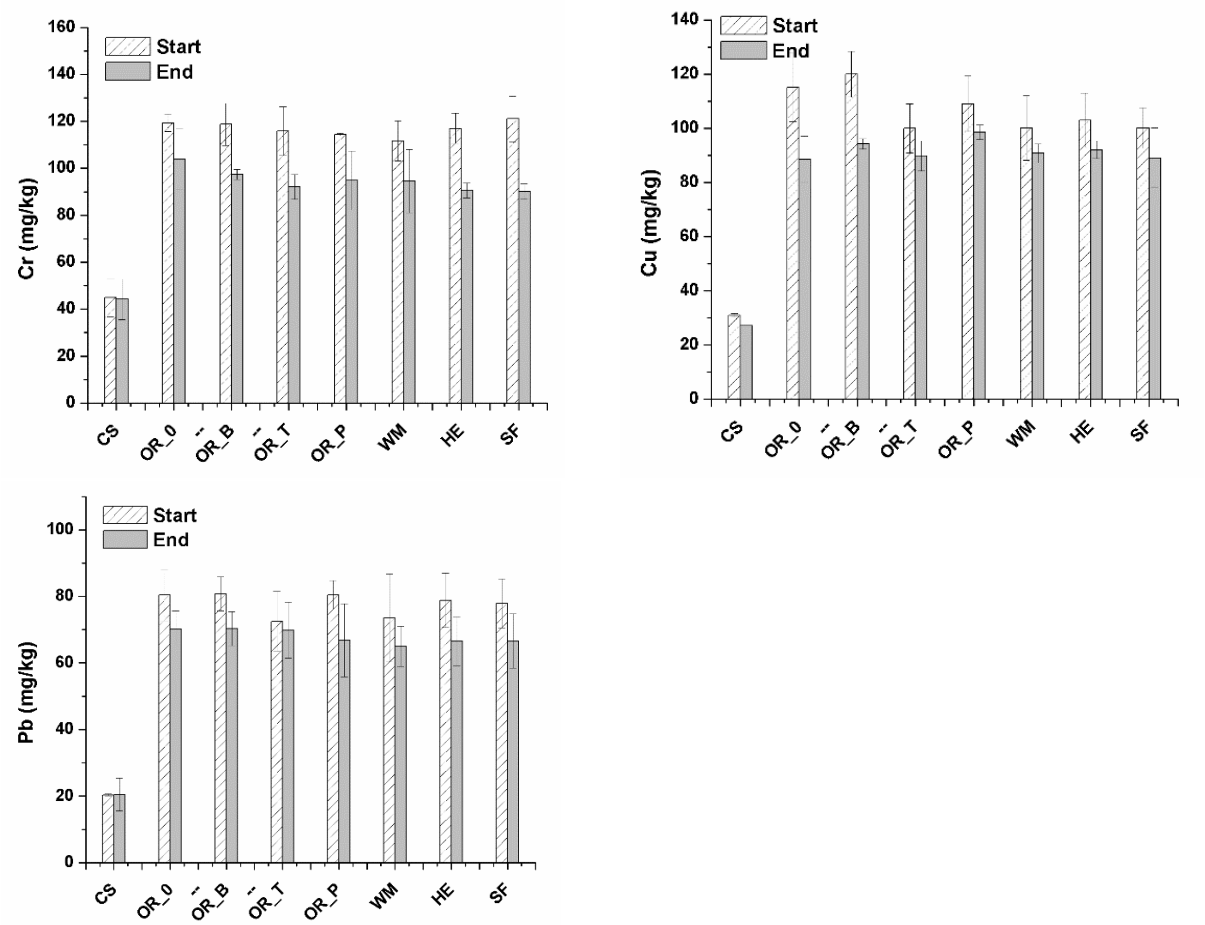


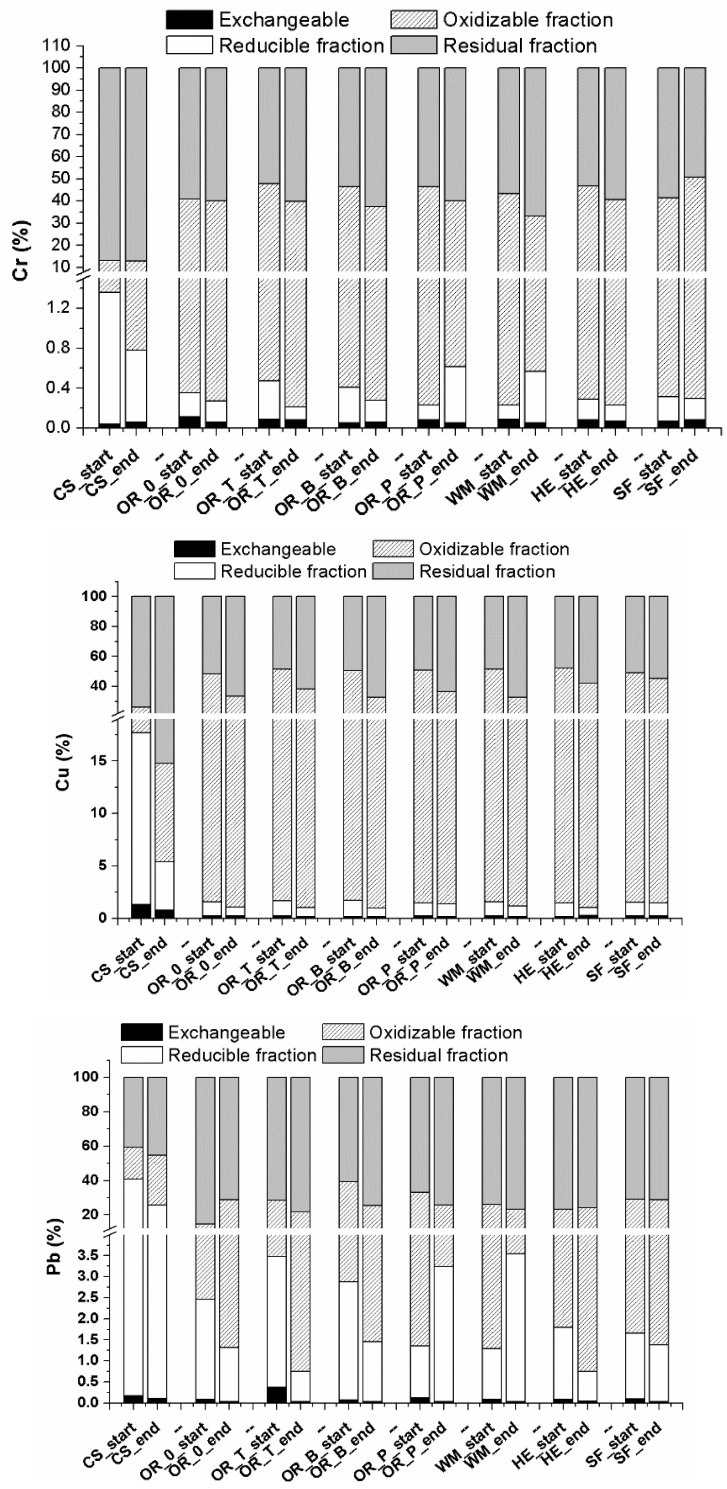
Figure 5.2. Metals and metalloids concentration in the soil during the port experiment

Total metal determination in soil/sediment is valuable information about the overall pollution levels, but is insufficient to estimate their biological effects, which depend on the chemical species of the metals in the soil and sediment. An experimental approach frequently employed to study the mobility, transport and bioavailability of metals in different types of environmental samples (soils, sediments), is the use of multi-step sequential extraction methods where the metals are distributed among the following fractions: exchangeable, carbonates-bound, Fe–Mn oxides-bound, organic matter-bound and residual. The more mobilizable metals correspond to the two first fractions, which can be released simply by increasing the ionic strength and by slight pH changes. The fractionation methods provide relevant information about the possible metal content that could be released on the environment.

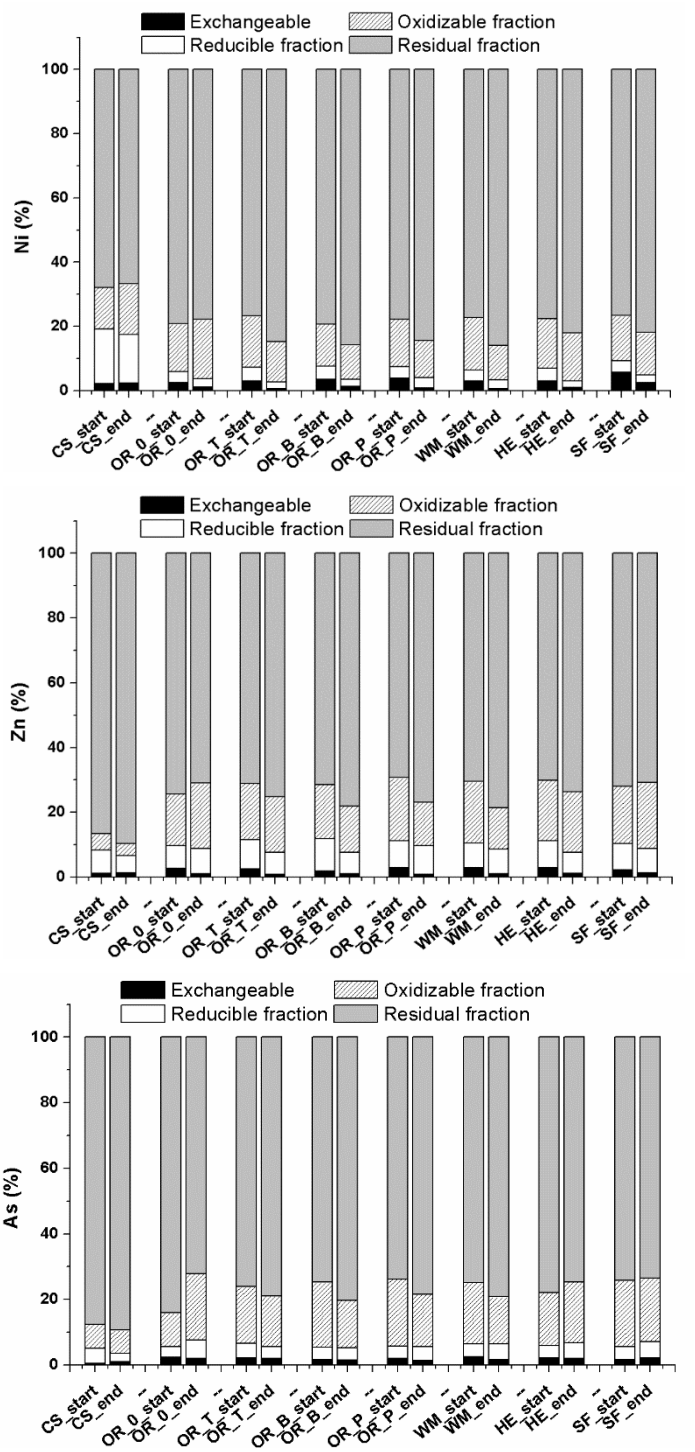
Based on the obtained results showed on the Figure 5.3 most of the present metal(oid)s are in non-available fraction (reducible and oxidizable). In general, available fraction of all metals in control sediment is higher than in contaminated sediment. In contaminated sediment the Cr, Cu, Pb, As and Ni bioavailable fraction are below 5%, and for Zn below 10%. Only in the case of Cd higher amount of the available fraction is observed, around 50%. This is in line with the content of the organic matter and clay in the soil. It is shown that this is two main regulating factor of metal bioavailability[29]. Also, chromium is found in a trivalent form, characterized by low solubility,

[29] Dubovina M., Krčmar D., Grba N., Watson M., Rađenović D., Tomašević D., Dalmacija B. (2018). *Environmental Pollution*, 236, 773-784.

reactivity, mobility, and thus toxicity to living organisms[30]. And cadmium is highly mobile, as it has similar physiochemical properties to essential micronutrients such as zinc, enabling it to be readily taken up by plants[31]. After the end of the pot experiments no significant changes in the metal distribution in different fraction has been observed. Except for Pb, where significant reduction has been obtained for the variant OR_0, OR_T, OR_B, HF and SF. Contrarily, in the variants with OR_P and WM increase of the Pb available fraction has been observed.



[30] Ahmad M. (2015), Journal of Genetic Engineering and Biotechnology 13 (1), 51-58.
[31] Coakley S., Cahill G., Enright A, O'Rurke B., Petti C. (2019), Sustainability, *11*(18), 5018.


Exchangeable
Oxidizable fraction
Reducible fraction
Residual fraction
Ni (%)
Zn (%)
As (%)
0
20
40
60
80
100
CS_start
CS_end
OR_0_start
OR_0_end
OR_T_start
OR_T_end
OR_B_start
OR_B_end
OR_P_start
OR_P_end
WM_start
WM_end
HE_start
HE_end
SF_start
SF_end

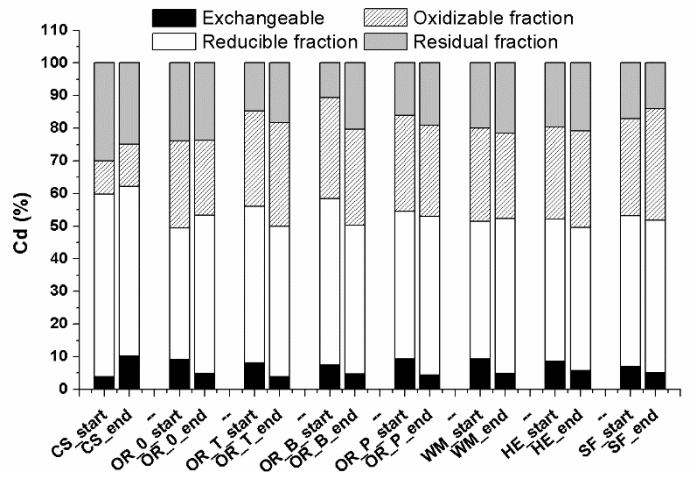
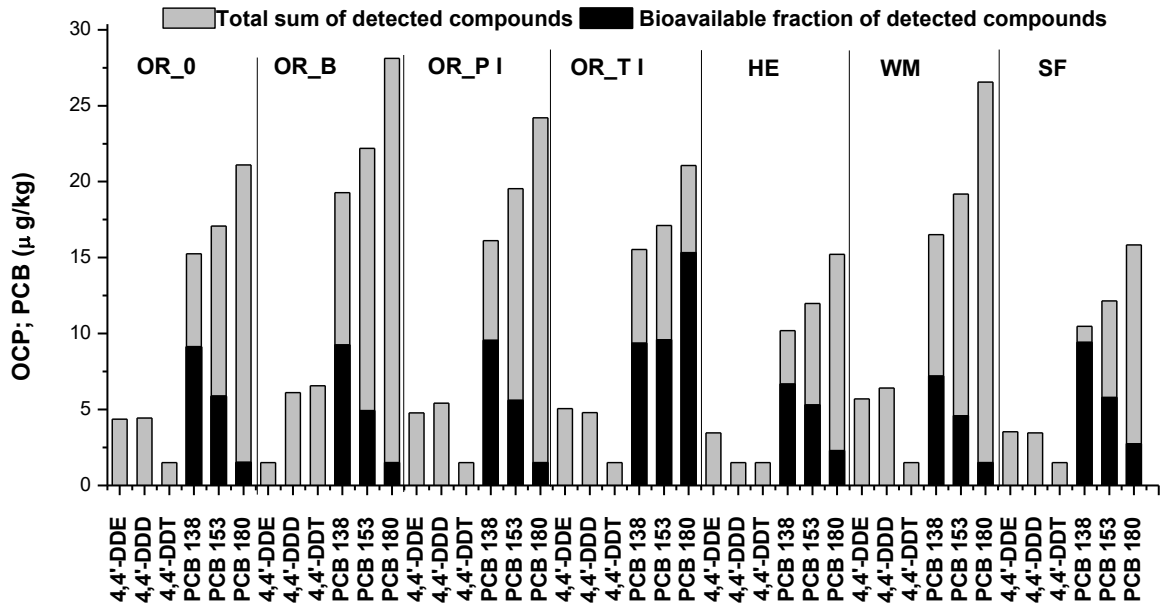


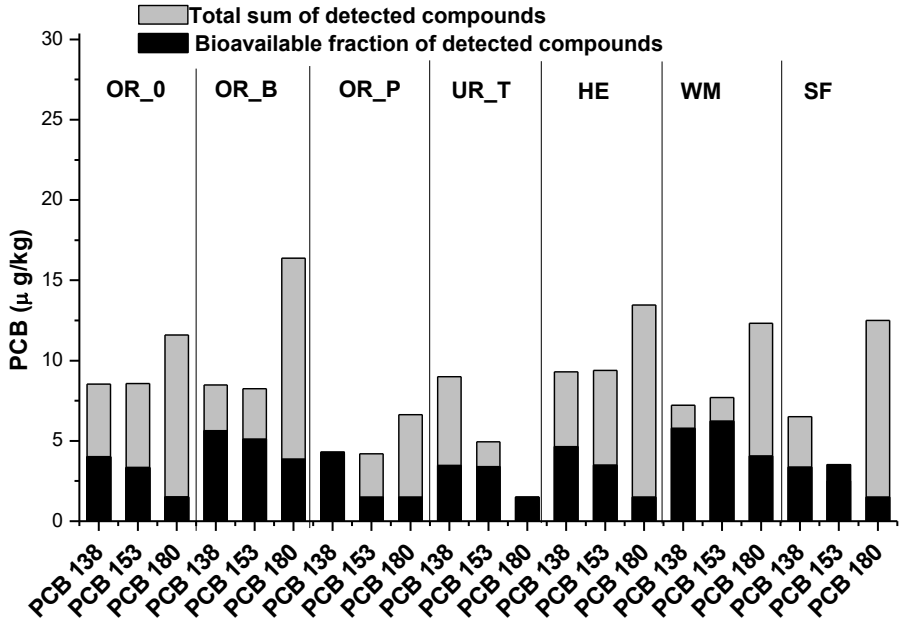
Figure 5.3 Results of the sequential metal(oid)s extraction - BCR

The quantitative results of the OCPs, PCBs, PAHs and TPH in soils analyses are presented in Figures 5.4 and expressed as total and bioavailable fractions at the start point and at the end of the experiments. ∑DDTs were the dominant OCPs and were in the range of 6.50 to 14.2 µg/kg at the start of the experiment. The highest ∑DDTs was observed for the pot experiment with OR_B where the detected concentration was about 14 µg/kg, while the lowest concentration was observed for pot with, HE. However, during the experiment, the concentration of DDTs decreased and was below the PQL in all samples at the end of the experiments.

Of the PCB congeners measured, the higher molecular weight (HMW) congeners were abundant and mostly dominated by hexa-, penta-PCB and in the range 38-70 µg/kg for both sampling seasons. This could be a consequence of the historical use of common technical PCB mixtures, such as Clophen A60 and Aroclors 1254 and 1268 since these commercial products mainly contain hexa-, hepta-PCB (PCB138, PCB153, and PCB180 as most abundant constituents). The highest concentration of PCBs was detected at the beginning of the experiments (37-70 µg/kg), while its concentration decreased by a factor of around two over the course of the experiment (ranged from 15 to 33 µg/kg). In both cases, the bioavailable fraction was in the range from 7 to 34 µg/kg and was higher in May at the beginning of the experiment. Taking into account all the interactions that are present in systems like this, including sorption onto the root systems of plants, additional PCBs diffusion into soil micropores and irreversible capture of hydrophobic PCBs, the phenomenon called aging effects could be the reason for the detected reduction in PCBs concentration. At the end of the experiment, the bioavailable fraction for ∑PCBs increased in the following order: UR_P < UR_T < UR_0 < SF < HE < UR_B < WM, indicating that the increase in the bioavailable fraction was not consistent with the ∑PCBs for the same period.



a)



b)

Figure 5.4. Total and bioavailable fraction of pesticides and PCBs at the a) start and b) end of the experiments

For all analyzed samples, TPHs were between 557 and 1273 mg/kg in May and decreased to 128 to 755 mg/kg in August (Figure 5.5). The median value of 337 mg/kg obtained at the end of the experiment was lower than the median value of 667 mg/kg obtained at the start, indicating a decrease of detected concentration over the 120 days. The lower TPHs could be a consequence of intensive sorption of TPHs on the root parts of the investigated plants. Additionally, leaching of organics in open-air pot experiments and exposure to different

weather conditions during the experiment could be a reason for the lower TPHs content detected.

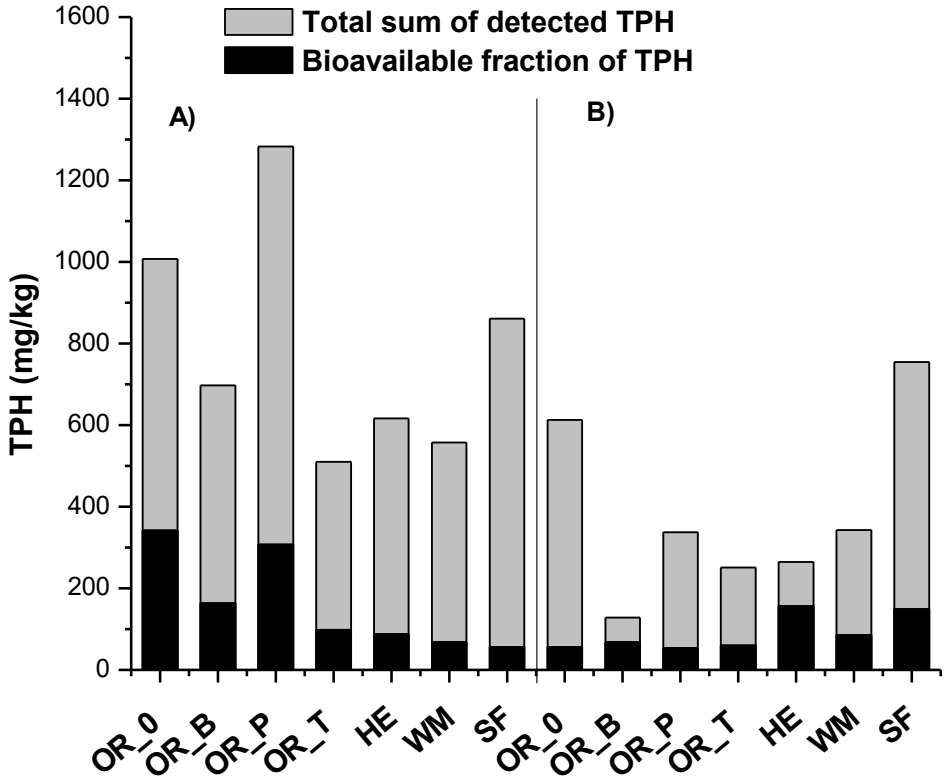
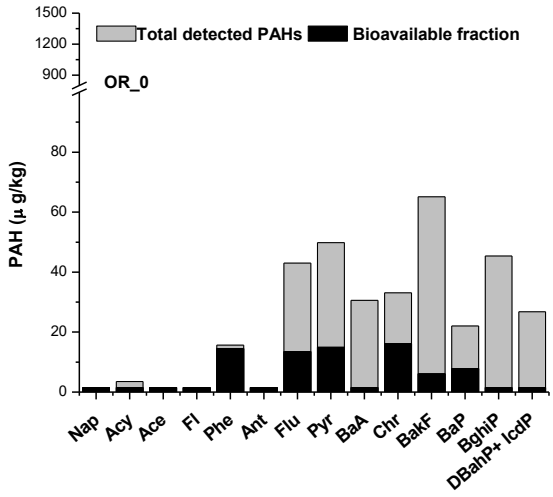
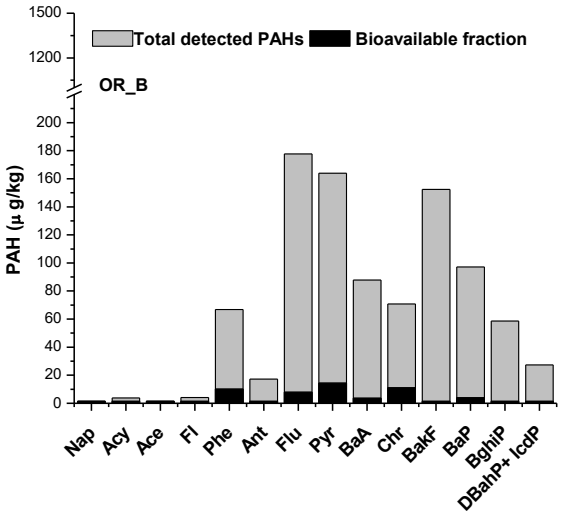
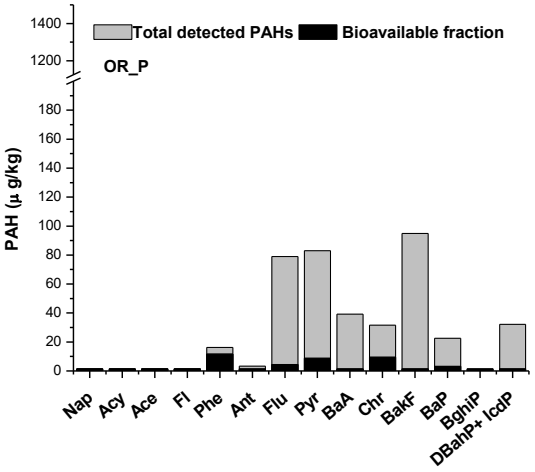


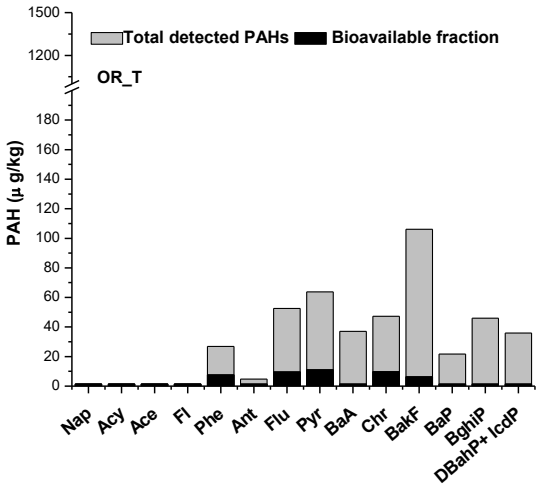
Figure 5.5. Total and bioavailable fraction of TPH at the A) start and B) end of the experiments

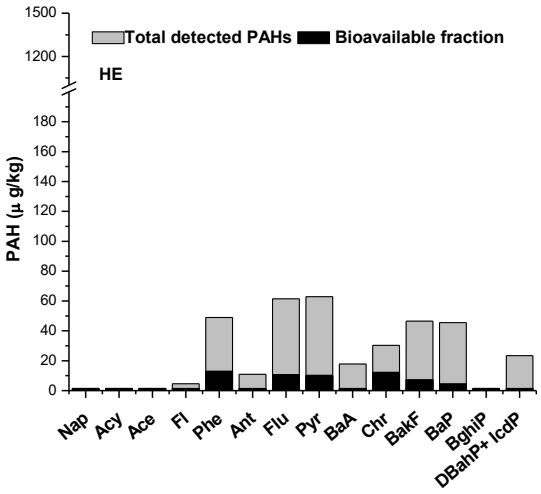
At the end of the pot experiment, the content of TPHs in the bioavailable fraction was investigated and generally increased in the following order OR_P < OR_0 < OR_T < OR_B < WM < SF < HE. The bioavailable fraction decreased during the experiment by a factor of two in comparison to the values obtained at the beginning. The Σ16 PAHs varied and ranged between 341 and 1395 µg/kg (mean: 633 µg/kg; median: 422 µg/kg) at the start (Figure 5.6) and decreased, ranging from 270 to 924 µg/kg (mean: 407 µg/kg; median 309 µg/kg) at the end of the experiment (Figure 5.7).

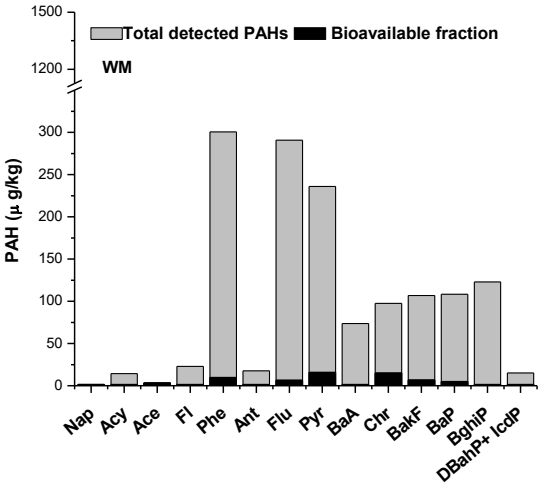

Total detected PAHs
Bioavailable fraction
OR_0
PAH (µ g/kg)
Nap
Acy
Ace
Fl
Phe
Ant
Flu
Pyr
BaA
Chr
BakF
BaP
BghiP
DBahP+ IcdP


Total detected PAHs
Bioavailable fraction
OR_B
PAH (µ g/kg)
Nap
Acy
Ace
Fl
Phe
Ant
Flu
Pyr
BaA
Chr
BakF
BaP
BghiP
DBahP+ IcdP


Total detected PAHs
Bioavailable fraction
OR_P
PAH (µ g/kg)
Nap
Acy
Ace
Fl
Phe
Ant
Flu
Pyr
BaA
Chr
BakF
BaP
BghiP
DBahP+ IcdP


Total detected PAHs
Bioavailable fraction
OR_T
PAH (µ g/kg)
Nap
Acy
Ace
Fl
Phe
Ant
Flu
Pyr
BaA
Chr
BakF
BaP
BghiP
DBahP+ IcdP


Total detected PAHs
Bioavailable fraction
HE
PAH (µ g/kg)
Nap
Acy
Ace
Fl
Phe
Ant
Flu
Pyr
BaA
Chr
BakF
BaP
BghiP
DBahP+ IcdP


Total detected PAHs
Bioavailable fraction
WM
PAH (µ g/kg)
Nap
Acy
Ace
Fl
Phe
Ant
Flu
Pyr
BaA
Chr
BakF
BaP
BghiP
DBahP+ IcdP

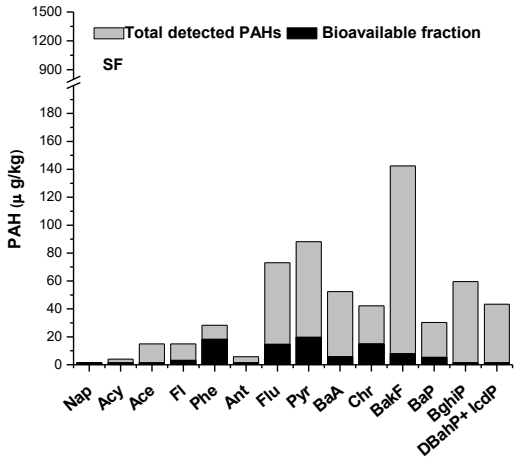
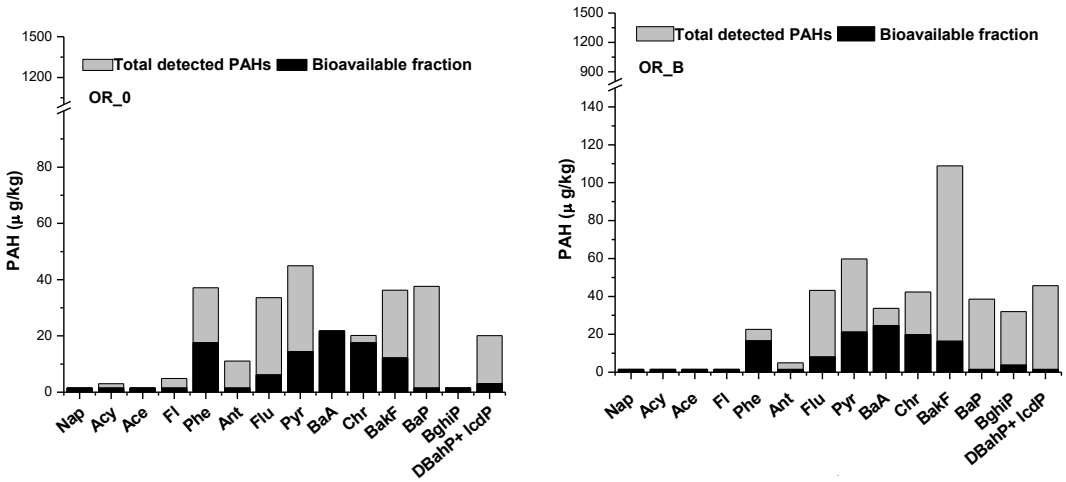


Figure 5.6. Total and bioavailable fraction of PAHs at the start of the experiments

Similarly, to the PCBs, for all analyzed samples during both sampling seasons, ∑16PAHs in soils was dominated by HMW PAHs (4, 5 and 6 rings) which were abundant and contributed on average from 74-95% of the ∑16PAHs. At the end of the experiment, ∑16PAHs decreased compared to those samples taken at the start. However, ∑16PAHs of the bioavailable fraction was in the range 44 to 89 µg/kg at the beginning of the experiment and slightly higher at the end of experiment 49-124 µg/kg, indicating that during the 120-day treatment and cultivation of the soil the bioavailable fraction of PAHs increased. The obtained values ∑16PAHs for the pot experiment at the end indicate that the bioavailable fractions increased in the following order OR_0 < OR_P < OR_T < HE < SF < OR_B < WM, whereby the greatest bioavailable fraction was obtained for WM and was about 124 µg/kg.



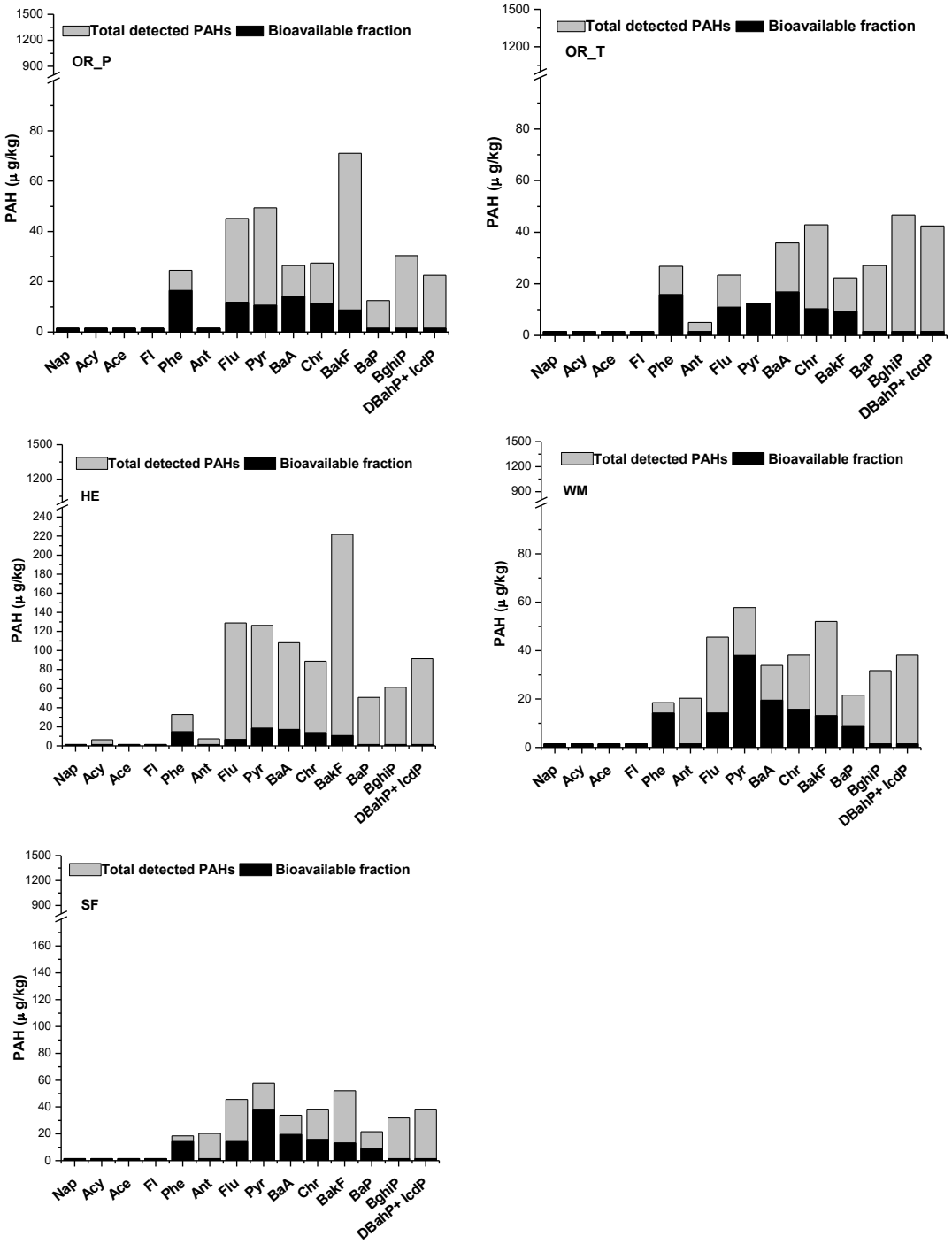


Figure 5.7. Total and bioavailable fraction of PAHs at the end of the experiments

It is assumed that the main mechanism accounting for these results is the sorption mechanisms of PAHs on the soil organic carbon and root systems of the cultivated plants, which are responsible for a decrease in PAHs concentrations in the soil at the beginning. Otherwise, sorption can be categorized as weak and reversible due to the formation of hydrogen bonds, hydrophobic and Van der Waals (VDW) interactions and as such could be a reason of the further increase in the bioavailable fraction during the experiment.

The microbiological characterization of the has been presented in the table 5.5. The most abundant was Amino-heterotrophs, and its abundance slightly decreased after the harvesting.

Generally, the abundance of analyzed microorganisms and dehydrogenase activity was similar on all treatments. Slight change after the harvest was observed.

Table 5.5 Microbiological characterization of the soil and sediment during the pot experiment

	Azotobacter spp. ×10	Amino-heterotrophs ×10^6	Total number of bacteria ×10^6	Free N-fixing bacteria ×10^5	Fungi ×10^3	Actino-bacteria × 10^3	Dehydrogenase activity
	Number of microorganisms (CFU/g soil)						mU/g soil
Before sowing							
HE	245.0	319.7	185.0	103.5	28.2	25.1	13.0
WM	223.4	257.2	251.3	129.7	39.4	34.1	13.6
SF	239.4	303.9	244.3	128.9	32.5	27.3	14.2
OR_0	179.3	182.3	182.1	93.80	21.8	19.3	12.9
OR_P	216.4	187.3	312.8	160.5	32.0	23.6	14.8
OR_T	226.8	279.4	334.2	208.3	24.8	31.2	13.8
OR_B	191.7	184.7	290.9	108.2	26.6	23.7	15.8
CS	81.20	204.0	264.4	289.9	29.1	25.0	15.5
After the harvest							
HE	179.4	133.2	160.1	194.5	28.6	29.9	9.70
WM	715.0	257.6	274.1	177.1	19.1	32.6	8.70
SF	166.2	298.1	401.0	329.2	21.8	56.5	10.8
OR_0	202.2	215.9	319.7	245.8	26.5	26.7	19.7
OR_P	182.6	166.0	269.2	149.2	26.5	20.7	19.9
OR_T	203.5	327.5	377.3	303.8	34.2	73.2	19.9
OR_B	212.9	236.7	447.9	322.3	42.2	50.5	16.9
CS	139.0	174.8	186.1	258.1	29.1	51.1	7.00

5.5.2 Energy crop samples

The biomass obtained for the tested plants is presented in the table 5.6. The below ground biomass of rapeseed in the contaminated sediment is marginally lower comparing to the control soil. Significantly lower below ground biomass has obtained for the rapeseed treated with Panorama bio plus. However, above ground rapeseed biomass is significantly higher for all rapeseed treatment in contaminated sediment compared to the control soil. The treatment with addition PGPR Trifender has the best performance from the all-rapeseed treatment in the respect of obtained biomass. White mustard shows the poor performance, low biomass yield, in comparison to the rapeseed treatment. Hemp and sunflower, however, have significantly higher biomass yield compared to the rapeseed treatments.

Table 5.6. Biomass obtained after pot test

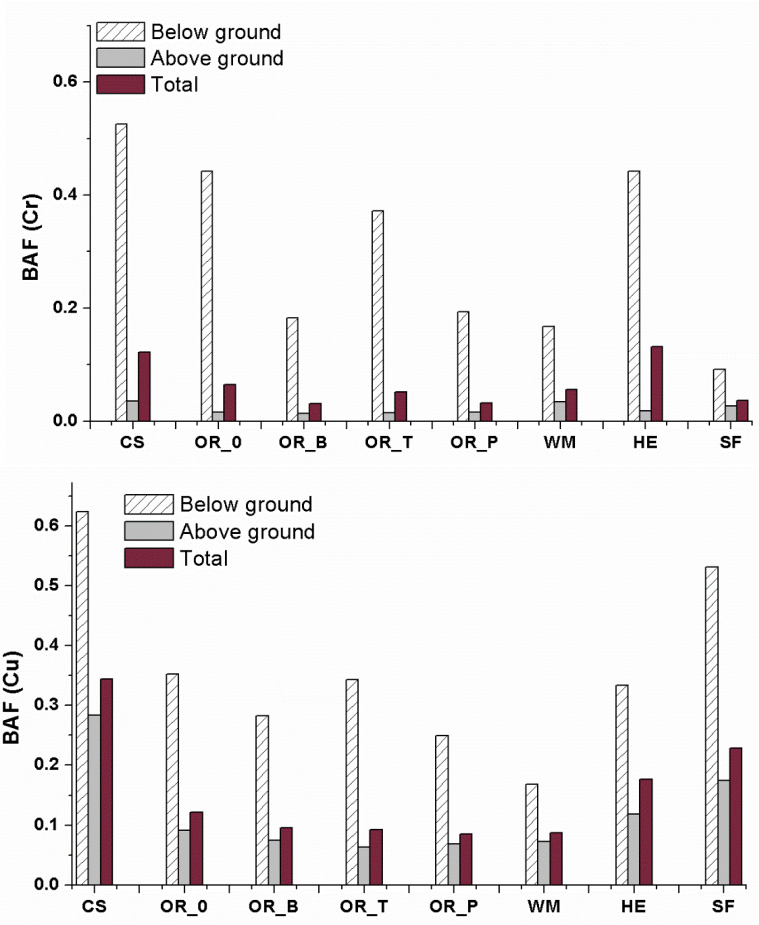
Pot test	Lebel	Below ground biomass (g)	Above ground biomass
Rapeseed- agricultural soil	CS	4.62±0.54	21.5±0.75
Rapeseed	OR_0	3.88±0.35	30.0±3.1
Rapeseed/BioEho	OR_B	3.31±0.87	29.2±4.3

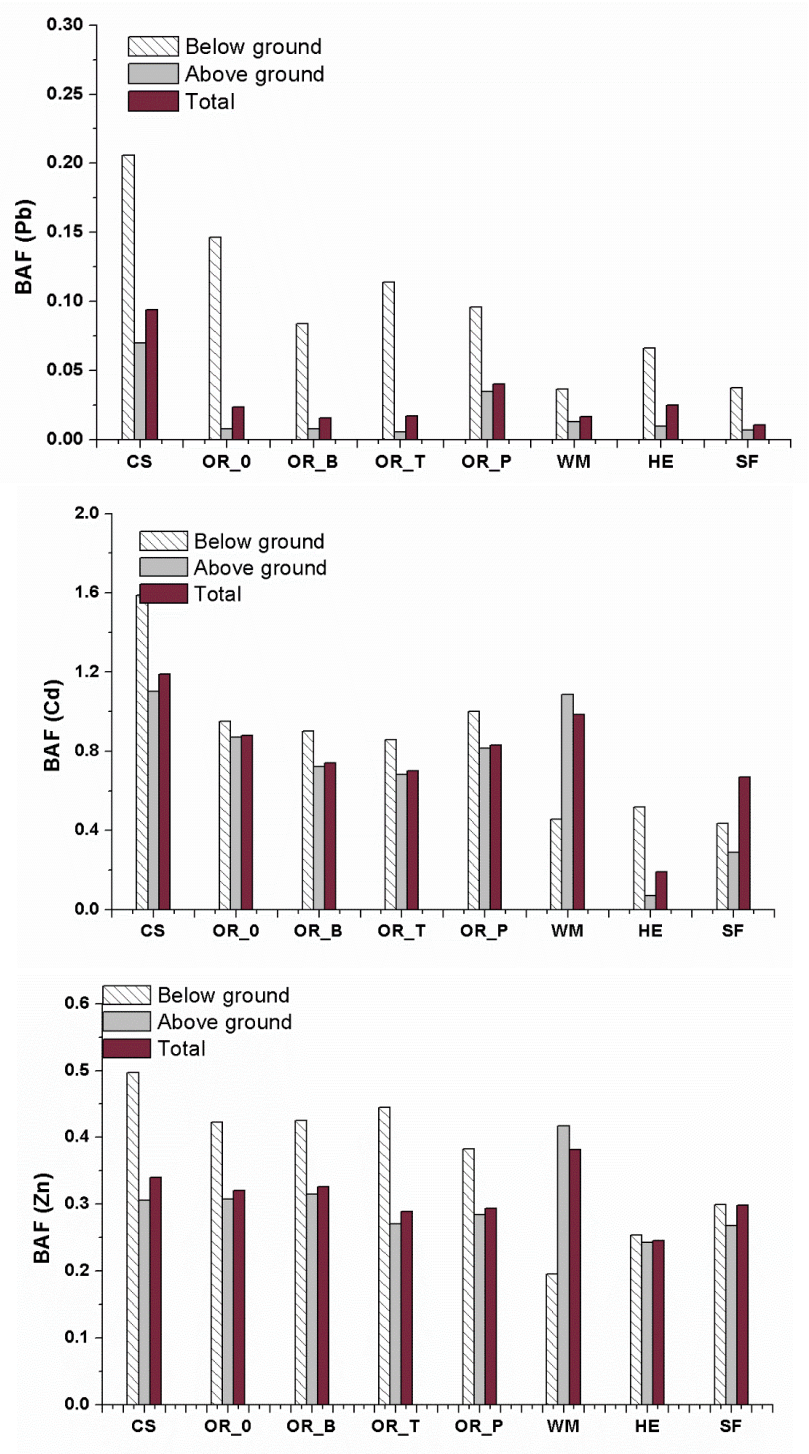
Table 5.6. Biomass obtained after pot test

Pot test	Lebel	Below ground biomass (g)	Above ground biomass
Rapeseed/Trifender	OR_T	4.62±1.21	39.8±0.56
Rapeseed/Panorama bio plus	OR_P	3.17±0.72	31.7±3.6
White mustard	WM	2.03±0.41	10.6±1.3
Hemp	HE	12.5±1.3	33.9±11.2
Sunflower	SF	8.96±0.75	59.8±2.0 (20.0 g of flower)

Bioaccumulation factor (BAF) for relevant toxic metal(oid)s is presented on the Figure 5.8, and translocation factor (TF) in table 5.7. Generally, BAF of the below ground biomass is significantly higher for all investigated metal(oid)s except in the case of Cd. For Cd BAF is approximately at the same level in above and below ground biomass which is in line with its high mobility explained above.

For all investigated treatments the rapeseed has highest BAF for all investigated metal(oid)s. And addition of PGPR didn't further increase BAF, except in the case of (1) chromium where highest BAF was observed for the OR_0, OR_T and HE; (2) copper where highest BAF was obtained for sunflower. The hemp showed the similar performance to the rapeseed regarding the BAF for most investigated metals. White mustard has lowest BAF for all investigated metals. His poor performance is in line with the lowest biomass obtained.




Below ground
Above ground
Total
BAF (Pb)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
CS
OR_0
OR_B
OR_T
OR_P
WM
HE
SF
Below ground
Above ground
Total
BAF (Cd)
2.0
1.6
1.2
0.8
0.4
0.0
CS
OR_0
OR_B
OR_T
OR_P
WM
HE
SF
Below ground
Above ground
Total
BAF (Zn)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CS
OR_0
OR_B
OR_T
OR_P
WM
HE
SF

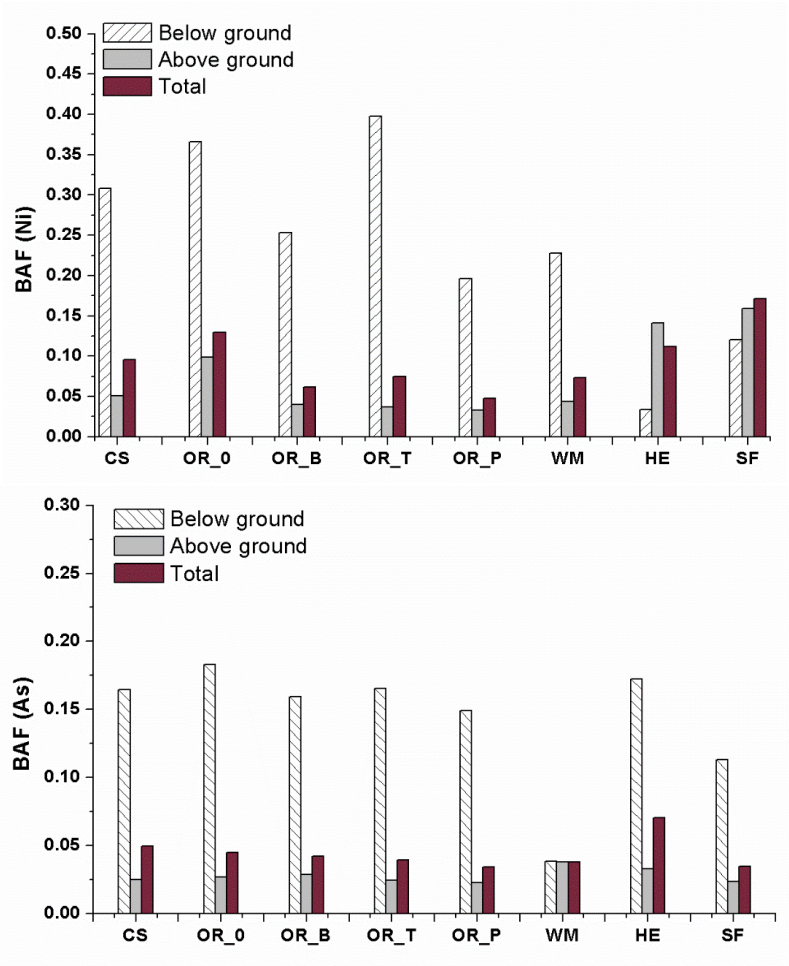


Figure 5.8. Bioaccumulation factor

The translocation factor is shown on the figure 5.7. The TF was <1 which indicate that the main mechanism of the metal(oid) removal is phytostabilisation and not phytoextraction[32]. TF above 1 was observed for the white mustard, but as was indicated above this plant generally accumulates low level of all metals. So seemingly higher TF is not of relevant importance. The lowest TF was observed from Cr, this is in line with its lowest mobility and consequently lowest potential for accumulation[33]. The low TF can be attributed also to the short grooving season in pot experiments (3 month). However, for pilot site remediation rapeseed is sown at the autumn (September) and will be harvested at summer (July). Therefore, longer contact of the plant with contaminated soil is expected to provide the highest translocation factor.

Table 5.7. Translocation factor

Pot	Cr	Ni	Cu	Zn	As	Cd	Pb
CS	0.068	0.16	0.46	0.62	0.15	0.69	0.34
OR_0	0.036	0.27	0.26	0.73	0.15	0.91	0.051
OR_B	0.076	0.16	0.26	0.74	0.18	0.80	0.095
OR_T	0.040	0.093	0.19	0.61	0.15	0.79	0.049

[32] Baker, A. J. M., (1981). Accumulators and excluders-strategies in the response of plants to heavy metals. Journal of Plant Nutrition, 3: 643-654.
[33] Ahmad M. (2015), Journal of Genetic Engineering and Biotechnology 13 (1), 51-58.

Table 5.7. Translocation factor

Pot	Cr	Ni	Cu	Zn	As	Cd	Pb
OR_P	0.081	0.17	0.27	0.74	0.15	0.81	0.36
WM	0.21	0.19	0.43	2.14	0.99	2.39	0.36
HE	0.040	4.18	0.35	0.96	0.19	0.14	0.15
SF	0.29	1.32	0.33	0.89	0.21	0.67	0.18

One of the main factors influencing heavy metals uptake by plants is metal bioavailability in soil. A lot of research has been conducted on how to increase metal uptake by *Brassica* plants by increasing its bioavailability. However, in most of the studies the increased bioavailability increased bioconcentration factor, but translocation factors, although increased, remained below or near to 1[34],[35],[36]. This phenomenon is particularly pronounced in multi-element contaminated soils due to limited capability of *Brassica* species to extract several metals simultaneously[9],[37],[38].

5.6 Conclusions

The degree of adsorption, i.e., accumulation of given pollutants within plant tissues is conditioned by a number of factors, the most important of which include availability of macro and micronutrients, organic matter and clay content, pH value (heavy metal uptake increases with decreasing soil pH) and availability of the present pollutants. The mechanisms of heavy metal uptake by plants are numerous and complex. The degree of accumulation of a metal in a plant is primarily determined by its physiological needs and not by the toxicity of the metal. That is, plants have relatively little selectivity when it comes to the uptake of elements and molecules.

Based on the obtained results main conclusions are:

- The conditions for plant grooving regarding the nutrient and water content was optimal.
- The content of the metal in the contaminated sediment has not changed significantly during the experiment.
- Bioavailable fraction of the metal(oid)s is very low, and most of the metal content is distributed in the oxidizable ad residual fractions (non-bioavailable fraction).
- The contaminated sediments didn't pose stress to the plant growth and obtained biomass.
- Rapeseed has, in general, best performance regarding the BAF, addition of PGPR Trifinder increase its biomass and BAF for Cr. Additionally, hemp has similar performance for the relevant metal(oid)s (As, Cr and Cu), and due to the higher biomass accumulate higher absolute amount of this metals.
- The TF was <1 which indicate that the main mechanism of the metal(oid) removal is phytostabilisation and not phytoextraction.

[34] Diarra, I.; Kotra, K.K.; Prasad, S. (2021) *Chemosphere*, *273*, 128483.
[35] Guo, D.; Ali, A.; Ren, C.; Du, J.; Li, R.; Lahori, A.H.; Xiao, R.; Zhang, Z.; Zhang, Z. (2019) *Ecotoxicol. Environ. Safe. 167*, 396–403.
[36] Bouquet, D.; Braud, A.; Lebeau, T (2017). Int. J. Phytoremediation, 19(5), 425–430.
[37] Gurajala, H.K.; Cao, X.; Tang, L.; Ramesh, T.M.; Lu, M; Yang, X. (2019). *Environ. Pollut. 254*, 113085.
[38] Mourato, M.P.; Moreira, I.N.; Leitão, I.; Pinto, F.R.; Sales, J.R.; Martins, L.L. (2015). *Int. J. Mol. Sci.*, *16(8)*, 17975–17998.

Further research should be focused on:

- Increasing bioavailability of metals by soil acidification by addition acid fertilisers, addition chelating agents for increasing the mobility of metals, addition of amendments for changing oxidoreduction state of the metals or similar.
- Testing different plants (i.e., Ricinus) which could potentially have higher accumulation potential, but same or higher biomass production as rapeseed.

6. LITHUANIAN SITE POT TRIALS EXERIMENTAL PLAN

6.1 Objectives

The main objective of the pot trial was to evaluate the potential to degrade petroleum hydrocarbons and other organics substances in contaminated soil using specially for this purpose designed combination of plants, biological additives, and nutrients.

It was planned to use three mixes of herbaceous plants in the pot trial, where a single mix is comprised of at least four different plant species. During the pot trial it was aimed to determine which mixes or even species are more suitable for PAH degradation, so that in the upcoming field trial only one mix with the highest degradation potential would be used.

6.2 Materials and methods

6.2.1 Materials

Contaminated soil was brought from a site in Šiauliai (Mid-North-Lithuania). The site is contaminated with TPH and other organics substances. The site was divided into three subplots based on the contamination depth. Subplot with the shallowest contamination (0-40 cm) was intended for Jerusalem artichoke; subplot with a deeper laying contamination (0-60 cm) was intended for amaranth; subplot with the deepest contamination (up to 100 cm) – for herbaceous plants. Such subdivision applies both for the pot experiment and the upcoming field trial.

Control (clean) soil, identified as a sandy loam, was taken from an arable land, and mixed with sand to resemble granulometric composition of the contaminated soil.

Biological additive is an industrially prepared powdered biological material consisting of a carefully selected blend of natural micro-organisms that can degrade all main classes of compounds in oil fractions. This biological supplement contains: *Bacillus subtilis 001* (<0.1%), *Bacillus subtilis 009* (<0.1%), *Bacillus licheniformis 002* (<0.1%), *Bacillus amyloliquefaciens 001* (<0.1%), *Pseudomonas putida 002* (<0.1%), *Pseudomonas putida 004* (<0.1%), *Pseudomonas fluorescens 002* (<0.1%), *Pseudomonas fluorescens 003* (<0.1%), *Pseudomonas fluorescens 005* (<0.1%), *Pseudomonas fluorescens 007* (<0.1%), *Rhodococcus rhodochrous 002* (<0.1%), wheat bran filler (<20.0%), distilled dried corn filler (to 100%).

Vermicast - is localy produced end-product of the breakdown of organic matter by earthworms by worm farm “Sliekynė.lt”. This vermicast contains: 45.23% dry matter, 34.42% organic matter and levels of NPK 1.55%-1.48%-2.13%.

Mineral fertilizers - agriculturally conventional amounts of macronutrients deriving from urea (N-46,2%), ammonium sulphate (N-21%; S24%), NPK(S) 15%-15%-15% (S11.5%), and KCl (K60%).

Tap water.

6.2.2 Methods

Collection and preparation of contaminated soil. Contaminated soil was collected from three subplots which exhibited different contamination patterns. The soil was pooled from at least three pits in every subplot. The pits were dug with mini excavator which allowed to reach depth of 1 meter. The soil was collected from varying depths at every pit. The soil was not mixed between different subplots. Large stones and roots were discarded, and the soil was sieved using mesh with 1.5x1.5 cm eyes. The soil was placed into plastic boxes and taken to the greenhouse facilities, where it was again thoroughly homogenized and portioned back into the boxes. Each box contained 60 kg of fresh (weight) soil.

Collection and preparation of clean soil. Clean soil was collected from an arable field near the greenhouse facilities. It was also sieved and portioned into the boxes. Each box contained 60 kg of fresh (weight) soil.

Application of nutrients. Aerated and loose vermicast (1.25 kg (DM)) + fertilizers were used by following directions:

- For herbaceous plant mixes: at 15cm depth top layer vermicast mixed with contaminated soil and NPKS fertilizers targeting to 5 t/ha of dry total biomass. According to agronomical calculations and previous studies, NPK (mg/kg) needs for 1 t of herbacious plant biomass NPK+S is 27,5 - 4 -25 + 3,06S. Additionally added 4 mg/kg of N for microbial activity.
  Total: NPK+S: 137.5-20-125 + 15,3S.
- For Jerusalem artichoke: vermicast to 15 cm depth top layer mixed with contaminated soil and NPKS fertilizers, targeting to 25 t/ha of dry total biomass. According to agronomical calculations and previous studies, NPK (mg/kg) needs for 1 t of Helianthus tuberosus biomass NPK+S is 4-1,5-7 + 1,15S. Additionally added 4 mg/kg of N for microbial activity.
  Total: NPK+S: 140-37.5-175 + 28,8S.
- For amaranth: vermicast to 15 cm depth top layer mixed with contaminated soil and NPKS fertilizers targeting to 15 t/ha of dry biomass. According to agronomical calculations and previous studies, NPK (mg/kg) needs for 1 t of A.Caudatus biomass NPK+S is 4-4-4 + 6,1S. Additionally added 4 mg/kg of N for microbial activity.
  Total: NPK+S: 100-60-60 + 91,6 S.

Weight of the soil was adjusted back to 60 kg after the addition of vermicast + fertilizers. Nutrients were used only in the experimental pots.

Application of biological additive. The additive was added to lukewarm water (ratio 1w:10w) and left for 1 hour to revive bacteria, then the slurry was poured onto contaminated soil (ratio 200 g of the additive to 1 t soil (DM)) surface and gently mixed into the soil to about 1 cm depth. The soil prior the addition of this biological additive was already homogenized, portioned and vermicast + nutrients were mixed in as well. Biological additive was used only in the experimental pots. Application of biological additive was followed 48 hours' rest period.

Seeding and sowing. Prepared and portioned soil was left in the greenhouse for 48 hours to accommodate the surrounding temperature and moisture. Then plants were sowed and seeded.

Four tubers of Jerusalem artichoke were planted in every pot. The tubers were about 5 cm x 3 cm large and weighted about 10-20 g each. There were three pots with Jerusalem artichoke in contaminated soil, and three pots with Jerusalem artichoke in control soil.

For amaranth, 226 seeds were seeded in every pot in triplicates for contaminated and for control soil.

For herbaceous plants, there were three different mixtures comprised of different species and proportions (Table 6.1). Each mix was sowed in triplicates in the pots with contaminated soil and with control soil.

Table 6.1 Names of the herbaceous plant species and proportions of it in the mixes

Mix	Species name (*in Latin*)	Percentage (%)
I	Tall fescue (*Festuca arundinacea)*	35
	Perennial ryegrass (*Lolium perenne)*	30
	Reed canary grass (*Phalaris arundinacea)*	10
	Red clover (*Trifolium pratense)*	25
II	Annual ryegrass (*Lolium multiflorum subsp. Italicum)*	10
	Meadow fescue (*Festuca pratensis)*	35
	Meadow foxtail (*Alopecurus pratensis)*	10
	Alfalfa (*Medicago sativa)*	25
	Common bird's-foot trefoil (*Lotus corniculatus)*	20
III	Festulolium (*Festulolium)*	20
	Tall fescue (*Festuca arundinacea)*	15
	Common bent (*Agrostis capillaris)*	10
	White clover (*Trifolium repens)*	20
	Honey clover (*Melilotus)*	35

6.2.3 Description of the set-up

55 L with the dimensions (Width x height x length) of 400 x 350 x 600 mm, internal dimensions (Width x height x length) are 345 x 350 x 500 mm. Surface area of soil in one box was 0.1725 m^2.

Pots in the greenhouse chamber were placed in a random order. Area of the chamber was 40 m^2 (8 m x 5 m) and 5 m (max) high. Temperature regime in the chamber was always maintained at 25° ± 5°. Air humidity was not controlled. Plants were watered on daily basis.

In total, there were 33 pots: 3 pots with amaranth in contaminated soil, and 3 pots with amaranth in control soil; 3 pots with Jerusalem artichoke in contaminated soil, and 3 pots with Jerusalem artichoke in control soil; 3 pots with herbaceous Mix I in contaminated soil, and 3 pots with herbaceous Mix I in control soil; 3 pots with herbaceous Mix II in contaminated soil, and 3 pots with herbaceous Mix II in control soil; 3 pots with herbaceous Mix III in contaminated soil, and 3 pots with herbaceous Mix III in control soil; 3 pots with contaminated soil (used for the trial with herbaceous plants only without any pre-treatments) without any plants (blank).

The pot experiment started on the 30th of April 2021.

6.3 Sampling campaign

6.3.1 Soil samples

Soil samples were collected on two occasions.

At the start, the soil samples were taken after homogenization, prior portioning it into the pots. Joint samples were pooled from different sides and depths of the pile of the homogenized soil for every experimental group (3 samples). Clean soil sample was collected following the same technique as well (1 sample).

Another sampling campaign was carried out right after harvest of the plants. The samples were taken from every pot at the varying depth from 5 to 30 cm. Blank soil samples from the pots that were left without plants, were taken as well.

In all cases, it was attempted to collect about 1 kg of fresh (weight) soil to ensure it is sufficient for all analysis. Soil samples were kept in freezer until further analysis, except the samples that were analyzed for microbial activity. This analysis was carried out within 2 days after sampling.

Soil samples were analyzed for:

- pH, electrical conductivity;
- macronutrients: P, K, total N, total C;
- Organic matter, microbial biomass;
- Contaminants: hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), perchloroethylenes (PCE).

6.3.2 Energy crop samples

Herbaceous plants were harvested, and samples collected on three occasions (1st cut - 14th of July 2nd cut - 25th of August 3rd cut - 27th of September (2021)). When harvesting, all above ground biomass was cut down with scissors and the fresh weight was recorded immediately. Then the biomass was brought to the lab where it was dried at room temperature to achieve dry weight.

During the first harvest in mid-July, herbaceous plant samples for morphometric analysis were collected as well. One plant from every species (see Table 6.1) was removed from all pots. Immediately, fresh weight of roots and aboveground part, plant height and root length were measured. Dry weight of roots and aboveground parts were determined later.

Morphometric analysis was not carried out during the following harvests because roots of the herbaceous plants developed and became entangled, thus an attempt to remove any plants might have caused too much damage to the entire root system. In addition, it would have been difficult to remove plants containing the full root length, so the morphometric analysis would have been inaccurate.

Amaranth was harvested on the 23rd of July. Aboveground biomass was cut with scissors and fresh weight of the plants recorded. The biomass was dried at room temperature to achieve the dry weight. Plant height was measured as well. Roots were left in the soil and, unexpectedly, started to sprout small stems with leaves.

Jerusalem artichoke was harvested on the 27th of September. As usual, fresh biomass was recorded. Plant height, number of stems per one tuber planted at the start of experiment, number

and weight of tubers were counted. Both aboveground parts and tubers were dried at room temperature to achieve the dry weight.

Note, that no chemical analysis was performed with the biomass, as organic contaminants typically do not accumulate in biomass.

6.4 Irrigation regime

Tap water was used to water the plants. Due to extremely hot summer in 2021, plants were watered on daily basis, most often in early morning. The volume of water used for every pot was not recorded, but the volume of water increased as the plants developed.

6.5 Monitoring plan

6.5.1 Soil characterization

Soil was not monitored or characterized in any means during the pot experiment. Soil sampling was done prior sowing/seeding and after the harvest as described above.

6.5.2 Energy crop characterization

Crops during the pot experiment were closely monitored. The evaluated parameters were the following: the percentage of germinated plants, which was assessed 5 times during the first 6 weeks; then the soil cover with plants, the plant density and the luxuriant of plants – the latter parameters were assessed every 10 days for 15 weeks.

The plants were inspected every day for pest and disease control.

6.5.2.1 Energy crop monitoring results

Germination rate was calculated as shown in the equation (1), and the results are presented in Table 6.2.

$$Germination = \frac{number\ of\ germinated\ seeds}{total\ number\ of\ seeds} \times 100 \qquad (1)$$

Germination of the herbaceous plant seeds in all cases was weaker in the contaminated soil in comparison to the seeds in the clean soil. Herbaceous plant Mix II had the highest germination rate both for the contaminated and the clean soil. While Mix III had the lowest germination rate, where some species, like honey clover and white clover did not germinate at all.

For Jerusalem artichoke, 4 tubers were planted in each pot, and all four tubers sprouted in all pots.

Germination rate for amaranth seeds in the contaminated soil was same to the one in the clean soil. The differences were insignificant.

Table 6.2 Average percentage (%) of germinated plants ± standard deviation (n=3)

Plants/soil	2021 05 21	2021 05 31	2021 06 04	2021 06 08	2021 06 11
Mix I, contaminated	16.4 ± 6.9	51.6 ± 24.2	44.2 ± 8.0	43.1 ± 8.6	44.3 ± 7.3
Mix I, clean	28.8 ± 7.4	59.1 ± 11.6	68.0 ± 2.0	66.4 ± 3.0	68.4 ± 3.6
Mix II, contaminated	26.0 ± 10.7	46.3 ± 0.9	58.6 ± 1.4	58.8 ± 1.2	59.5 ± 0.2
Mix II, clean	27.1 ± 5.0	38.3 ± 8.9	56.5 ± 3.6	56.8 ± 2.9	61.7 ± 6.4
Mix III, contaminated	4.9 ± 0.8	7.5 ± 4.3	10.9 ± 3.4	11.9 ± 2.4	14.3 ± 1.3
Mix III, clean	13.7 ± 5.0	18.8 ± 0.5	20.9 ± 1.5	21.0 ± 1.6	21.8 ± 0.7
J. artichoke, contam.	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
J. artichoke, clean	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Amaranth, contam.	23.4 ± 3.8	43.6 ± 8.3	36.6 ± 8.3	37.1 ± 8.1	42.9 ± 0.8
Amaranth, clean	21.4 ± 1.0	35.6 ± 23.9	40.1 ± 5.8	42.4 ± 7.7	46.6 ± 4.7

Soil cover with plants was assessed every 10 days until the soil in the pots was fully covered. The assessment was as follow: 20% - covered no more than 1/20 soil; 40% - covered more than 1/20 soil; 60% - covered from ¼ to ½ soil; 80% - covered from ½ to ¾ soil; 100% - covered more than ¾ soil. The results are given in Table 6.3.

All trials reached >90% soil cover by plants in the first 9 weeks, until the 1st of July 2021, except Mix III. This can be explained by lower germination rate and by the fact that 2 species in the mix did not germinate at all. For herbaceous mixes a decrease in soil cover can be noticed on the 22nd of July and the 2nd of September which was due to herbaceous plant harvests.

Development and soil cover with Jerusalem artichoke and amaranth was very similar for the plants grown on contaminated and on the clean soil.

Table 6.3 Average percentage (%) of soil cover by plants ± standard deviation (n=3)

Plants/soil	2021 05 19	2021 05 25	2021 06 10	2021 06 21	2021 07 01	2021 07 12	2021 07 22
Mix I, contam.	21.7 ± 2.9	23.3 ± 2.9	45.0 ± 5.0	88.3 ± 7.6	96.0 ± 1.7	98.3 ± 1.2	60.0 ± 15.0
Mix I, clean	58.3 ± 7.6	61.7 ± 10.4	75.0 ± 13.2	91.7 ± 5.8	97.0 ± 1.7	99.0 ± 1.7	81.7 ± 10.4
Mix II, contam.	30.0 ± 0.0	31.7 ± 2.9	51.7 ± 7.6	81.7 ± 7.6	93.3 ± 2.9	96.7 ± 1.5	53.3 ± 5.8
Mix II, clean	60.0 ± 10.0	65.0 ± 15.0	71.7 ± 2.9	80.7 ± 6.0	92.3 ± 10.0	96.3 ± 2.3	86.7 ± 7.6
Mix III, contam.	25.0 ± 5.0	25.0 ± 5.0	43.3 ± 10.4	58.3 ± 10.4	76.7 ± 16.1	84.7 ± 14.5	51.7 ± 16.1
Mix III, clean	63.3 ± 5.8	65.0 ± 8.7	83.3 ± 5.8	93.3 ± 2.9	97.7 ± 2.5	99.3 ± 1.2	90.0 ± 0.0
J. artichoke, contam.	40.0 ± 10.0	50.0 ± 10.0	73.3 ± 5.8	88.3 ± 7.6	95.0 ± 0.0	95.7 ± 1.2	100.0 ± 0.0

Table 6.3 Average percentage (%) of soil cover by plants ± standard deviation (n=3)

Plants/soil	2021 05 19	2021 05 25	2021 06 10	2021 06 21	2021 07 01	2021 07 12	2021 07 22
J. artichoke, clean	53.3 ± 20.8	63.3 ± 20.8	70.0 ± 10.0	86.7 ± 7.6	95.0 ± 0.0	96.3 ± 1.2	100.0 ± 0.0
Amaranth, contam.	21.7 ± 2.9	21.7 ± 2.9	91.7 ± 2.9	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Amaranth, clean	33.3 ± 5.8	33.3 ± 5.8	81.7 ± 2.9	88.3 ± 2.9	99.3 ± 1.2	99.3 ± 1.2	100.0 ± 0.0

Plants/soil	2021 08 02	2021 08 12	2021 08 23	2021 09 02	2021 09 12	2021 09 23
Mix I, contam.	83.3 ± 5.8	88.3 ± 7.6	95.0 ± 5.0	85.0 ± 5.0	93.3 ± 2.9	94.7 ± 4.0
Mix I, clean	93.3 ± 11.5	98.3 ± 2.9	100.0 ± 0.0	95.0 ± 0.0	98.3 ± 2.9	97.7 ± 2.5
Mix II, contam.	80.0 ± 0.0	88.3 ± 5.8	94.0 ± 3.6	71.7 ± 17.6	80.0 ± 10.0	84.3 ± 9.0
Mix II, clean	93.3 ± 5.8	97.3 ± 2.5	100.0 ± 0.0	95.0 ± 0.0	98.3 ± 2.9	98.3 ± 2.9
Mix III, contam.	71.7 ± 14.4	76.7 ± 15.3	88.3 ± 10.4	70.0 ± 15.0	81.7 ± 12.6	84.3 ± 14.0
Mix III, clean	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	95.0 ± 5.0	100.0 ± 0.0	99.3 ± 1.2
J. artichoke, contam.	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	98.3 ± 2.9	93.3 ± 2.9
J. artichoke, clean	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	98.3 ± 2.9	98.3 ± 2.9
Amaranth, contam.	43.3 ± 2.9	48.3 ± 2.9	63.3 ± 11.5	70.0 ± 13.2	75.0 ± 13.2	61.7 ± 18.9
Amaranth, clean	45.0 ± 0.0	53.3 ± 5.8	61.7 ± 2.9	66.7 ± 2.9	66.7 ± 2.9	58.3 ± 2.9

Plant density was assessed every 10 days and was evaluated by points: 1 point – very rare; 3 - rare; 5 – medium; 7 – dense; 9 – very dense. The results are given in Table 6.4.

Plant density is closely related to the soil cover by plants. Herbaceous mixes in all cases reached the state of a "very dense" in the first two weeks of July, which was the same time when soil cover by plants reached >90%. Cutting down herbaceous plants reduced the plant density accordingly.

Density of Jerusalem artichoke was the highest in July (7-8), and then began to drop as the plants started to transfer more energy and nutrients to tubers rather than aboveground part.

Amaranth grown on the contaminated soil showed a slightly higher density than grown on the clean soil. In both cases it was point 8-9. It can be noticed that the density dropped to 4-5 in August and did not recover. This occurred after the amaranth was harvested, and, unexpectedly, started to grow young sprouts.

Table 6.4 Average plant density (in points) ± standard deviation (n=3)

Plants/soil	2021 05 21	2021 05 31	2021 06 10	2021 06 21	2021 07 01	2021 07 12	2021 07 22
Mix I, contam.	4 ± 1	4 ± 1	5 ± 1	6 ± 1	8 ± 1	8 ± 1	6 ± 1
Mix I, clean	7 ± 1	7 ± 1	7 ± 1	8 ± 1	8 ± 0	8 ± 0	7 ± 1
Mix II, contam.	5 ± 1	5 ± 1	6 ± 1	7 ± 1	8 ± 0	8 ± 0	5 ± 1
Mix II, clean	8 ± 0	8 ± 1	8 ± 1	8 ± 1	8 ± 1	8 ± 1	7 ± 0
Mix III, contam.	3 ± 0	3 ± 0	3 ± 1	6 ± 1	6 ± 1	8 ± 1	4 ± 1
Mix III, clean	8 ± 1	8 ± 1	8 ± 1	9 ± 0	9 ± 0	9 ± 0	8 ± 1
J. artichoke, contam.	5 ± 0	5 ± 1	6 ± 1	6 ± 1	7 ± 1	7 ± 1	7 ± 1
J. artichoke, clean	7 ± 2	6 ± 1	6 ± 1	6 ± 1	7 ± 1	8 ± 1	8 ± 1
Amaranth, contam.	4 ± 1	5 ± 2	7 ± 1	8 ± 1	8 ± 0	8 ± 0	9 ± 0
Amaranth, clean	4 ± 1	4 ± 1	6 ± 1	7 ± 1	8 ± 1	8 ± 1	8 ± 0

Plants/soil	2021 08 02	2021 08 12	2021 08 23	2021 09 02	2021 09 12	2021 09 23
Mix I, contam.	6 ± 1	6 ± 1	7 ± 1	7 ± 1	7 ± 1	7 ± 0
Mix I, clean	6 ± 1	7 ± 1	7 ± 1	8 ± 0	8 ± 0	8 ± 0
Mix II, contam.	5 ± 0	6 ± 0	6 ± 0	5 ± 1	6 ± 1	6 ± 1
Mix II, clean	7 ± 0	7 ± 0	7 ± 0	8 ± 1	8 ± 0	8 ± 0
Mix III, contam.	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1
Mix III, clean	7 ± 0	8 ± 0	8 ± 0	8 ± 1	8 ± 1	8 ± 1
J. artichoke, contam.	7 ± 1	5 ± 1	6 ± 1	6 ± 1	5 ± 1	5 ± 1
J. artichoke, clean	8 ± 1	7 ± 0	7 ± 0	7 ± 0	7 ± 0	7 ± 0
Amaranth, contam.	9 ± 0	4 ± 1	4 ± 1	4 ± 1	4 ± 1	4 ± 1
Amaranth, clean	8 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0	5 ± 0

Luxuriant (lushness) of the plants was also assessed every 10 days. It was evaluated by points as follow: 1 point - plants are very small, 3 points - plants are small, 5 points - plants of medium luxuriant, 7 points - plants are luxuriant, 9 points - plants are very luxuriant. The results are presented in Table 6.5.

For herbaceous plants the luxuriant was the highest (8) before the first harvest. After that it was only about 6-7. The luxuriant for plants grown on the contaminated soil and on the clean one did not differ, meaning that contaminants in the soil did not affect plant development. In addition,

Jerusalem artichoke and, especially, amaranth grew more luxuriant in the contaminated than in the clean one.

Table 6.5 Average luxuriant (in points) of the plants ± standard deviation (n=3

Plants/soil	2021 05 21	2021 05 31	2021 06 10	2021 06 21	2021 07 01	2021 07 12	2021 07 22
Mix I, contam.	4 ± 1	4 ± 1	5 ± 2	7 ± 0	8 ± 0	8 ± 0	6 ± 0
Mix I, clean	6 ± 0	6 ± 1	7 ± 1	7 ± 0	8 ± 0	8 ± 0	5 ± 0
Mix II, contam.	3 ± 0	4 ± 1	5 ± 1	8 ± 1	8 ± 1	8 ± 1	5 ± 0
Mix II, clean	5 ± 1	7 ± 1	6 ± 1	7 ± 0	7 ± 0	8 ± 1	5 ± 1
Mix III, contam.	3 ± 0	4 ± 0	4 ± 0	8 ± 0	8 ± 0	8 ± 1	6 ± 1
Mix III, clean	6 ± 1	6 ± 1	6 ± 1	7 ± 0	8 ± 1	8 ± 1	5 ± 0
J. artichoke, contam.	9 ± 0	8 ± 1	8 ± 0	8 ± 0	9 ± 0	9 ± 0	9 ± 0
J. artichoke, clean	7 ± 0	6 ± 1	7 ± 0	7 ± 0	8 ± 1	8 ± 0	8 ± 0
Amaranth, contam.	5 ± 0	6 ± 1	8 ± 1	9 ± 0	9 ± 0	9 ± 0	9 ± 0
Amaranth, clean	6 ± 1	6 ± 1	7 ± 1	7 ± 1	7 ± 0	7 ± 0	7 ± 0

Plants/soil	2021 08 02	2021 08 12	2021 08 23	2021 09 02	2021 09 12	2021 09 23
Mix I, contam.	5 ± 0	7 ± 0	7 ± 0	7 ± 0	7 ± 1	7 ± 0
Mix I, clean	6 ± 1	7 ± 1	7 ± 1	7 ± 0	7 ± 0	6 ± 0
Mix II, contam.	6 ± 1	8 ± 1	8 ± 1	7 ± 0	7 ± 0	7 ± 1
Mix II, clean	7 ± 0	7 ± 0	7 ± 0	6 ± 1	7 ± 1	7 ± 1
Mix III, contam.	6 ± 1	7 ± 1	7 ± 1	7 ± 0	7 ± 0	8 ± 0
Mix III, clean	7 ± 0	7 ± 0	7 ± 0	6 ± 1	6 ± 1	7 ± 1
J. artichoke, contam.	9 ± 0	8 ± 0	8 ± 0	8 ± 0	8 ± 0	6 ± 0
J. artichoke, clean	8 ± 0	7 ± 0	7 ± 0	7 ± 0	7 ± 0	5 ± 0
Amaranth, contam.	9 ± 0	5 ± 1	6 ± 1	7 ± 1	7 ± 1	6 ± 1
Amaranth, clean	7 ± 0	4 ± 0	4 ± 0	5 ± 0	5 ± 0	4 ± 1

6.6 Results

In the WP2 package, the pot trial had two practical tasks. Firstly, to determine if it is possible to ensure the required amount of biomass for the TCR reactor feed. Secondly, to determine if it is possible to reach sufficient rate of phytoremediation capacity. To fulfil these tasks, plant morphology, biomass output, and phytoremediation capacity was evaluated, and the results from the pot experiment are presented in this chapter.

The pot experiment has started on the 30th of April 2021 and finished on the 27th of September 2021.

6.6.1 Plants morphology

Plants morphological analysis in experimental and control groups consisted of:

- Aboveground part height and aboveground part dry weight;
- Root length and root dry weight.

Morphology of herbaceous plants was evaluated during the first harvest in mid-July. Jerusalem artichoke and amaranth were evaluated after the harvest.

The morphology of herbaceous plants Mix I is presented in Table 6.6. Plants grown on the contaminated soil developed slightly better than the ones grown on the clean soil. However, in most cases the differences were insignificant. Results from morphological analysis are well in line with evaluation of the luxuriant (Table 6.5). Red clover did not germinate in the contaminated soil at all. Tall fescue and perennial ryegrass showed the best biomass potential. Furthermore, tall fescue produced almost 3 times higher aboveground part biomass in the contaminated soil as compared to the tall fescue grown in the clean soil.

Table 6.6 Average morphological parameters of the plants in Mix I ± standard deviation (n=3)

Contaminated soil				
Species	**Aboveground part height, cm**	**Root length, cm**	**Root weight, g (DM)**	**Aboveground part weight, g (DM)**
Tall fescue	53.0 ± 7.4	13.4 ± 3.4	0.77 ± 0.06	0.95 ± 0.70
Perennial ryegrass	43.9 ± 5.5	13.5 ± 3.8	0.03 ± 0.01	0.98 ± 0.62
Reed canary grass	63.6 ± 1.8	13.3 ± 0.8	0.05 ± 0.04	0.71 ± 0.51
Red clover	*Did not germinate*			
Clean soil				
Species	**Aboveground part height, cm**	**Root length, cm**	**Root weight, g (DM)**	**Aboveground part weight, g (DM)**
Tall fescue	37.9 ± 9.1	11.1 ± 4.1	0.04 ± 0.04	0.35 ± 0.26
Perennial ryegrass	35.9 ± 6.0	12.5 ± 1.7	0.08 ± 0.03	0.90 ± 0.17
Reed canary grass	46.1 ± 10.1	14.4 ± 6.9	0.05 ± 0.03	0.37 ± 0.04
Red clover	21.8 ± 6.5	13.3 ± 6.1	0.06 ± 0.03	0.55 ± 0.32

The morphology of herbaceous plants Mix II is presented in Table 6.7. In all cases plants grown on the contaminated soil developed better, were higher, had longer root and produced more biomass per plant. The most productive species, regarding aboveground biomass, was annual ryegrass. Meadow foxtail did not germinate nor in the contaminated soil, neither in the clean one.

Table 6.7 Average morphological parameters of the plants in Mix II ± standard deviation (n=3)

Contaminated soil				
Species	**Aboveground part height, cm**	**Root length, cm**	**Root weight, g (DM)**	**Aboveground part weight, g (DM)**
Annual ryegrass	50.8 ± 3.4	14.7 ± 1.2	0.14 ± 0.08	2.52 ± 1.60
Meadow fescue	27.8 ± 4.3	8.4 ± 3.1	0.03 ± 0.01	0.15 ± 0.05
Meadow foxtail	*Did not germinate*			
Alfalfa	54.1 ± 9.8	20.7 ± 8.8	0.18 ± 0.15	0.73 ± 0.48
Birdsfoot trefoil	52.7 ± 8.1	11.6 ± 4.2	0.01 ± 0.01	0.67 ± 0.36

Table 6.7 Average morphological parameters of the plants in Mix II ± standard deviation (n=3)

Clean soil				
Species	**Aboveground part height, cm**	**Root length, cm**	**Root weight, g (DM)**	**Aboveground part weight, g (DM)**
Annual ryegrass	40.0 ± 0.5	13.5 ± 1.8	0.12 ± 0.09	1.56 ± 0.54
Meadow fescue	35.2 ± 3.0	9.6 ± 1.7	0.01 ± 0.01	0.25 ± 0.10
Meadow foxtail	Did not germinate			
Alfalfa	38.7 ± 1.8	25.5 ± 3.1	0.47 ± 0.27	0.52 ± 0.07
Birdsfoot trefoil	28.0 ± 1.3	10.2 ± 2.1	0.01 ± 0.01	0.14 ± 0.01

The morphology of the Mix III is presented in Table 6.8. Common bent and honey clover did not germinate at all and the germination of white clover was negligible as well. Tall fescue and *Festulolium* produced the highest aboveground part biomass output. Tall fescue grown on the contaminated soil produced more than 4 times more biomass than tall fescue grown on the clean soil.

Table 6.8 Average morphological parameters of the plants in Mix III ± standard deviation (n=3)

Contaminated soil				
Species	**Aboveground part height, cm**	**Root length, cm**	**Root weight, g (DM)**	**Aboveground part weight, g (DM)**
Festulolium	43,467 ± 8,719	13,133 ± 0,764	0,077 ± 0,047	1,26 ± 0,953
Tall fescue	70,1 ± 6,245	15,467 ± 4,934	0,087 ± 0,055	1,26 ± 0,749
Common bent	*Did not germinate*			
White clover	54,15 ± 9,829	54,15 ± 9,830	54,15 ± 9,833	
Honey clover	*Did not germinate*			
Clean soil				
Species	**Species**	**Species**	**Species**	**Species**
Festulolium	32,567 ± 5,962	13,433 ± 2,060	0,147 ± 0,158	0,99 ± 0,678
Tall fescue	41,667 ± 5,754	11,567 ± 3,910	0,04 ± 0,04	0,37 ± 0,07
Common bent	*Did not germinate*			
White clover	14,167 ± 6,667	10,4 ± 1,345	0,023 ± 0,040	0,15 ± 0,174
Honey clover	*Did not germinate*			

Table 6.9 presents morphological parameters of Jerusalem artichoke. The plants were of similar height, but there were fewer stems (per planted tuber) when Jerusalem artichoke grew on the contaminated soil in comparison to the ones grown on the clean soil. This resulted that aboveground biomass output per plant was significantly higher for plants from the contaminated soil. This result is well in line with the results from lushness monitoring. Similar phenomenon was observed for tubers. Plants grown on the clean soil had deeper root, while the ones grown on the contaminated soil, had a significantly shorter root. This could be related to the fact that vermicast + nutrients were inserted into the contaminated soil only into the top layer (20-30 cm), and the roots did not grow deeper into soil without nutrients. However, Jerusalem artichoke from the contaminated soil produced larger tubers. Although, the number of tubers per plant was higher in the clean soil, average weight of a single tuber from the contaminated soil was 3.06 g, whereas single tuber from the clean soil on average weighted only 1.74 g.

Table 6.9 Average morphological parameters of J. artichoke ± standard deviation (n=3)

Soil	Aboveground part height, cm	Number of stems	Root length, cm	Root weight, g (DM)	Aboveground part weight, g (DM)	Number of tubers	Tuber weight, g (DM)
Contaminated	160.3 ± 14.8	3 ± 2	18.5 ± 2.8	26.2 ± 12.5	86.4 ± 32.2	13 ± 5	39.8 ± 22.1
Clean	160.7 ± 28.3	5 ± 1	24.5 ± 4.6	10.4 ± 2.7	36.9 ± 8.3	16 ± 5	27.9 ± 9.3

Table 6.10 presents average morphological parameters of amaranth. All measured morphological parameters but root length was significantly higher for the plants grown on the contaminated soil. This is well in line with the results from the lushness monitoring. It was also noticed that plants grown on the clean soil started to blossom about one week earlier than the plants grown on the contaminated soil. This can explain why the lushness of the plants differed by 2 points towards the end of the pot experiment. In general, plants before blossoming transfer high portion of water, nutrients, and energy into the blossom area, whereas other parts (stem, leaves) receive less, thus start to dry out and shed leaves.

Table 6.10 Average morphological parameters of amaranth ± standard deviation (n=3)

Soil	Aboveground part height, cm	Root length, cm	Root weight, g (DM)	Aboveground part weight, g (DM)
Contaminated	185.6 ± 20.8	25.4 ± 6.4	4.5 ± 1.1	57.1 ± 32.3
Clean	135.5 ± 32.0	24.6 ± 5.9	1.5 ± 0.8	11.8 ± 5.6

6.6.2 Potential biomass output

Potential biomass output was evaluated and compared between plants grown on the contaminated soil and on the clean one. Fresh biomass was measured right after cutting it down followed by a drying phase at a room temperature to reach dry weight and then measured again. Only aboveground part was evaluated for herbaceous plants and amaranth. Both aboveground biomass and tubers were evaluated for Jerusalem artichoke.

Biomass of herbaceous plants was evaluated individually after the first harvest on the 14th of July, second harvest on the 25th of August and third harvest on the 27th of September 2021. The total biomass output, achieved during the entire pot test experiment, was calculated after the last harvest.

The potential biomass of herbaceous Mix I is presented in Table 6.11. Plants grown on the contaminated soil developed significantly better and the total dry weight biomass output was by nearly 30% higher than from the plants grown on the clean soil.

Total biomass yield for herbaceous plants Mix I cultivated on contaminated soil, according to pot experiment results is 1,384.86 kg/ha (DM). Cultivated on clean soil – 1,088.57 kg/ha (DM).

Table 6.11 Biomass output of the herbaceous plants in Mix I ± standard deviation

Soil	Cut	Mix I, average aboveground biomass weight, kg (DM), n=3	Mix I, total aboveground biomass weight, kg (DM), n=9
Contaminated	1st	0.034 ± 0.002	,0.215
	2nd	0.021 ± 0.003	
	3rd	0.016 ± 0.002	
Clean	1st	0.020 ± 0.000	0.169
	2nd	0.025 ± 0.008	
	3rd	0.012 ± 0.002	

The potential of biomass for herbaceous Mix II is presented in Table 6.12. Plants grown on the contaminated soil developed only slightly better and the total dry weight biomass output was just 4.5% higher than from the plants grown on the clean soil.

Total biomass yield for herbaceous plants Mix II cultivated on contaminated soil, according to pot experiment results is 1,294.69 kg/ha (DM). Cultivated on clean soil – 1,236.71 kg/ha (DM).

Table 6.12 Biomass output of the herbaceous plants in Mix II ± standard deviation

Soil	Cut	Mix II, average aboveground biomass weight, kg (DM), n=3	Mix II, total aboveground biomass weight, kg (DM), n=9
Contaminated	1st	0.034 ± 0.003	0.201
	2nd	0.021 ± 0.003	
	3rd	0.012 ± 0.003	
Clean	1st	0.027 ± 0.004	0.192
	2nd	0.021 ± 0.001	
	3rd	0.016 ± 0.008	

The potential of biomass for herbaceous Mix III is presented in Table 6.13. Plants grown on the contaminated soil developed better and the total dry weight biomass output was 15% bigger than from the plants grown on the clean soil.

Total biomass yield for herbaceous plants Mix III cultivated on contaminated soil, according to pot experiment results is 1,191.63 kg/ha (DM). Cultivated on clean soil – 1,037.04 kg/ha (DM).

Table 6.13 Biomass output of the herbaceous plants in Mix III ± standard deviation

Soil	Cut	Mix III, average aboveground biomass weight, kg (DM), n=3	Mix III, total aboveground biomass weight, kg (DM), n=9
Contaminated	1st	0.030 ± 0.004	0.185
	2nd	0.018 ± 0.004	
	3rd	0.014 ± 0.001	
Clean	1st	0.028 ± 0.003	0.161
	2nd	0.019 ± 0.005	
	3rd	0.013 ± 0.002	

In general, plants grown on the clean soil had higher density and higher soil cover with plants in comparison to the plants grown on the contaminated soil. However, the lushness of plants was

higher for the ones grown on the contaminated soil. Dominant species in all mixes were fescue, perennial ryegrass and *Festulolium*, and in all cases, weight per single plant was higher when grown on the contaminated soil. Furthermore, since clover (red, white, honey), meadow foxtail, and common bent did not germinate, it resulted that Mix II and Mix III had lower biomass output than Mix I, which contained lush tall fescue (35%) and perennial ryegrass (30%). It is noteworthy, to say, that plants on the contaminated soil probably didn't develop better due to the contamination, but rather to the addition of nutrients through vermicast + fertilizers (see Table 14) and addition of soil bacteria.

Biomass output of Jerusalem artichoke was evaluated after harvesting it on the 27th of September 2021. Table 6.14 presents biomass output of Jerusalem artichoke. Plants grown on the contaminated soil developed significantly better. Jerusalem artichoke, grown on contaminated soil, reached the total aboveground biomass of 0.867 kg (DM) per 3 replications (pots) and it was by 2.25 times higher than the aboveground biomass (0.384 kg) of plants grown on the clean soil. The average biomass per replicate (pot) when grown on the contaminated soil was 0.289 ± 0.035 kg (DM), while the ones grown on clean soil was only 0.128 ± 0.013 kg (DM). Tuber of Jerusalem artichoke, grown on the contaminated soil, biomass per 3 replications (pots) reached 0.348 kg (DM) and it was by 1.5 times higher as compared to Jerusalem artichoke plants grown on the clean soil (0.230 kg DM). Tubers of the plants grown on contaminated soil on average dry weight per replicate was 0.116 ± 0.003 kg, while the ones grown on the clean soil was 0.077 ± 0.006kg. Total (aboveground + tubers) Jerusalem artichoke grown on the contaminated soil biomass was 1.216 kg (DM) per 3 replications (pots), compared to the biomass of the plants grown on the clean soil – 0.614 kg, which is 1.9 lesser.

Total biomass yield for Jerusalem artichoke cultivated on contaminated soil, according to pot experiment results is 23497.58 kg/ha (DM). Cultivated on clean soil – 11,864.73 kg/ha (DM)

Table 6.14 Biomass output of the Jerusalem Artichoke ± standard deviation (n=3)

Soil	Average aboveground biomass, kg (DM)	Average tuber biomass, kg (DM)	Total aboveground biomass, kg (DM)	Total tubers biomass, kg (DM)	Total biomass, kg (DM)
Contaminated	0.289 ± 0.035	0.116 ± 0.029	0.867	0.348	1.216
Clean	0.128 ± 0.013	0.077 ± 0.006	0.384	0.230	0.614

Amaranth was evaluated after harvest on the 23rd of July 2021. After unexpected regrowth of plants after the first harvest, 2nd harvest was performed on the 27th of September 2021. Table 6.15 presents biomass output of amaranth. Plants grown on the contaminated soil developed significantly better as compared to the ones grown on the clean soil.

After the first and main cut, amaranth grown on the contaminated soil exhibited total aboveground biomass of 1.319 kg (DM) per 3 replications (pots) which was by 2.73 times higher than amaranth plants grown on the clean soil (0.482 kg DM). Average biomass of the plants grown on the contaminated soil was 0.440 ± 0.056 kg (DM) per replicate (pot), while the ones grown on the clean soil was 0.161 ± 0.018 kg (DM) per replicate.

After regrowth and second cut, amaranth grown on the contaminated soil, had aboveground biomass of 0.087 kg (DM) per 3 replications (pots) and it was 1.55 times higher compared to amaranth plants grown on the clean soil (0.054 kg DM). Average biomass of the plants grown

on the contaminated soil was 0.029 ± 0.011 kg (DM) per replicate (box), while the ones grown on the clean soil was 0.018 ± 0.002 kg (DM) per replicate.

Total biomass yield for Amaranth cultivated on contaminated soil, according to the pot experiment results is 27,169.08 kg/ha (DM). Cultivated on clean soil – 10,357.49 kg/ha (DM).

Table 6.15 Biomass output of amaranth ± standard deviation (n=3)

Soil	Cut	Average aboveground biomass, kg (DM)	Aboveground biomass weight, kg (DM)
Contaminated	1st	0.440 ± 0.056	1.319
	2nd	0.029 ± 0.011	0.087
Clean	1st	0.161 ± 0.018	0.482
	2nd	0.018 ± 0.002	0.054

6.6.3 Phytoremediation potential

To assess the phytoremediation potential, contaminated and clean soil samples prior the pot experiment and after the harvest of plants were evaluated. Analytes, such as polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), total petroleum hydrocarbons (TPH), and halogenated organic compounds (PCE) were determined as well as general soil parameters, such as pH, electrical conductivity, total C and total N, NPK and Mg.

Organic contaminants in the clean soil were determined only in soil before the experiment. As all of them were below instruments detection limits, and thus below maximum permissible concentrations (MPC), these analytes weren't determined in the clean soil after the pot experiment.

Analytes, like PAH, PCB, and PCE in the contaminated soil in most cases were below instruments detection limits. In some cases, it was possible to determine slight increases, but none were above of even close to the MPC, thus PAH, PCB and PCE here will not be discussed here in detail.

6.6.4 Herbaceous plants

Table 6.16 presents average general soil parameters prior and after the pot experiment with three herbaceous mixes. For the clean soil, parameters are given as determined at the start of the experiment (prior sowing), and at the end, i. e., after harvest, as average value of all three mixes because the differences were insignificant. For the contaminated soil, end-values are given individually for all three mixes. Furthermore, because the trial where contaminated soil was left blank without any plants contained soil from the same subplot as herbaceous plants, end-values are presented in the Table 6.16 as well.

Table 6.16 Average general soil parameters prior and after the pot experiment with three herbaceous mixes ± standard deviation (n=3)

Analyte	Unit	Herbaceous plants						
		Clean soil		Contaminated soil				
		Start	End, all mixes	Start	End, Mix I	End, Mix II	End, Mix III	End, blank soil*
Electrical Conductivity, 25°C	mS/m	14.6 ± 3.0	44.8 ± 2.8	30.3 ± 6.1	67 ± 13.9	53.0 ± 15.2	62.1 ± 17.8	36.0 ± 9.9
pH (H_2O)	-	8.3 ± 0.1	9.0 ± 0.3	9.3 ± 0.1	9.0 ± 0.2	8.9 ± 0.2	8.9 ± 0.2	8.0 ± 0.3
Organic Dry Mass	% DM	3.1 ± 0.2	3.4 ± 0.01	3.9 ± 0.2	5.4 ± 1.0	4.67 ± 1.2	5.3 ± 0.8	5.6 ± 3.5
Dry matter, 105°C	%	94.1 ± 5.6	90.1 ± 0.7	92.5 ± 5.6	87.6 ± 1.7	87.9 ± 2.5	87.0 ± 0.8	91.5 ± 1.0
Total Carbon	% DM	1.38 ± 0.2	1.6 ± 0.01	6.4 ± 1.0	7.7 ± 1.5	6.8 ± 1.2	7.1 ± 1.1	4.9 ± 06
Total Nitrogen as N	mg/kg DM.	789 ± 161	824 ± 43	946 ± 192	1521 ± 781	1320 ± 504	1253 ± 215	905 ± 303
Magnesium	mg/kg DM.	1030 ± 206	379 ± 45	958 ± 192	754 ± 236	677 ± 78	879 ± 287	635 ± 202
Phosphorus	mg/kg DM	39.1 ± 8.1	51.8 ± 5.4	19.9 ± 4.5	62.4 ± 18.5	56.4 ± 18.7	58.0 ± 15.5	56.9 ± 26.0
Potassium	mg/kg DM	560 ± 112	546 ± 34	271 ± 54	366 ± 148	321 ± 76	354.0 ± 39.3	516 ± 110
Zinc	mg/kg DM	3.28 ± 0.7	-	32.7 ± 6.5	-	-	-	14.3 ± 1.3
Microbial activity	CFU/g	$3.0x10^5 \pm 0.3x10^5$	$1.6x10^6 \pm 0.3x10^6$	$1.9x10^5 \pm 0.6x10^5$	$2.1x10^6 \pm 0.3x10^6$	$1.7x10^6 \pm 0.5x10^6$	$1.7x10^6 \pm 0.5x10^6$	$4.7 \times 10^4 \pm 1.0x10^4$
Microbial activity, blank soil*	CFU/g	-	-	$5.7x10^4 \pm 1.2x10^4$	-	-	-	

*n=2. One replicate was identified as outlier and eliminated.

Major changes and differences between the clean and contaminated soils are as follow:

- Electrical conductivity in all cases increased, except in the blank soil, where it remained the same as in the contaminated soil at the start. Changes in pH were insignificant.
- Organic dry mass increased in all cases, even in the blank soil.
- Total carbon and total nitrogen increased in all cases, except in the blank soil, where it decreased. Contaminated soil had higher content of total C and N than the clean one. Significantly higher increase of N in the contaminated soil at the end of experiment, can be attributed to the addition of vermicast + fertilizers.
- Magnesium in all cases decreased. This could be attributed to the fact that plants "consumed" a share of it. In addition, it is likely that a fraction of Mg leached out during watering, as decrease of Mg was determined in the blank soil as well. This can be observed in the case of Zn as well.

- Potassium and phosphorus content initially was higher in the clean soil than in the contaminated one. Phosphorus content in all cases increased. The increase was significantly higher in the contaminated soil and can be attributed to the addition of vermicast + fertilizers. It is unclear why the content of P increased in the blank soil. Potassium in the clean soil slightly decreased throughout the pot experiment. While, in the contaminated soil, it was higher due to the addition of vermicast + fertilizers. Here as well, it is unclear why K increased this much in the blank soil.

Figure 6.1 presents levels of contamination with petroleum hydrocarbons at the start of the experiment and at the end when growing three different herbaceous plant species mixes. Maximum permissible value, according to Lithuanian legislative document LAND 9-2009, is 200 mg/kg for TPH (taking into account that the Šiauliai site is classified as sensitive). The highest contamination was with petroleum hydrocarbon fractions C10-C16 and C16-35, i. e., with diesel, oil and residual range of organic contaminants. The Šiauliai site is known as a former oil base. Total petroleum hydrocarbon concentration in the contaminated soil at the start of the pot experiment was about **6790 mg/kg** (DM). After the pot experiment, decrease of all fractions was observed in all cases.

Relative phytoremediation potential was calculated as shown in equation (2). The potential for Mix I was = 6.3, for Mix II = 5.6, for Mix III = 5.4. This is well in line with other results: plants from the Mix I had higher biomass per plant and in total. Whereas plants from Mix III had the lowest biomass output, which was influenced by the selection of plant species.

$$Phytoremediation\ potential = \frac{C_{TPH\ (start)}}{C_{TPH\ (end)}} \qquad (2)$$

Surprisingly, decrease of contaminants was observed in the blank soil as well. A possible reason for this phenomenon could be the fact that the soil was aerated when excavating and transporting to the greenhouse. Also, during pot test experiment, blank soil pots were watered with tap water same as all group pots. As a consequence, a share of TPH either volatilized and evaporated into the air, undergone chemical degradation or leached out of the soil.

As it can be seen from the Figure 6.1, lighter fractions decreased more as compared to the heavier fractions. Although, it was not possible to achieve that the TPH would decrease below the MPC value after only one growing season, the results show high potential to clean up the soil with extra 1-2 years.

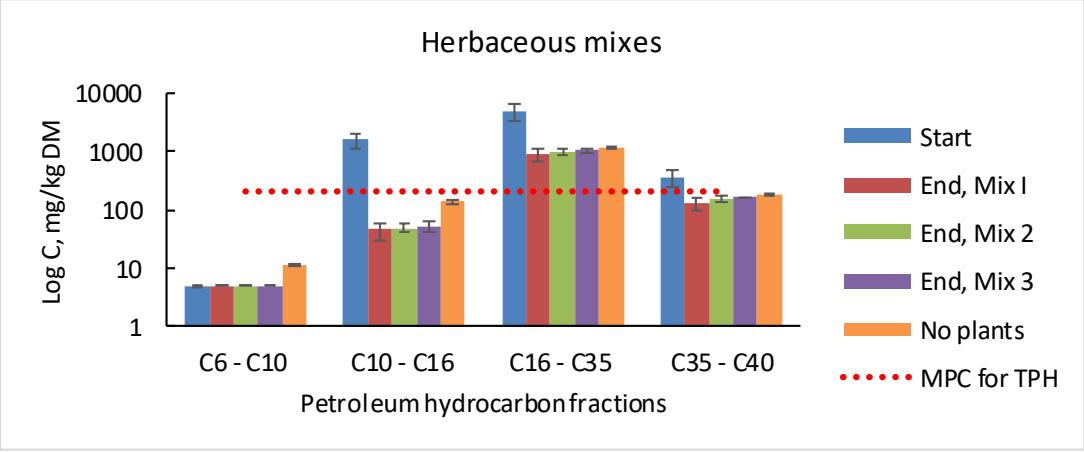


Figure 6.1 TPH concentrations in the contaminated soil at the start and at the end of the pot experiment with herbaceous plants (n=3). Note the logarithmic scale

6.6.4.1 Amaranth

Table 6.17 presents average general soil parameters prior and after the pot experiment with amaranth. The main observations are as follow:

- Electrical conductivity in all cases increased, whereas changes in pH were insignificant.
- Organic dry matter did not change in the clean soil, but there was a significant increase in the contaminated soil.
- Contaminated soil had higher content of total C and N than the clean one. Significantly higher increase of N in the contaminated soil at the end of experiment can be attributed to the addition of vermicast + fertilizers.
- Magnesium in all cases decreased. This could be attributed to the fact that plants consumed a share of it. In addition, it is likely that a fraction of Mg leached out during watering.
- Phosphorus content was initially higher in the contaminated soil. At the end, P content significantly increased in the clean soil. Whereas in the contaminated soil the changes were negligible even though the contaminated soil received addition of N through vermicast + fertilizers.
- Potassium content was about the same in both soils at the start, and it decreased in both cases at the end despite the addition of vermicast + fertilizers to the contaminated soil.

Table 6.17 Average general soil parameters prior and after the pot experiment with amaranth ± standard deviation (n=3)

Analyte	Unit	Amaranth			
		Clean soil		Contaminated soil	
		Start	End	Start	End
Electrical Conductivity, 25°C	mS/m	14.6 ± 3.0	34.8 ± 2.9	21.1 ± 4.3	86.6 ± 24.4
pH (H_2O)	-	8.3 ± 0.1	9.2 ± 0.2	8.3 ± 0.2	8.7 ± 0.4
Organic Dry Mass	% DM	3.1 ± 0.2	3.1 ± 0.2	3.6 ± 0.2	5.7 ± 0.6
Dry matter, 105°C	%	94.1 ± 5.6	82.1 ± 0.6	90.8 ± 5.4	85.9 ± 1.6
Total Carbon	% DM	1.38 ± 0.2	1.48 ± 0.1	4.5 ± 0.7	4.6 ± 0.4
Total Nitrogen as N	mg/kg DM	789 ± 161	718 ± 41	1,060 ± 214	1,563 ± 156
Magnesium	mg/kg DM	1,030 ± 206	347 ± 48	503 ± 101	455 ± 83
Phosphorus	mg/kg DM	39.1 ± 8.1	57.8 ± 9.0	106 ± 21	118 ± 30
Potassium	mg/kg DM	560 ± 112	457 ± 125	497 ± 99	277 ± 91
Zinc	mg/kg DM	3.28 ± 0.7	-	18.1 ± 3.6	-
Microbial activity	CFU/g	$3.0x10^5$ ± $0.3x10^5$	$7.5x10^5$± $1.6x10^5$	$1.7x10^5$ ± $0.3x10^5$	$1.9x10^6$ ± $0.3x10^6$

Figure 6.2 presents levels of contamination with petroleum hydrocarbons at the start of the experiment and at the end growing amaranth. Maximum permissible value, according to Lithuanian legislative document LAND 9-2009, is 200 mg/kg for TPH. Total petroleum

hydrocarbon concentration in the contaminated soil at the start of the pot experiment was about **1275 mg/kg** (DM). After the pot experiment, decrease of all fractions was observed. The relative phytoremediation potential of amaranth at the given conditions was 1.69. Here, as well as in the case of herbaceous plants, heavier petroleum hydrocarbon fractions were degraded to a lesser extent, and only one year of phytoremediation was insufficient to achieve values of TPH below MPC.

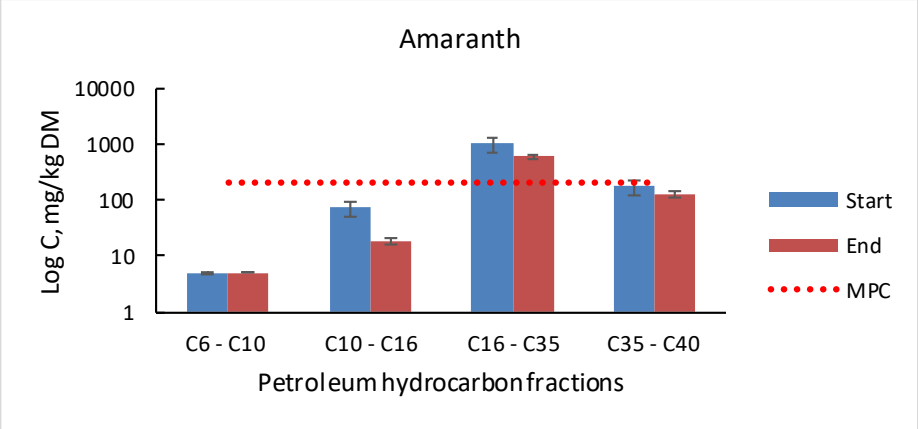


Figure 6.2. TPH concentrations in the contaminated soil at the start and at the end of the pot experiment with amaranth (n=3). Note the logarithmic scale

6.6.4.2 Jerusalem artichoke

Table 6.18 presents average general soil parameters prior and after the pot experiment with Jerusalem artichoke. The main observations are as follow:

- Electrical conductivity in all cases increased, whereas changes in pH were insignificant.
- Organic dry matter did not change in the clean soil, but there was a significant increase in the contaminated soil.
- Total C was about the same in both soils, and it remained at the same level in end in the clean soil, whereas in the contaminated soil it slightly increased. The same can be said about the total content of N, but the increased at the end of experiment in the contaminated soil can be attributed to the addition of vermicast + fertilizers.
- Magnesium content was significantly higher in the clean soil and it decreased about 5 times throughout the experiment. Unlike in the case of herbaceous plants and amaranth, here Mg content was higher at the end of experiment in the contaminated soil.
- Phosphorus content was initially higher in the contaminated soil. At the end, P content slightly increased in the clean soil. Whereas, in the contaminated soil the increase was more than 3 times, due to addition of vermicast + fertilizers.
- Potassium content was about the same in both soils at the start, and it decreased in both cases at the end despite the addition of vermicast + fertilizers to the contaminated soil.

Table 6.18 Average general soil parameters prior and after the pot experiment with Jerusalem artichoke ± standard deviation (n=3)

Analyte	Unit	Jerusalem artichoke			
		Clean soil		Contaminated soil	
		Start	End	Start	End
Electrical Conductivity, 25°C	mS/m	14.6 ± 3.0	54.3 ± 8.0	14.8 ± 3.0	113 ± 5.1

Table 6.18 Average general soil parameters prior and after the pot experiment with Jerusalem artichoke ± standard deviation (n=3)

Analyte	Unit	Jerusalem artichoke			
		Clean soil		Contaminated soil	
		Start	End	Start	End
pH (H_2O)	-	8.3 ± 0.1	9.5 ± 0.2	8.4 ± 0.2	8.2 ± 1.1
Organic Dry Mass	% DM	3.1 ± 0.2	3.1 ± 0.1	2.6 ± 0.1	5.4 ± 0.4
Dry matter, 105°C	%	94.1 ± 5.6	85.1 ± 0.6	92.5 ± 5.6	91.4 ± 0.8
Total Carbon	% DM	1.38 ± 0.2	1.5 ± 0.01	1.6 ± 0.2	2.8 ± 0.3
Total Nitrogen as N	mg/kg DM	789 ± 161	643 ± 38	815 ± 166	1193 ± 156
Magnesium	mg/kg DM	1,030 ± 206	298 ± 16	309 ± 62	407 ± 100
Phosphorus	mg/kg DM	39.1 ± 8.1	43.5 ± 4.3	70.2 ± 14.2	216 ± 126
Potassium	mg/kg DM	560 ± 112	415 ± 35	475 ± 95	471 ± 243
Zinc	mg/kg DM	3.28 ± 0.7	-	-	-
Microbial activity	CFU/g	$3.0x10^5$ ± $0.3x10^5$	$6.6x10^5$± $1.3x10^5$	$4.1x10^4$ ± $1.3x10^4$	$4.9x10^5$ ± $1.1x10^5$

Figure 6.3 presents levels of contamination with petroleum hydrocarbons at the start of the experiment and at the end growing Jerusalem artichoke. Maximum permissible value, according to Lithuanian legislative document LAND 9-2009, is 200 mg/kg for TPH. Total petroleum hydrocarbon concentration in the contaminated soil at the start of the pot experiment was about **625 mg/kg** (DM). After the pot experiment, decrease of all fractions was observed. The relative phytoremediation potential of amaranth at the given conditions was 1.26. Here, as well as in the case of herbaceous plants and amaranth, heavier petroleum hydrocarbon fractions were degraded to a lesser extent, and only one year of phytoremediation was insufficient to achieve values of TPH below MPC.

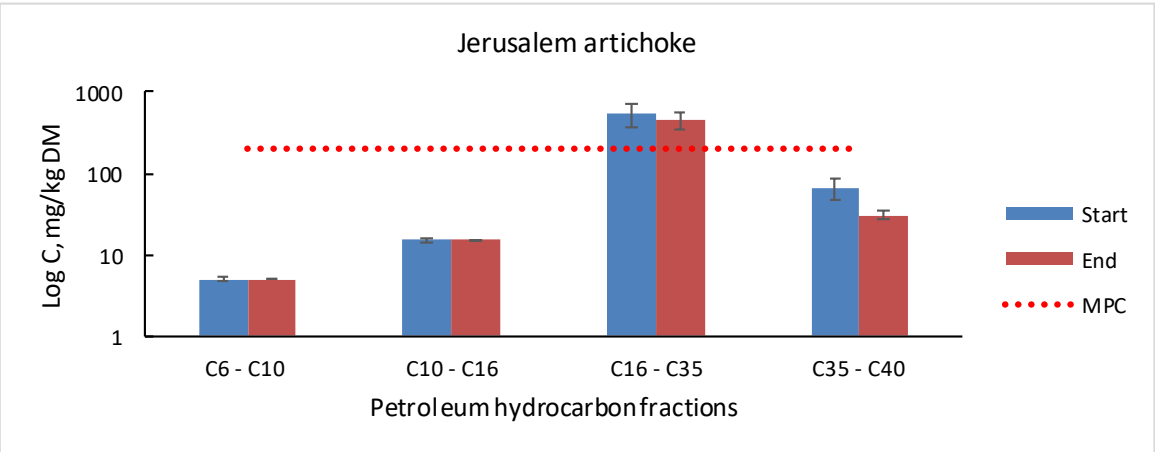


Figure 6.3. TPH concentrations in the contaminated soil at the start and at the end of the pot experiment with J. artichoke (n=3). Note the logarithmic scale

6.7 Conclusions

6.7.1 Plant development and biomass output

Trifolium species (red, white and honey clovers), meadow foxtail and common bent did not germinate in the contaminated soil and were considered as not suitable for the field trials. Tall fescue, annual ryegrass, meadow fescue and *Festulolium* produced the highest above ground biomass yield per plant. Thus, these species will be sown in the field trial. Herbaceous plants grown on contaminated soil were as luxuriant as the ones grown on the clean soil. Jerusalem artichoke grown on the contaminated soil had fewer stems and tubers per planted tuber, but the dry weight was significantly higher than for plants grown on the clean soil. Amaranth grown on the contaminated soil developed significantly better than the plants grown on clean soil.

Jerusalem artichoke and amaranth grown on the contaminated soil had biomass output of 1. 216 kg (DM) and 1.319 kg (DM), respectively, per 3 replicates (0.1725 m^2 x 3). While the ones grown on the clean soil had biomass output of 0.614 kg (DM) and 0.482 kg (DM), respectively, per 3 replicates. Herbaceous mixes per 3 replicates and 3 cuts (total 9 replicates, 0.1725 m^2 x 9) grown on the contaminated soil had biomass output as follow: Mix I – 0.215 kg (DM), Mix II - 0.201 kg (DM), Mix III - 0.185 kg (DM). Herbaceous mixes per 3 replicates grown on the clean soil had biomass output as follow: Mix I - 0.169 kg (DM), Mix II - 0.192 kg (DM), Mix III - 0.161 kg (DM).

According to the "Biovala" (BVA) chosen phytoremediation plants cultivation strategy on contaminated soil, were obtained following total biomass outputs:

- Total biomass yield for herbaceous plants Mix I cultivated on contaminated soil, according to pot experiment results is 1384.86 kg/ha (DM). Cultivated clean soil - 1088.57 kg/ha (DM).
- Total biomass yield for herbaceous plants Mix II cultivated on contaminated soil, according to pot experiment results is 1294.69 kg/ha (DM). Cultivated on clean soil – 1236.71 kg/ha (DM).
- Total biomass yield for herbaceous plants Mix III cultivated on contaminated soil, according to pot experiment results is 1191.63 kg/ha (DM). Cultivated on clean soil - 1037.04 kg/ha (DM).
- Total biomass yield for Jerusalem artichoke cultivated on contaminated soil, according to pot experiment results is 23497.58 kg/ha (DM). Cultivated on clean soil – 11864.73 kg/ha (DM).
- Total biomass yield for Amaranth cultivated on contaminated soil, according to the pot experiment results is 27169.08 kg/ha (DM). Cultivated on clean soil - 10357.49 kg/ha (DM).

The findings clearly indicate that the chosen plant species can be successfully grown on the contaminated soil at the given contamination levels, according to the chosen cultivation strategy. Furthermore, plants cultivated on contaminated soil with appropriate strategy can produce equal and if not higher biomass output.

6.7.2 Phytoremediation potential

The phytoremediation potential was achieved using herbaceous plants, Mix I in particular. The phytoremediation potential for all plant species used in the pot experiment can be summarized as follow: Mix I (6.3) > Mix II (5.6) > Mix III (5.4) > amaranth (1.7) > Jerusalem artichoke (1.3).

It was observed that the lighter petroleum hydrocarbon fractions were degraded easier. Whereas heavier fractions (diesel, oil, residual), that were dominant in the contaminated soil, were degraded to a lesser extent, but the decrease in concentration of all fractions was observed in all cases.

Although, one growing season was insufficient to degrade TPH below maximum permissible values in this specific case, the obtained results show a large potential for a successful clean-up of the contaminated site in Šiauliai city just in a few years.

7. ARGENTINIAN SITE POT TRIALS EXPERIMENTAL PLAN

7.1 Objectives

The aims of this study are: 1) to evaluate the acute and chronic toxicity of a soil contaminated with mining waste on *Plectrocarpa tetracantha*, *Bulnesia retama*, *Larrea cuneifolia* and *Prosopis flexuosa*; 2) to evaluate the metal(loid) bioaccumulation capacity of *Plectrocarpa tetracantha*, *Bulnesia retama*, *Larrea cuneifolia* and *Prosopis flexuosa* growing on a soil contaminated with and without amendment application.

7.2 Materials and methods

7.2.1 Description of the set-up

INTA is carrying out a phytoremediation strategy in a relevant mining zone until the early 1970's in La Planta (31º10'24,38" S, 67º52'57,26" W), San Juan, Argentina. The study area is in an arid environment which corresponds to the "Monte" phytogeographic province. It has a dry and warm climate with mainly summer (December-March) rainfall of a torrential nature, ranging between 80 and 200 mm per year (Poblete and Minetti, 1999; Cabrera, 1994). Temperatures are very high and reach an absolute maximum of 46ºC (Dalmasso and Anconetani, 1993). Regarding geomorphology, the area is located in an extensive alluvial plain of the Bermejo river. Primary and secondary streams are often dry and only have water during certain seasons (Dalmasso and Anconetani, 1993). Vegetation is uniform both in its appearance and in its diversity of species (Montenegro et al., 2007), changing according to the topography. This area presents the appearance of an open forest in which species such as "black carob" (Prosopis flexuosa) and "broom" (Bulnesia retama) predominate (Dalmasso and Anconetani, 1993). In this sense, the primary productivity of this kind of environment is limited.
Based on background of the INTA's research team, we have proposed to evaluate the metal(loid) bioaccumulation capacity of native shrubs and trees, such as *Plectrocarpa tetracantha*, *Bulnesia retama*, *Larrea cuneifolia* and *Prosopis flexuosa*. For this, three experiments were carried out in controlled conditions: 1) Acute exposure test; 2) Chronic exposure test; and 3) Chronic exposure of contaminated soil amended with dolomite and compost.

Family: Zygophyllaceae

- *Plectrocarpa tetracantha*: woody shrub, 1.6 to 2 m in height. Endemic species of the Argentinean northwestern region (Fig. 7.1) (Ruiz Leal, 1972).

Figure 7.1. *Plectrocarpa tetracantha*

• *Bulnesia retama*: shrub or tree, reaches up to 4 m in height. South American species that inhabits the entire arid western region of Argentina (Fig. 7.2). Currently, this species is protected because in the past it was highly exploited for its wood for vineyard posts and for the wax on its branches (Palacios and Hunziker, 1984).

Figure 7.2. *Bulnesia retama*

• *Larrea cuneifolia*: resinous shrub, reaches up to 2 m in height (Fig. 7.3). In Argentina, this species inhabits between the provinces of Salta (Northwest) and Chubut (South). Blooms in early October and fructifies in late November. Historically, this species has been widely used for firewood (Hunziker et al., 1972).

Figure 7.3. *Larrea cuneifolia*

Family: Fabaceae

• *Prosopis flexuosa*: tree, 2 to 8 m in height. Inhabits the central western area, mainly present in the "Monte" phytogeographic province, in the Arid Chaco region and with less density in the "Espinal" Province. The seeds germinate between September and October. The wood can be used as firewood or for carpentry (posts and rods for livestock infrastructure). Fruiting begins in late December and ends in late January. Fruits and leaves are used to feed livestock (Fig. 7.4). In addition, the fruit is used to make a diuretic drink, "patay" (human food), "aloja" (alcoholic drink) and "añapa" (non-alcoholic drink) (Alvarez, 2002).

Figure 7.4. *Prosopis flexuosa*

Although these species are adapted to this polluted environment, the production of plant biomass is limited. For this reason, we have recently proposed the following contingency plan in order to achieve the requirements of plant biomass (DM) for the biofuel generation and metal recovery: incorporation of the quinoa crop (*Chenopodium quinoa*) to the Argentine Pilot Site. Quinoa is a flowering plant in the amaranth family. Fig. 7.5 shows a representative illustration of the quinoa crop. It is an herbaceous annual plant grown as a crop primarily for its edible seeds. Seeds are rich in protein, dietary fibre, B vitamins, and dietary minerals in amounts greater than in many other grains. Quinoa is related to spinach and amaranth (*Amaranthus* spp.), and originated in the Andean region of northwestern South America. In addition, the INTA's research team has experience in this crop (Roqueiro et al., 2020) and there are several studies that report the use of quinoa in phytoremediation strategies (Amjad et al., 2021; Bhargava et al., 2008; Parvez et al., 2020; Guarino et al., 2020) and biofuel production (Lisý et al., 2020; Matías et al., 2021).

Figure 7.5. Representative illustration of the quinoa crop (*Chenopodium quinoa*).

7.2.2 Sampling campaign

7.2.2.1 Soil sampling

Soil samples were taken from two sites: contaminated (Site 1) and reference site (Site 2). Figure 7.6 shows several pictures from each study site. Samples were taken at random from the first 20 cm of soil, composed for 4 subsamples.

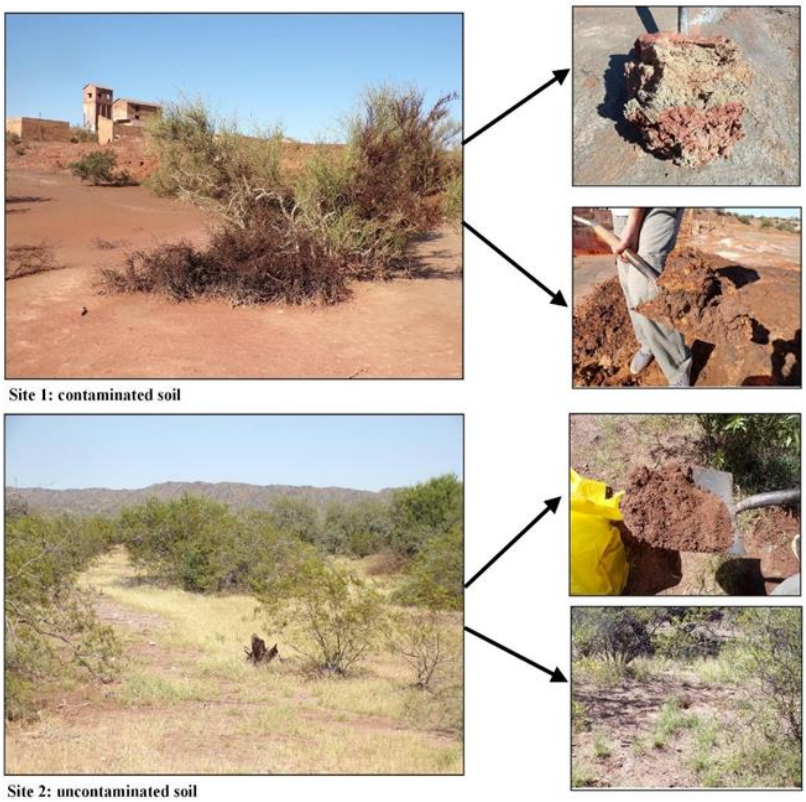


Figure 7.6. Sampling sites used to study the contaminated and control soils.

7.2.2.2 Experiments in controlled conditions

Acute exposure test

A seed germination and root elongation toxicity test were carried out on *P. flexuosa* and *P. tetracantha* for 7 days and on *L. cuneifolia* and *B. retama* for 12 days. Six treatments were conducted mixing samples of contaminated soil (Site 1) and reference soil (Site 2) in different concentrations, according to USEPA (1998): 0%, 10%, 25%, 50%, 70% and 100% contaminated soil. Five replicates were carried out for each treatment. Experimental unit consisted of 20 seeds on 25 g of soil into a Petri dish. A total of 100 seeds per treatment were used (600 seeds of each species). Seeds were previously cleaned and sterilised with 20% sodium hypochlorite for 10 min and rinsed with deionised water. Toxicity test was carried out in a germination chamber with controlled conditions: darkness, humidity (45%), and temperature (25°C).

Chronic exposure test

Seeds were collected around the pilot site and plants were grown for 3 months in a greenhouse. Plants were watered by automated drip irrigation, while light and temperature were not controlled. Three-months plants were exposed to different mixtures of contaminated and reference soil in greenhouse conditions for 90 days. The treatments used were: 0%, 1%, 10%, 50%, and 100% contaminated soil.

Chronic exposure of contaminated soil amended with dolomite and compost

Based on results obtained in chronic exposure test, we applied two amendments (compost and dolomite) to soil in order to regulate the acid pH and increase the nutrient and organic matter contents in soil.

We carried out a preliminary assay to calculate the dosage of dolomite. For this, we mixed three concentrations of dolomite, 5% of compost, and contaminated soil to reach a pH value of 6.5: 10%, 20% and 40% dolomite. Treatments are shown in Table 7.1.

Table 7.1 Mixture of contaminated soil and amendments

Treatment	Contaminated soil (g)	Dolomite (g)	Compost (g)
T0	190	0	10
T1	170	20	10
T2	150	40	10
T3	110	80	10

Each treatment was watered and maintained in darkness at 25°C for 15 days (optimum period to regulate the pH in soil).

7.3 Irrigation regime

In the chronic exposure tests with and without amendments, plants were watered by automated drip irrigation, while light and temperature were not controlled.

7.4 Monitoring program

7.4.1 Soil characterization

The physicochemical parameters measured in both sites were: pH, electrical conductivity (EC), texture, volume of sedimentation (VS), gravel percentage (Gr), organic matter (OM), C:N ratio, nitrogen (N), phosphorus (P), potassium (K), metal(loid)s (Cu, Cd, Zn and As), and major cations and anions. In addition, guideline values proposed by the Argentine Law no. 24,051 are also compared.

Soil samples were dried at ambient temperature and sieved through a 1.5 mm mesh to determine the concentration of Cu, Cd, Zn and As. Subsequently, they were treated with 3 different chemical agents in order to study the bioavailability of metals(oid):

- Microwave-assisted digestion: A total digestion of the soil samples was carried out to obtain the total fraction of metals(oid). Briefly, 0.25 g soil sample was weighed and placed in individual reactors. A START-D model microwave digestion system from Milestone (Sorisole, Italy) and polytetrafluoroethylene (PTFE) reactors were used. Aliquots were treated with 4 ml of 65% HNO_3, 1 ml of 30% H_2O_2, and 3 ml of 40% hydrofluoric acid. The dissolution was carried out at an increasing temperature of 10 min to 200 °C and was kept for a further 20 min. The microwave power used was up to 1000 W (Martínez et al., 2018).
- Diethylenetriaminepentaacetic acid (DTPA) - metal chelator agent: A soil mixture was made with an extracting solution of DTPA to determine the extractable or mobilisable fraction (0.005 M DTPA, 0.01 M $CaCl_2$ and 0.1 M triethanylamine (TEA)) in a 1:2 w:v ratio. After 2 h of stirring, the supernatant was filtered (Lindsay and Norvell, 1969; Maiz et al., 1997).
- Deionised water (aqueous extract): A sample of soil was mixed with deionized water (1:4 w:v ratio) for 30 min to determine the soluble fraction. After 60 min, the supernatant liquid was filtered (USEPA,1998) and used to measure pH and EC.

7.4.2 Energy crops characterization

In acute exposure test, the parameters determined were mean germination time (MGT), germination percentage, root and hypocotyl length. Using these data, toxicological endpoints and phytotoxicity indexes were estimated: NOEC (*No Observed Effect Concentration*), LOEC (*Lowest Observed Effect Concentration*), IC50 (*Inhibitory Concentration 50%*), RGI (*Relative Growth Index*), and GI (*Germination Index*).

In the chronic exposure tests with and without amendments, morphological variables were determined such as stem height, number of green and necrotic leaves, and stem diameter.

7.5 Results

7.5.1 Soil characterization

The physicochemical soil characteristics of both sites are shown in Table 7.2.

Table 7.2 Physicochemical parameters of rhizospheric and non-rhizospheric soil samples from the contaminated site (Site 1) and reference site (Site 2)

	Site 1	Site 2	Guideline values*	
			A	R
EC (mS cm^{1})	41.2	5.4		
pH	2.6	7.5		
Cations [mg kg^{-1}]				
Ca^{+2}	nd	1787.6		
Mg^{+2}	nd	73.0		
Na^{+}	128.8	4046.8		
Anions [mg kg^{-1}]				
CO_3H^{-}	13538.1	12226.4		
Cl^{-}	18981.7	2801.3		
SO_4^{-2}	nd	96.06		
N [mg kg^{-1}]	256.0	241.0		
P [mg kg^{-1}]	6.0	46.0		
K [mg kg^{-1}]	34.0	160.0		
As [mg kg^{-1}]	6516.3	20.3	20	30
Cu [mg kg^{-1}]	239.5	17.7	150	100
Cd [mg kg^{-1}]	75.9	0.8	3	5
Zn [mg kg^{-1}]	1122.6	78.19	600	500
OM [%]	0.99	0.2		
C/N	22.0	4.0		
Gr [%]	nd	30.6		
VS [ml g^{-1}]	78	84		
Texture	sandy loam	sandy loam		

A: agricultural use. **R:** residential use. nd: no detected. * Argentine Law no. 24,051.

The results of the bioavailability analysis of metal(loid)s are shown in Figure 7.7.

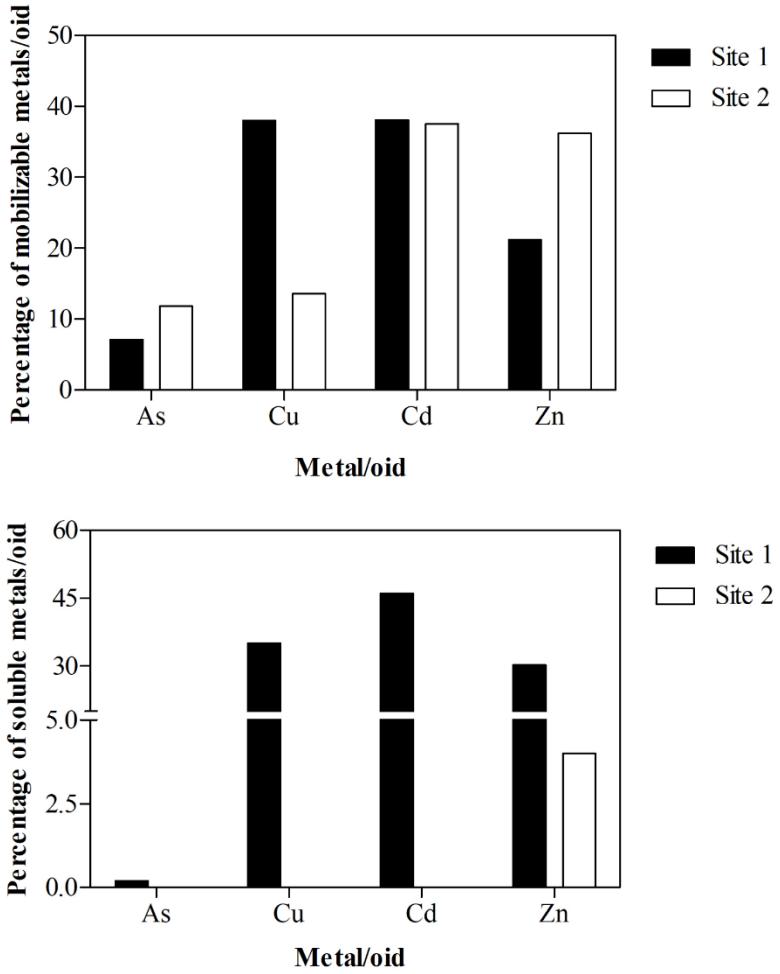


Figure 7.7 Percentage of extractable and available metal(loid)s in relation to total concentration

7.5.2 Acute exposure

Table 7.3 shows the total concentrations of metal(loid)s in each acute exposure treatment.

Table 7.3 Total metal(loid) concentration in each acute exposure treatment

Treatment	As	Cd	Cu	Zn
0%	nd	nd	nd	46.04
10%	626.36	1.13	27.86	1,032.31
25%	1,619.17	17.26	59.75	2,948.37
50%	3,060.54	40.19	114.10	5,489.60
70%	4,568.55	54.72	176.99	7,625.90
100%	6,608.27	89.78	259.81	10,891.99

nd: no detected.

Toxicological endpoints and phytotoxicity indexes are shown in Figures 7.8-7.13 and Table 7.4.

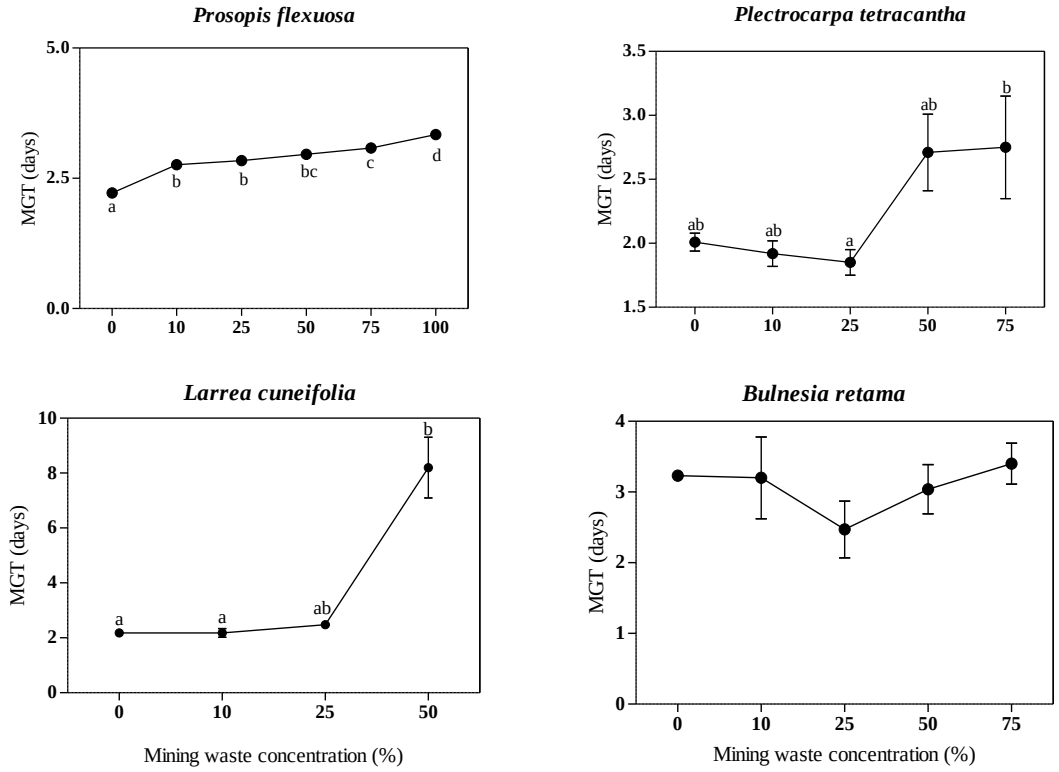


Figure 7.8. Mean (± SE) value of the mean germination time (days) in relation to the concentration of contaminated soil. Different letters indicate significant differences ($p<0.001$) between treatments.

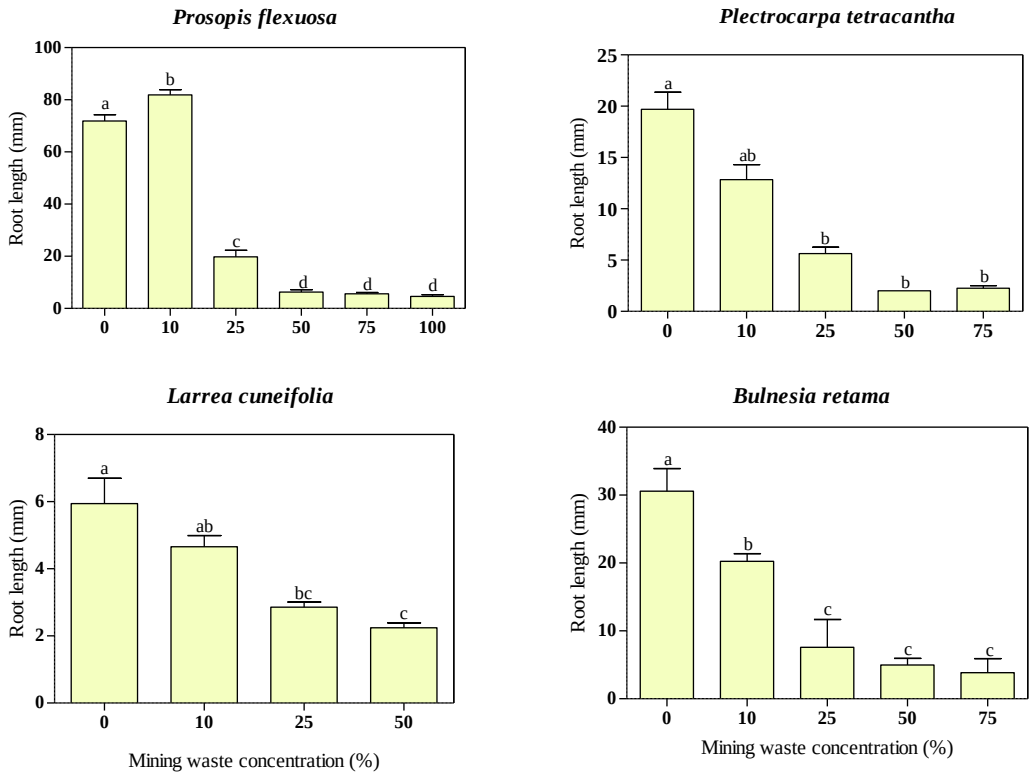


Figure 7.9. Mean (± SE) root length (mm) in relation to the concentration of contaminated soil. Different letters indicate significant differences ($p<0.001$) between treatments

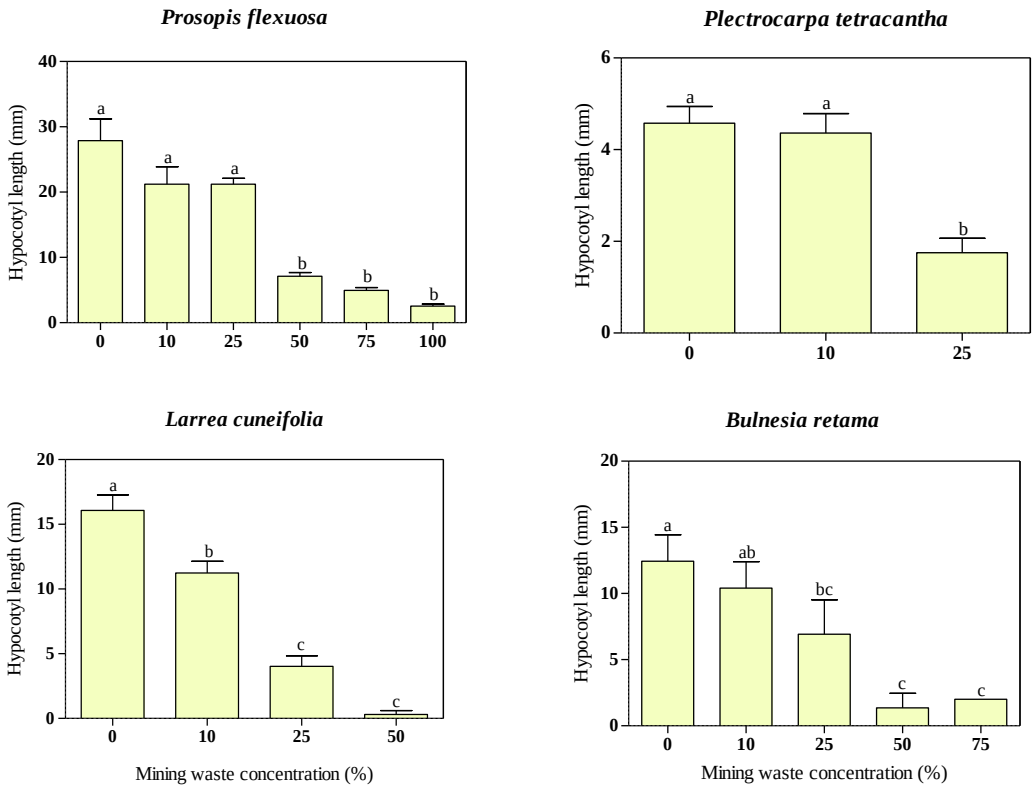


Figure 7.10. Mean (± SE) hypocotyl length (mm) in relation to the concentration of contaminated soil. Different letters indicate significant differences ($p<0.001$) between treatments

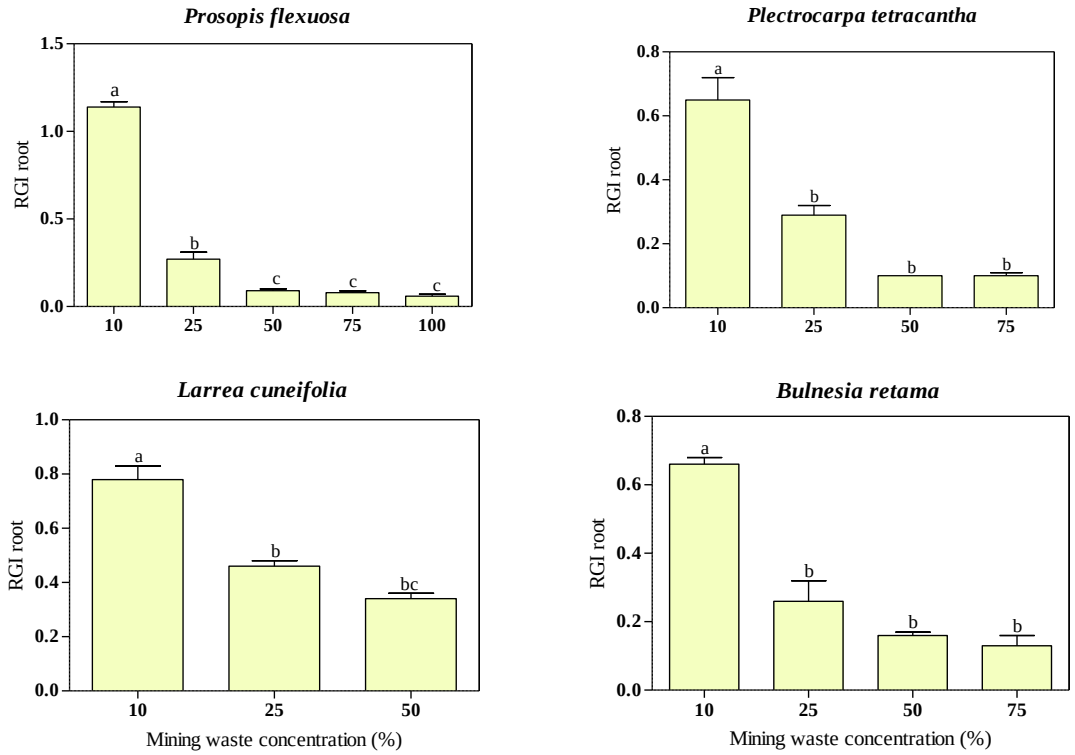


Figure 7.11. Mean (± SE) value of the root relative growth index in relation to the concentration of contaminated soil. Different letters indicate significant differences ($p<0.001$) between treatments

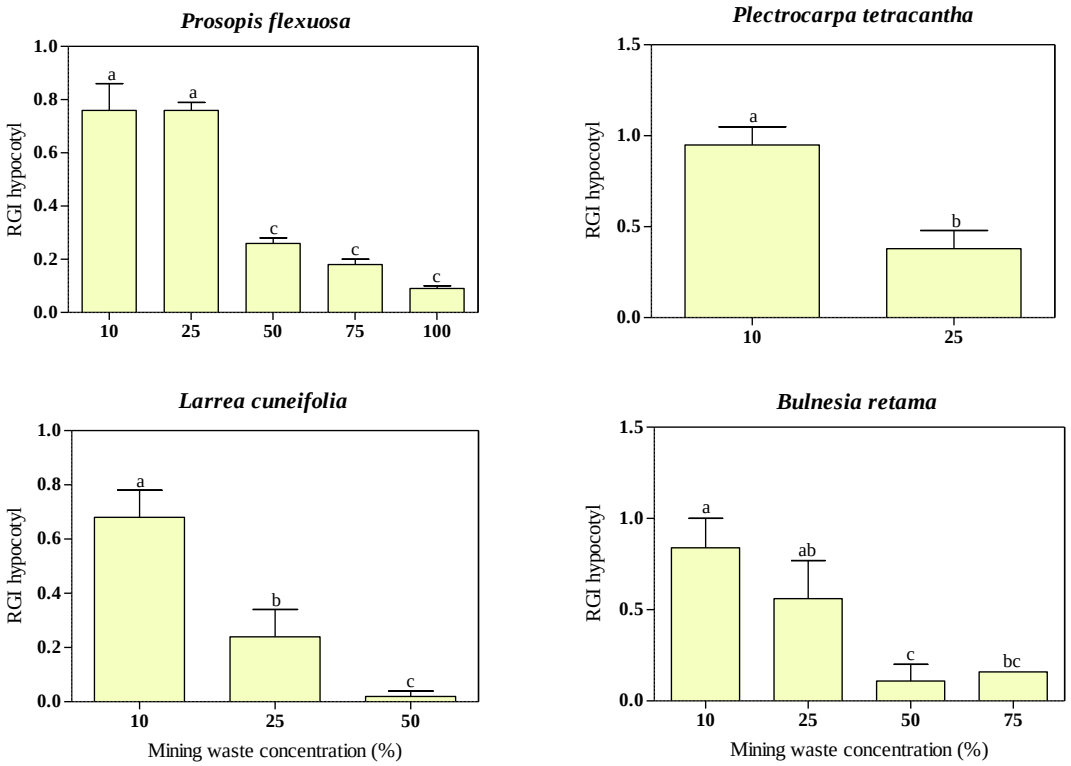


Figure 7.12. Mean (± SE) value of the hypocotyl relative growth index in relation to the concentration of contaminated soil. Different letters indicate significant differences ($p<0.001$) between treatments

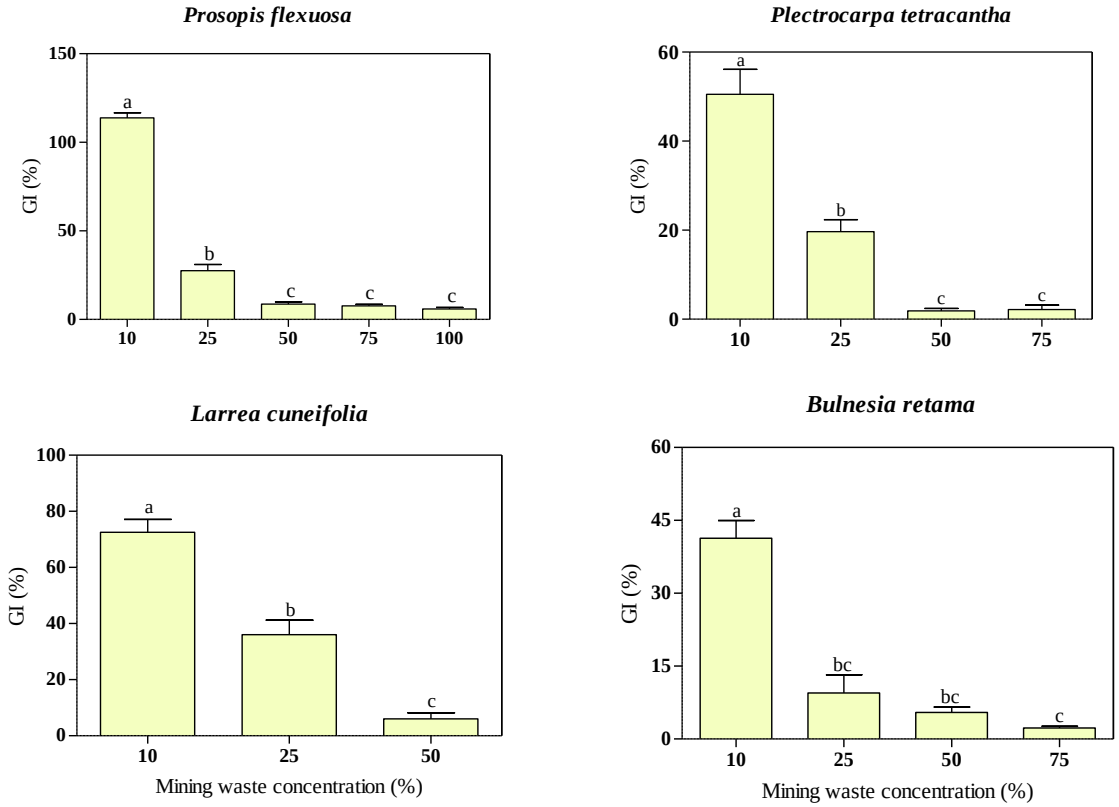


Figure 7.13. Mean (± SE) value of the germination index (%) in relation to the concentration of contaminated soil. Different letters indicate significant differences ($p<0.001$) between treatments

Table 7.4 Toxicological endpoints estimated for acute toxicity test

Endpoint	*P. flexuosa*	*P. tetracantha*	*L. cuneifolia*	*B. retama*
Seed germination inhibition				
NOEC	nd	25%	25%	<10%
LOEC	nd	50%	50%	10%
IC50	nd	35.64% (R^2=0.90)	33.74% (R^2=0.96)	15.19% (R^2=0.81)
Root length inhibition				
NOEC	10%	10%	10%	<10%
LOEC	25%	25%	25%	10%
IC50	21.1% (R^2=0.99)	16.12% (R^2=0.95)	27.70% (R^2=0.92)	14.71% (R^2=0.90)
Hypocotyl length inhibition				
NOEC	25%	10%	10%	10%
LOEC	50%	25%	25%	25%
IC50	40.29% (R^2=0.90)	21.89% (R^2=0.99)	15.1% (R^2=0.98)	28.39% (R^2=0.99)

nd: no determined (low toxicity).

7.5.3 Chronic exposure

Results are shown in Figures 7.14-7.16 and Tables 7.5-7.6. Stem elongation of all the evaluated species was inhibited at 10% of contaminated soil after 75 d of exposure. All the species tested did not survive at concentrations higher than 10% of the contaminated soil.

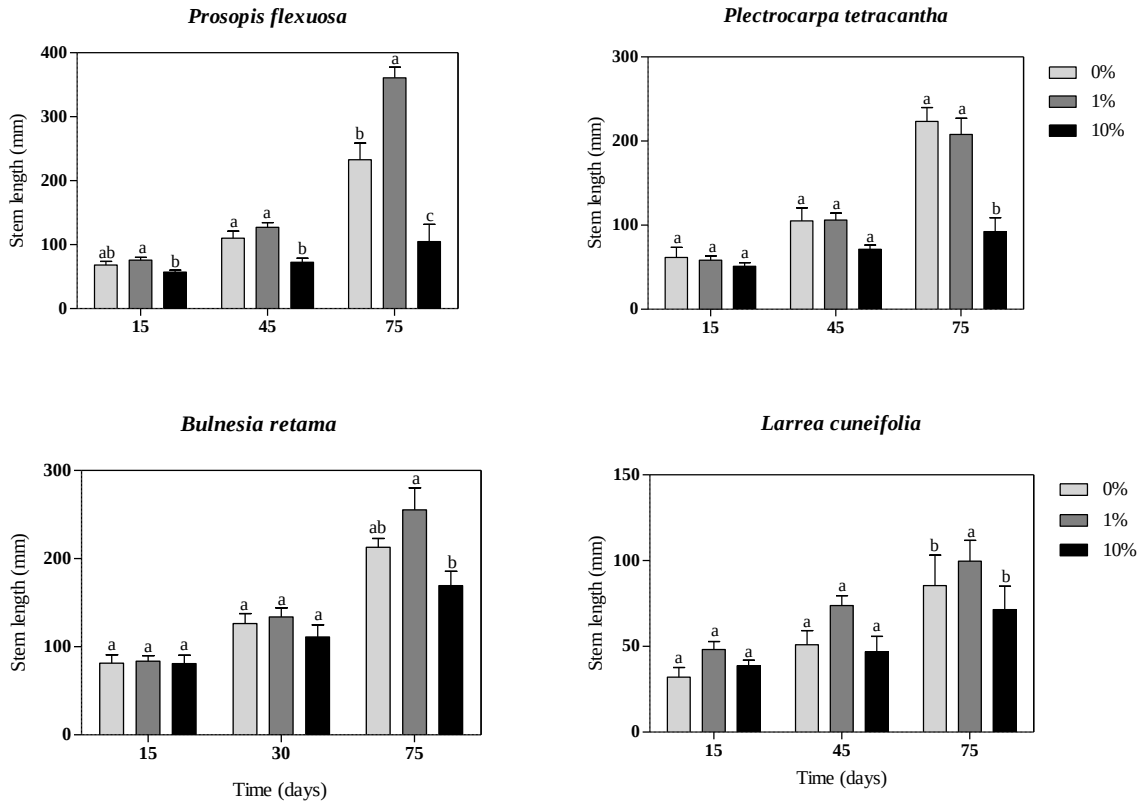


Figure 7.14. Mean (± SE) stem length in different exposure times. Different letters indicate significant differences ($p<0.05$) between treatments for each exposure time

Table 7.5 Toxicological endpoints estimated for stem length

Endpoint	*P. flexuosa*	*P. tetracantha*	*L. cuneifolia*	*B. retama*
15 days				
NOEC	10%	10%	10%	10%
LOEC	>10%	>10%	>10%	>10%
45 days				
NOEC	1%	10%	10%	10%
LOEC	10%	>10%	>10%	>10%
75 days				
NOEC	1%	1%	10%	10%
LOEC	10%	10%	>10%	>10%

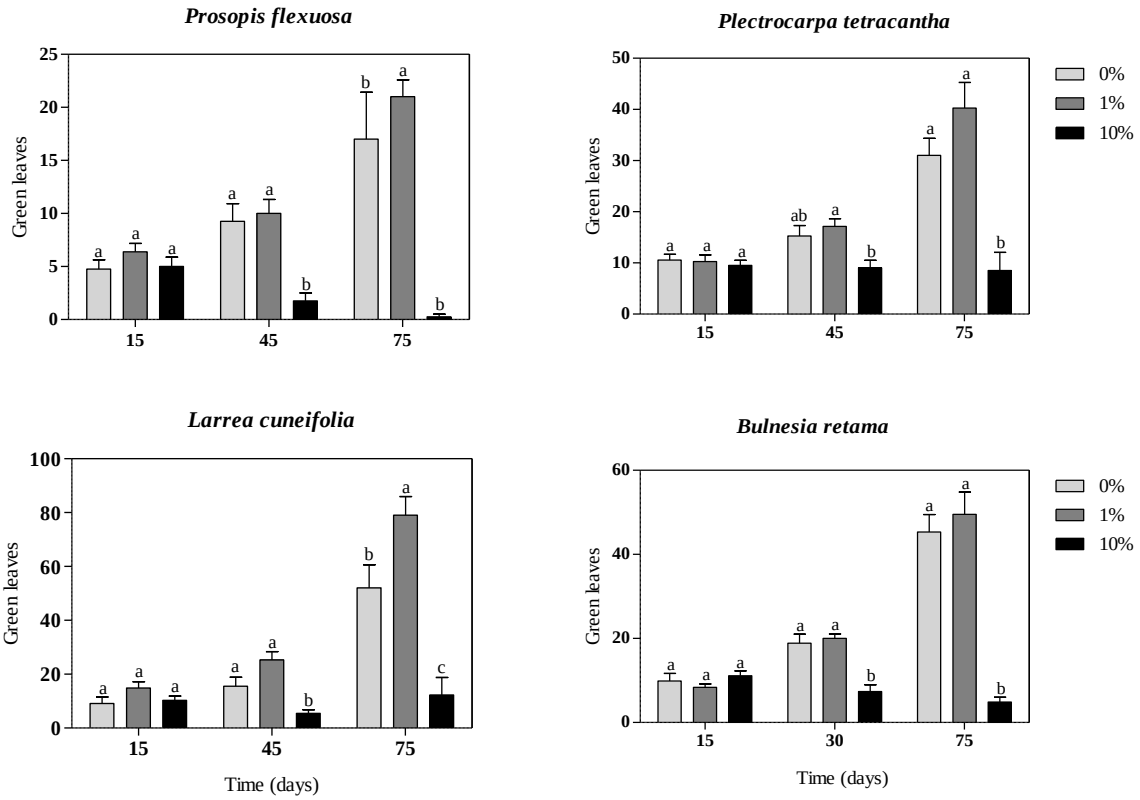


Figure 7.15. Mean (±SE) number of green leaves in different exposure times. Different letters indicate significant differences ($p<0.05$) between treatments for each exposure time

Table 7.6 Toxicological endpoints estimated for number of green leaves

Endpoint	*P. flexuosa*	*P. tetracantha*	*L. cuenifolia*	*B. retama*
15 days				
NOEC	10%	10%	10%	10%
LOEC	>10%	>10%	>10%	>10%
45 days				
NOEC	1%	10%	1%	1%
LOEC	10%	>10%	10%	10%
75 days				
NOEC	<1%	1%	<1%	1%
LOEC	1%	10%	1%	10%

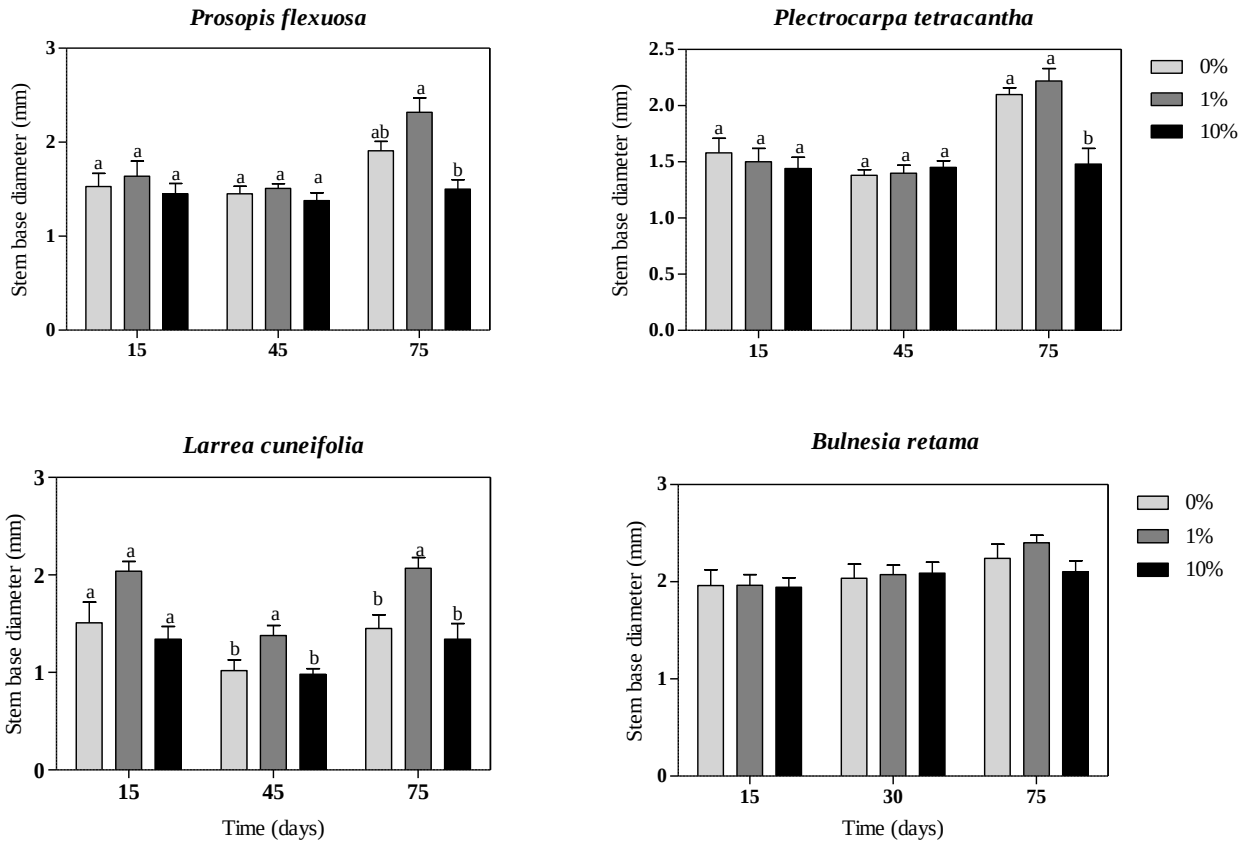


Figure 7.16. Mean (±SE) stem base diameter in different exposure times. Different letters indicate significant differences ($p<0.05$) between treatments for each exposure time.

Table 7.7 Toxicological endpoints estimated for stem diameter

Endpoint	*P. flexuosa*	*P. tetracantha*	*L. cuneifolia*	*B. retama*
15 days				
NOEC	10%	10%	10%	10%
LOEC	>10%	>10%	>10%	>10%
45 days				
NOEC	10%	10%	10%	10%
LOEC	>10%	>10%	>10%	>10%
75 days				
NOEC	10%	1%	10%	10%
LOEC	>10%	10%	>10%	>10%

7.5.4 Chronic exposure of contaminated soil amended with dolomite and compost

After soil incubation, pH and EC were determined (Table 7.8).

Table 7.8 Mean (± SE) values of pH and conductivity at each treatment

Treatment	pH	EC (mS cm^{-1})
T0	4.1 ± 0.3	4.2 ± 0.5
T1	5.8 ± 0.1	3.8 ± 0.2
T2	6.5 ± 0.03	3.6 ± 0.2
T3	6.4 ± 0.1	3.7 ± 0.2

EC: electrical conductivity

Using the pH data obtained in the different concentrations of dolomite, a linear regression analysis was done (Figure 7.12). We are using 18.5% of dolomite plus 5% of compost in the chronic exposure with amendments at the moment. This experiment is being carried out under greenhouse conditions. Until now, plants look healthy.

7.6 Conclusions

The series of experiments conducted allow us to conclude that the chronic exposure to contaminated soil causes mortality of the plants at concentrations higher than 10 %. This effect was reverted by the use of dolomite and compost. Also, the bioavailability of metal(loid)s was reduced due to the increase of the pH value, which reduce the inhibition in the plant growth.

8. INDIAN SITE POT TRIALS EXPERIMENTAL PLAN

Due to the COVID situation, partners from the Indian site were not able to provide their contributions to D2.2. Therefore, the following section is not completed at the time the first version of this deliverable is submitted. It will be completed once a contingency plan will be defined.

9. ACKNOWLEDGEMENTS

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