EDAPHOS

# D3.1 Optimized plant/microbe assemblages

## WP3 – Task 3.1

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Authors: M. Lebrun (UMLP), L. Ciadamidaro (UMLP), M. Chalot (UMLP), Y. Boisson (UMLP), F. Tatin-Froux (UMLP) P. Madejon (CSIC), E. Madejon (CSIC), W. Zegada Lizarazu (UNIBO), K. Iordanoglou (CRES), A. Zgorska (GIG), H. Castillo-Gonzales (UMLP), E. Alexopoulou (CRES), N. Manier (INERIS), J. Parelle (UMLP), P. Welters (PHYTOWELT), G. Felix (PHYTOWELT), G. Karakatsanis (EVO).

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# Executive summary

Deliverable D3.1 - *Report on optimized plant/microbe assemblage* - is the first deliverable of WP3 - *Implement and demonstrate the effectiveness of NBS* at EU contaminated soils of the EDAPHOS project. D3.1 is a result of Task 3.1 - *Plant and microbe selection greenhouse experiments* – and Task 3.2 - *Upscaling of crops into NBS field testing (including plant microbe consortium)*.

This document intends to give a compilation of the data coming from greenhouse and field tests, performed with the aim to: (i) select the most appropriate poplar hybrid for each of the seven case studies (CSs) (CS1, CS2, CS3, CS4, CS5, CS6, and CS7) at laboratory scale; (ii) establish the growth capacities of the companion species on the contaminated soils coming from the 7 CSs at laboratory scale; (iii) improve phytoremediation capacities of the tested species (poplar and companion species) by the addition of biological (microbial strain consortia) and organic (*e.g.*, vinasse, activated carbon) amendments at laboratory scale; and (iv) confirm the results obtained under greenhouse conditions by a preliminary field experiment.

The overarching goal of Work Package 3 (WP3) is to select crops and microbes to decontaminate polluted soils. In this regard, EDAPHOS partner UMLP has been characterizing bacterial and fungal communities associated with soil and roots of relevant plants at 2 CSs (CS1 and CS6) to explore their diversity and function in contaminant sequestration (T3.1). The EDAPHOS project, via WP3, will design and enhance plant/microbial systems based on an agroforestry concept to optimize biological performances and improve natural pathways and activities to safeguard and ameliorate soil quality and fertility. The microbial consortium inoculation will be optimized, taking into consideration the specificities of the polluted ecosystems and testing a novel strategy based on the production of site-specific microbial consortia rather than using commercial inoculums that may be not adapted to the site conditions. The most promising microbes, based on the characterization and feedback from the computational analysis (performed in the frame of the HE BIOSYSMO project), are used to establish a collection dedicated to contaminated soils (M3.1). They are initially tested under greenhouse conditions as consortium inoculated to the selected plants (T3.1) and will be later tested in the field (T3.3).

The results obtained within WP3 -Tasks 3.1 and 3.2- will then be used as key input data for the implementation of the field experiment in which monitoring of soil, plant and microbial indicators will be followed (Task 3.3) and a portfolio of site-specific best practices and Living Lab Experimental Sites implementation will be proposed (Task 3.4 – Deliverable 3.4). Collected data also serves other WPs of the project.

Finally, the obtained data will enable us to establish the effectiveness of Nature Based Solutions (NBS) proposed for the 7 CSs within the project and provide site-specific best practices for other contaminated sites across Europe for the different stakeholders.

# Keywords

Poplar hybrid, Companion species, Microbial inoculation, Heavy metals, Amendments.

# Abbreviations and acronyms

| Acronym | Description |
|---|---|
| ABA | Abscisic acid |
| ACC | 1-aminocyclopropan-1-carboxylic acid |
| Ag | Silver |
| As | Arsenic |
| BTEX | Benzene, toluene, ethylbenzene, xylenes |
| Cd | Cadmium |
| Co | Cobalt |
| Cr | Chrome |
| CS | Case study |
| Cu | Copper |
| EPS | Exopolysaccharide |
| ERA | Ecological risk assessment |
| ESS | Ecosystem Service |
| EU | European Union |
| GA | Gibberellic acid |
| Hg | Mercury |
| HM | Heavy metal |
| IAA | Indole-3-acetic acid |
| Mn | Manganese |
| NBS | Nature Based Solution |
| Ni | Nickel |
| OM | Organic matter |
| PAH | Polyaromatic hydrocarbon |
| PGP | Plant growth promoting |
| Pb | Lead |
| SOD | Superoxide dismutase |
| UMLP | Université Marie et Louis Pasteur |
| WP | Work Package |
| Zn | Zinc |

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# 1 Introduction

## 1.1 The overall concept

Soils are the backbone of healthy and vibrant ecosystems, providing substrates together with physical, chemical, and biological functions essential for life. But soils are constantly threatened by pollution, creating a significant risk to human health together with terrestrial and aquatic ecosystems. With the emergence of new products (*e.g.,* nanoparticles, pharmaceuticals, pesticides), new questions arise in the field of terrestrial and aquatic ecotoxicology research, as well as concerning the disruption of ecological systems' behavior. Furthermore, the continued development of human activities and the effects of climate change are expected to generate additional pressures. Between 1970 and 2008, global biodiversity has declined by 30 %, with no signs of slowing down nowadays. This represents a faster and unprecedented rate of decline. The total annual economic cost of biodiversity loss and ecosystem degradation was estimated at between 2400 and 4500 billion US dollars in 2008 (*Biodiversity Lost and Ecosystem Degradation*). Therefore, halting biodiversity loss and the degradation of ecosystem services (ESSs) remains one of the main goals of the European Union (EU) (*Biodiversity Strategies – Environment*). Today, EU's long-term policy is increasingly adopting a more systemic perspective, linking environment, health, and human well-being (*Europe's Environment – The Fourth Assessment*). It is thus necessary to find NBS that are cost-effective and that simultaneously provide environmental, social, and economic benefits, while helping to strengthen ecosystem resilience (*Nature-Based Solutions – Environment;* Triest *et al.,* 2016).

The EU Soil Strategy 2030 sets out a framework of concrete measures to protect, restore and ensure that soil is used sustainably. It sets out a vision and targets to achieve healthy soils by 2050, with concrete actions to be implemented by 2030. Phytoremediation strategies represent an attractive NBS to other conventional physical and chemical remediation technologies, due to their relatively low cost, potential effectiveness and environmental benefits, and the inherently aesthetic aspect of using plants to clean up contaminated sites, particularly in situations where there is no immediate time pressure (Pilon-Smits, 2005). Nowadays, one of the main strategies for soil decontamination is phytoextraction, *i.e.*, the use of herbaceous and woody plants to extract heavy metals (HMs) from soil, which however faces some limitations to become successful at commercial scale and then, often generate skepticism regarding its usefulness (Payá & Rodriguez, 2018). To be successful, the phytoextraction process must be adapted and optimized to the local conditions. A recent paper highlighted that soil element depletion occurred for a range of HMs, after 10 years of poplar growth (Chalot *et al*., 2020). This approach influences positively other ESSs, providing valuable sources of renewable biomass for the bio-based- economy, *e.g.,* bioenergy, bio-catalysis and platform molecules, and eco-materials (Kidd *et al*., 2015).

Accordingly on previous statements, ideal plant species for phytoextraction should be hardy, produce significant biomass, tolerate the toxic effects of HMs and other contaminants, be easy to cultivate and harvest, have a high uptake capacity, and not be attractive to herbivores (Adesodun *et al.*, 2010; Sahabani & Sayadi, 2012; Sakakibara *et al*., 2011). In many cases, plant species alone prove insufficiently effective and require aids to enhance their phytoremediation performance. These aids include soil amendments such as biochar and compost (Paz-Ferreiro *et al*., 2014; Huang *et al*., 2016), microbial inoculation (Mench *et al*., 2010) or even transgenic transformation of plants (Abhilash *et al*., 2009; Kawahigashi, 2009). These interventions support the phytoextraction process by reducing pollutant toxicity, increasing their bioavailability, and/or stimulating plant growth. However, comprehensive information is still lacking regarding the optimization of each phytoextraction mechanism's contribution—through appropriate plant selection—and on the means to improve the performance of phytoextraction strategies.

Over the past two decades, there has been a growing use of various tree/plant species in combination with microbial biotechnologies (Mench *et al.*, 2010). Bioremediation is increasingly recognized in developed countries due to the strong connections between pollutants from industrial, agricultural, and urban activities (Adriano, 2001). Several studies have highlighted the benefits of symbioses between microorganisms and plants cultivated on sites contaminated by HMs, aiming to promote biotechnological applications such as phytoremediation (Gadd, 2010; Kozdrój *et al*., 2007; Krupa & Kozdrój, 2007). It is now widely acknowledged that rhizospheric microorganisms can play an active role in phytoremediation processes and may be useful in expanding this approach to other polluted sites (Kuffner *et al.,* 2008; Moreira *et al*., 2016). Indeed, studies have shown that associations with mycorrhizal fungi promote plant growth in contaminated soils (Ciadamidaro *et al*., 2017), improve the vitality of habitats disturbed by HMs, and reduce HM toxicity by modulating their uptake and accumulation in plant tissues (Phanthavongsa *et al.,* 2017). More specifically, mycorrhizal fungi enhance plant resistance to unfavorable conditions, including HM-induced toxicity, by influencing the bioavailability of HMs in the soil and facilitating their absorption and translocation from root to leaf (Yilmaz & Perlak, 2011; Meier *et al.,* 2012). Therefore, selecting appropriate tree species and microorganisms is essential to optimize phytomanagement strategies (Ciadamidaro *et al.,* 2017). Adaptability and effectiveness under complex environmental conditions often limit the success of fungal inoculation in the field (Hart *et al.,* 2017; O'Callaghan *et al.,* 2022). To reduce the risk of inoculant poor performance, one effective approach is to prepare a synthetic consortium that, unlike single-strain inoculants, capitalizes on functional complementarity and microbial interactions to enhance metabolic versatility and ecological resilience (De Lorenzo, 2008; Thomloudi *et al.,* 2019). Indeed, microorganisms behave differently in pure cultures compared to when they exist within complex communities. Moreover, different fungal species may produce distinct enzymes and metabolites that collectively promote nutrient solubilization, pathogen suppression, and pollutant degradation (Lacercat-Didier *et al.,* 2016; Sabra *et al.,* 2018). The cooperative interactions within fungal consortia can expand the overall catabolic potential beyond the sum of individual members by sharing diffusible metabolic intermediates and coordinating complementary biochemical pathways (Cecchi *et al.,* 2021; De Lorenzo, 2008). This metabolic network not only improves contaminant breakdown but also strengthens the plant's ability to withstand abiotic stresses, such as HM toxicity and drought (Lacercat-Didier *et al.,* 2016; Rehmann *et al.,* 2022).

## 1.2 Objectives of the work related to D3.1 and links with other WPs and other Deliverables

In EDAPHOS, the integration of multiple plant layers using HM-accumulating trees, HM-(hyper)accumulating and HM-tolerant herbaceous plants in co-cropping strategies will represent an innovative research pathway towards the development of a comprehensive strategy for soil restoration. Poplars will be co-cropped with (hyper)accumulating plant species for accelerated demonstrations of the agroforestry solution, using well-known plant species (*e.g*., *Chrysopogon zizanoides*) as well as underutilized species such as *Lablab purpureus* reported to be tolerant to several HMs (Ruthrof *et al.,* 2018) and qualified as a climate smart crops (Rai *et al.,* 2021), and *Brassica juncea* reported to (hyper)accumulate HMs (Visconti *et al.* 2020).

The use of plant microbial communities in a bioremediation strategy allows to tackle multiple contaminations as faced in real environments since microbes are able to degrade organic contaminants and accumulate organic ones, while some plants are experts in decreasing HM soil content by extracting the pollutants from the soil. According to this statement, WP3's overall scope is to create the basis for a sustainable remediation strategy of contaminated lands that support the provision of ESSs.

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One of the specific objectives is to select crops and microbes to decontaminate polluted soils. In this goal, EDAPHOS partner UMLP is characterizing bacterial and fungal communities associated to soil and roots of plants of interest coming from 2 CSs (CS1 and CS6) to explore their diversity and function in contaminant dynamics (T3.1). The microbial consortia inoculation has been optimized in the on-going Horizon project BIOSYSMO, taking into consideration the specificities of the polluted ecosystems and testing a novel strategy based on the production of site-specific microbial consortia rather than using commercial inoculums that may be not adapted to the site conditions. The most promising microbes, based on the characterization and feedback from the computational analysis performed within the project BIOSYSMO, are being used in EDAPHOS to test them in the 2 CS soils (M3.1). The microbial strains have been tested under greenhouse conditions as consortia inoculated to the selected co-cropping plants (T3.1), and will be later tested in field (T3.2).

The EDAPHOS project via WP3 aims to design and enhance plant/microbe systems based on an agroforestry concept to optimize biological performances and improve natural pathways and activities to safeguard and ameliorate soil quality and fertility. This WP3 is tighly linked to four other WPs of the project (more details about such link is given in section 0):

- **WP1**. The field and laboratory measurements of plants (*e.g.*, pigments, HM concentrations) are confronted to the multispectral and hyperspectral measures, to build empirical and prediction models.
- **WP2**. The initial and regular samples of soils are and will be used for ecological risk assessment (ERA) (D2.1 & D2.2), and to determine key ecotoxicological indicators to monitor soil health and NBS performance (D2.3).
- **WP4**. All the data acquired during the greenhouse and field tests are used to perform socio-economical assessments of the polluted soils and their rehabilitation (D4.1).
- **WP5**. All the data will be computed to build a decision support tool (D5.1).

Within the EDAPHOS project, in WP3, we are developing two approaches:

- *Laboratory tests* (T3.1). Greenhouse trials were conducted to select the hybrid poplar species best suited to each CS. The companion species were also tested in controlled greenhouse conditions to ensure their capacities to adapt and develop in soils coming from our CSs. In addition, optimization of the phytoextraction was tested on the 2 French CSs (CS1 & CS6) using microbial inoculation, with two consortia elaborated based on their properties and compatibility, and amendments, especially vinasse, which is a waste material rich in sulphur and able to acidy soil and thus mobilize HMs.
- *Field tests* (T3.2 & T3.3). Partners responsible for site management performed the site preparations, and take care of the site maintenance during the project. To ensure uniform and efficient monitoring of CS, protocols and templates were shared with all site managers (CS leaders). A preliminary field test was performed for a short period to test NBS at five CSs. For the final field test, poplars plantations were set up on all CSs, in late winter in Southern countries and in early spring in Western and Central Europe CS.

This document will first give background information on the biological materials used in the WP3, then present the methodology used to answer the objectives, and finally present the results.

# 2 Background information on plant species and microbial strains

## 2.1 *Populus* sp.

*Populus spp.,* also known as poplar, aspen, or cottonwood, is a genus belonging to the Salicaceae family, and composed of about 25 to 30 species. Poplars are fast growing deciduous trees, who originate from the Northern hemisphere, more specifically the temperate and boreal regions. The general characteristics of poplars are summarized below:

- **Family:** Salicaceae;
- **Life form:** Fast-growing deciduous trees;
- **Main use:** Biomass production, pulpwood, land reclamation, and wood-based manufacturing;
- **Used plant part:** Primarily stem wood and bark;
- **Growth habit:** Upright with extensive root systems and suckering capacity;
- **Height:** 15–50 meters;
- **Bark:** Smooth and light-colored with prominent lenticels in young trees; rough and fissured with age in some species;
- **Leaves:** Spirally arranged; variable in shape (triangular, circular, lobed); long petiole
- **Reproduction:** Both sexually (seeds) and asexually (suckering and cuttings).

Thanks to their wide distribution, high adaptability and fast biomass production, poplars have a significant economical and ecological impact. More specifically, poplars have a rapid growth and are easy to propagate, thus they are often selected in short rotation coppice forestry systems as well as in restoration projects (Hall & Heybroek, 1997). It is particularly the case in Europe, North America and Asia, where several species of poplar are cultivated for forestry, biomass and industrial uses (Fang *et al.,* 1999). Poplars are depicted by a high genetic and morphological diversity, with individuals that can go from medium size trees to large specimens of more than 40 m height and 2.5 m trunk diameter. In addition to this important aerial biomass, which has an economic value, poplars are also characterized by a vigorous root system, extending up to 40 m from the trunk. They can reproduce clonally, through root suckers or branches. Those properties allow for poplars to colonize large areas and to form extensive clonal stands (Eckenwalder, 1996; Pregitzer, 2008), reinforcing their interest in forestry and industrial uses. In Phytowelt, a poplar breeding program has been developed, with a high degree of hybridation, offering a large potential to not only identify but also develop genotypes with enhanced stress tolerance (*e.g.,* drought, pollution), making those hybrids/genotypes suitable for a wider range of environmental conditions.

To implement a sustainable poplar plantation, one must first handle and plant the cuttings properly. The best time to plant poplar cuttings is through the spring season, after the risk of frost has passed. This will limit the stress towards the plants and ensure optimal regrowth conditions. Poplars have a high demand for both water and sunlight, therefore, plantations should be established in open fields and with the least competing vegetation possible. Such precautions reduce the competition for resources and thus maximize early plant growth. Another preventive measure to take when establishing a poplar plantation concerns the depth of cutting planting. Cutting lengths vary from 20 cm to 100 cm depending on the soil conditions, and should be buried about two-thirds into the ground, with at least one viable bud above soil surface. Regarding the maintenance of the plantation, regular mowings between rows should be made to suppress any competing grass and to ensure that poplars receive enough sunlight and water.

In terms of long-term sustainability, poplar exhibits a strong regenerative capacity. Trees up to five years old can be coppiced, cut down near the base, stimulating the development of multiple new shoots. Repeated cutting promotes a bushier growth form, resulting in a higher number of stems. Over time, this not only increases biomass yield but also enhances the plant's ability to take up HMs and other pollutants from the environment. Consequently, repeated coppicing in short rotation cycles

contributes both to sustainable biomass production and to improved phytoremediation efficiency. However, this cannot be tested in EDAPHOS as the duration of the project is less than a rotation period (4 to 7 years for poplars).

Phytowelt has successfully developed non-genetically modified hybrid poplars, with different genotypes, including diploid, triploid (3n), tetraploid (4n), and somatic hybrid lines recovered from *in vitro* cultivation. These genotypes show promising potential for improved phytoremediation performance. The hybrids are produced through somatic hybridization, employing protoplast electrofusion as an innovative alternative to conventional breeding. The resulting somatic hybrids incorporate high growth potential from *P. maximowiczii*, superior wood quality from *P. nigra*, and drought resistance and low soil nutrient requirements from *P. alba*. Electrofusion of protoplasts, as established by Phytowelt, enables the generation of stable somatic hybrids with low incidence of mixoploidy. In field trials, these cultivars outperformed the Max4 control line in terms of vegetative growth, stem height, and leaf area, indicating an increase in photosynthetic capacity. Similar to the Max4 parent, these hybrids are capable of vegetative propagation through wood cuttings. Although these hybrid genotypes have demonstrated strong agronomic traits, their performance under soil contamination stress has not yet been evaluated. Given the contribution of *P. alba* to their genome, these somatic hybrids are expected to exhibit tolerance to poor soil conditions and HM contamination, making them strong candidates for phytoremediation applications.

## 2.2 Companion species

In addition to the tree layer, another one can be implemented, such as the herbaceous layer. One of the advantages of this layer is that it covers a wider surface on the soil. Moreover, many herbaceous species have been described to be tolerant to HMs and capable of accumulating them. When co-cropping systems are used, mixing both trees and herbaceous plants, we can choose to assemble trees with hyperaccumulator plants and/or nitrogen-fixing plants, which will enrich the soil.

- One known hyperaccumulator is *Brassica juncea*, a natural hybrid between the black mustard and the turnip mustard, which is also widespread in Europe. It is commonly grown for oil production and condiments (Nepal *et al.* 2024), but it also has an important biomass production, which could be used for bioenergy production (Jeyasundar *et al.* 2021). Published studies have demonstrated its tolerance to HMs as well as its (hyper)accumulating capacities towards arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) (Clemente *et al.,* 2005; Hsiao *et al.,* 2007; Huang *et al.,* 2020; Jeyasundar *et al.,* 2021; Picchi *et al.,* 2022; Visconti *et al.*, 2020).
- *Lablab purpureus* is a legume plant which can tolerate stress, including HM-stress, and can adapt to many soil conditions such as intense drought (Aguilar-Garrido *et al.,* 2023). Although yet underutilized, few studies demonstrated its potential in phytoremediation (Aguilar-Garrido *et al.,* 2023; Meyer *et al.,* 2024).
- *Chrysopogon zizanoides* is a hydrophilic plant highly used in wastewater treatment to remove in particular nitrogen and phosphorus but also HMs (Mahmoudpour *et al.,* 2021). It has the advantage of being tolerant to harsh conditions and to grow particularly well under elevated temperatures (25 °C to 30 °C), which makes it a suitable candidate in Mediterranean hot countries, such as CS5. Although studies were mainly performed in the context of wastewater treatment, the potential of vetiver to accumulate HMs (Kiiskila *et al.,* 2019; Masinire *et al.,* 2021; Otunola *et al.,* 2023) could be taken to our advantage for the phytomanagement of polluted soils.

## 2.3 Microbial strains

### 2.3.1 Introduction

Bioremediation is highly recognized for the rehabilitation of contaminated soils. Microorganism can form symbiosis with plants and play an active role in the dynamics of soil pollutants (*e.g.*, transformation, accumulation, (im)mobilization). Furthermore, they are known to help plants develop faster and to produce more biomass. This makes them good candidates in phytomanagement systems.

Our study takes advantage of the work made within the BIOSYSMO project (D3.1 and 3.5, publicly available on August 31st, 2025) in the isolation of the strains from the soil, their characterization and the elaboration of the consortia.

### 2.3.2 List of the microbial strains selected and literature review

Candidate strains were prioritized based on a comprehensive set of relevant traits, including tolerance and transformation of HMs such as Cd, Zn, and Pb; plant growth promoting (PGP) activities, such as Indole-3-acetic acid (IAA) production, siderophore-mediated iron chelation, and phosphate solubilization; enzymatic capacities involving lignocellulolytic enzymes, laccases, and peroxidases critical for organic matter (OM) degradation and pollutant breakdown; symbiotic potential to establish endophytic relationships with poplar roots; non-pathogenicity ensuring absence of risk to humans, animals, or plants; and cultivability and scalability, demonstrated by robust growth under laboratory and greenhouse conditions facilitating practical inoculum production. Beyond functional screening, each strain was characterized morphologically (colony morphology, spore production) and genetically (Internal transcribed spacer). These methods confirmed taxonomic identity and provided insights into their functional repertoire.

All selected strains are known to exhibit rapid colonization and prolific spore production, enabling efficient lab-scale escalation and reliable rhizosphere establishment. According to relevant literature, these strains also express multiple PGP traits and potential for bioremediation, which are developed in Table 1.

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| UFC 003 |
| --- |
| Multiecological roles, *e.g.,* pathogenicity; limited direct pest control efficacy; biological control agent which efficacy depends on soil temperature, timing, and interactions with soil microbes.<br>Minimal and transient impact on the microbial communities, making it a low ecological risk.<br>Production of hormones, solubilization of nutrients.<br>Endophyte with plants, enhance drought resilience and improve plant growth and fruit quality (*e.g., Capsicum chinense*, papya). |
| Baraja-Méndez *et al.*, 2022 ; Canfora *et al.,* 2023 ; Chan-Cupul *et al.,* 2025; Jaber & Enkerli, 2016 ; Kessler *et al.,* 2003 ; Razinger *et al.,* 2014 ; Toscano-Verduzco *et al.,* 2019 |
| **UFC 006** |
| Production of IAA, ABA, ACC deaminase, and siderophore.<br>Enhancement of plant tolerance and root growth, nutrient uptake.<br>Accumulation and detoxification of HMs (*e.g.*, Cd).<br>Formation of long lasting association (*e.g.*, in grapevines), improving plant vigor. |
| Molnár *et al.,* 2023 ; Varga *et al.,* 2021 ; Wang & Zhuang, 2020 |
| **UFC 008** |
| Metal-tolerant ericoid mycorrhizal fungus.<br>Tolerance and accumulation of HMs (*e.g.*, intracellular Zn sequestration thanks to a specific Zn-efflux transporter).<br>Production of mucilage and pigments which help HM sequestration, reducing toxicity.<br>Endophytic behavior (*e.g.*, *Quercus ilex*), saprobe (*e.g.*, *Sphagnum* peat)<br>Synthesis of tryptophan, indole-3-pyruvate, IAA, jasmonic acid, salicylic acid, brassinolide-type compounds and plant cell wall degrading enzymes.<br>Secretion of extracellular phytases and proteases, which participle to the solubilization of phosphorus and other nutrients, promoting plant growth (*e.g.*, *Rhododendron spp., Vaccinium spp.*) |
| Bergero *et al.,* 2000 ; Chiapello *et al.*, 2015 ; Daghino *et al.,* 2025 ; Khouja *et al.*, 2013 ; Martino *et al.,* 2000 Mikheev et al., 2023 ; Rice & Currah, 2007 ; Wei *et al.*, 2020 ; Wei *et al.*, 2022 |
| **UFC 009** |
| Primarily found in forest ecosystems.<br>Significant medical, economic and ecological value.<br>Potential biocontrol agent (*e.g.*, inhibition of nematode reproduction).<br>Improvement of plant growth. |
| Chen *et al.,* 2023 ; Wang *et al.,* 2021 |
| **UFC 013** |
| Melanized ascomycetes, known for ecological versality and diverse lifestyle.<br>Occupation of a wide range of habitats (*e.g.*, plant, soil, polyextreme rock environment, BTEX-contaminated soil).<br>Utilize toluene and related hydrocarbons as a carbon and energy sources; complete mineralization of toluene.<br>High tolerance to HMs (*e.g.*, Pb, Cu).<br>High accumulation of Pb.<br>Improvement of plant growth and resistance (*e.g.*, strawberries, tomato).<br>Secretion of extracellular ferricrocin, contributing to iron acquisition.<br>Secretion of enzymes (*e.g.*, cytochrome P450 monooxygenases, laccases), participating to PAH degradation.<br>Some strains with pathogenic potential. |
| Bailão *et al.,* 2023 ; Badali *et al.*, 2011 ; Blasi *et al.,* 2017; Da Silva *et al.,* 2023 ; Das *et al.,* 2019 ; Feng *et al.,* 2013 ; Fu *et al.*, 2023 |
| **UFC 016** |
| Resistance to HMs, *e.g.*, Ni, Cu (energy dependent uptake and biomineralization).<br>Degradation of organic pollutants (*e.g.*, triphenylmethane dyes, PAHs).<br>Formation of synergistic biofilms with *Pseudomonas fluorescens,* enhancing HM uptake and plant growth. |
| Crusberg, 2004 ; Gadd & White, 1985; Rosatto *et al.*, 2019 ; Saraswathy & Hallberg, 2005; Shedbalkar *et al.*, 2008 ; Shedbalkar & Jadhav, 2011; Rosatto *et al.*, 2021 |

| UFC 017 |
| --- |
| Widely distributed (*e.g.*, China, Brazil, Germany, Italy, South Africa, Thailand). |

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| |
|---|
| Ecological versality (*e.g.*, endophytes in woody plants, saprobes in soil and litter, insect symbionts); some are known pathogens (*e.g.*, causing stem cankers in trees, infecting raspberry, causing leaf spot on date palm).<br>Production of bioactive compounds (*e.g.*, danthron, bergammotane sesquiterpenoids, furanones), having antibacterial properties.<br>Genomic studies predict over 650 carbohydrate-active enzymes:<br>• Strong capacity to synthetize, modify and degrade complex carbohydrates and glycoconjugates;<br>• Support of nutrient acquisition and hyphal growth;<br>• Selective catalytic transformations of environmental pollutants (*e.g.*, decolorization of synthetic dyes);<br>Oxidation/Reduction, *e.g.*, Mn (formation of Mn oxides), Se (formation of Se nanoparticles).<br>Accumulation of HMs, *e.g.*, Mn, Co, Cu (transformation of dissolved metals into solid biominerals).<br>PAH degradation.<br>Promotion of plant growth (*e.g., Lactuca sativa*).<br>Synthesis of IAA.<br>Antitumor, antibacterial and antifungal activities. |
| Almeida *et al.,* 2014 ;Anisha *et al.,* 2017 ; Ashrafi *et al.,* 2013 ; Azami *et al.,* 2025 ; Damm *et al.,* 2008 ; Doydora *et al.,* 2024 ; Fu *et al.,* 2022 ; Guarnaccia *et al.,* 2022 ; Guo *et al.,* 2015 ; Hkiri *et al.,* 2023 ; Korkmaz & Blumenstein, 2025 ; Ligoxiagakis *et al.,* 2013 ; Lu *et al.,* 2022 ; Mizradeh *et al.*, 2014 ; Rosenfeld *et al.,* 2020 ; Wang *et al.,* 2021 ; Yu *et al.*, 2013 |
| **UFC 019** |
| Soil dwelling fungus.<br>Production of enzymatic activities, participating to OM decomposition and nutrient mobilization.<br>*In vitro* antagonistic activity against phytopathogenic fungi, showing a potential for biocontrol application.<br>Detection of mycoviral elements, showing a potential opportunistic behavior. |
| Benítez *et al.,* 2004 ; Chen & Zhuang, 2017 ; Liu *et al.,* 2023 ; Odoñez Casso, 2023 ; Quispe Bautista, 2024 |
| **UFC 020** |
| Ecologically versatile fungi.<br>Production of bioactive secondary metabolites (*e.g.*, chaetoglobosins, azaphilones, xanthones, chaetochromones A & B, epipolythiodioxopiperazines) with antibacterial, antifungal, cytotoxic, antitumor and enzyme-inhibitory properties.<br>Many strains work as anti-pathogens.<br>Increase of available phosphorus and potassium, improving plant growth (*e.g., Brassica*).<br>Improvement of disease resistance (*e.g.*, sugarcane, maize, cucumber).<br>Tolerance and accumulation of HMs, *e.g.*, Cu, Zn, Pb, Ag (live-cell biosorption, bioaccumulation).<br>Degradation of PAHs via secretion of ligninolytic peroxygenase and laccases.<br>Secretion of enzymes (*e.g.*, manganese peroxidase, lignin peroxidase, laccase, peroxygenase) which participates in the degradation of textile dyes, the breakdown of recalcitrant plant biomass, and cellulose degradation. |
| Dwibedi *et al.,* 2023 ; Elshahawy & Khattab, 2022 ;Feng *et al.,* 2023 ; Goswami *et al.,* 2024 ; Hkiri *et al.,* 2023 ; Li *et al.,* 2020 ; Lu *et al.,* 2013 ; Manai *et al.,* 2016 ; Tian *et al.,* 2022 ; Yu *et al.,* 2025 |
| **UFC 026** |
| Versatile fungi exhibiting a dual endophytic-saprotrophic lifestyle and having ecological plasticity.<br>Phosphate solubilization, siderophore production, ACC deaminase activity, promoting plant growth, nutrient acquisition, mitigating drought stress, and increasing ecological resilience and plant protection.<br>Production of bioactive secondary metabolites (*e.g.*, antibiotics, isocoumarins, betulin, echinocandins) with antifungal and antibacterial properties. |
| Bruyant *et al.,* 2025 ; Dasila *et al.,* 2024 ; Lin *et al.,* 2022 ; Shu *et al.,* 2024 ; Yue *et al.,* 2018 |
| **UFC 035** |
| Production of bioactive secondary metabolites (*e.g.*, saintopin, leucinostatins) with anticancer properties, antibiotic activity.<br>Biodegradation of xenobiotics.<br>Strong antioxidant responses (upregulation of enzymes, *e.g.,* SOD, catalase, glutathione, ascorbate)<br>Tolerance & accumulation HMs, *e.g.*, Zn, Cu, Pb (binding to cell wall); detoxification of HMs (alteration of fatty acid profile).<br>Production of IAA, GA, ABA, solubilization of phosphorus, promoting plant growth (*e.g.*, maize, Chinese cabbage). |
| Baron *et al.,* 2020 ; Radios *et al.,* 1987 ; Rossi *et al.,* 1987 ; Słaba *et al.,* 2012 ; Słaba & Długonski, 2004 ; Wieder *et al.,* 2025 ; Zheng *et al.,* 2024 a b |

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| UFC 038 |
|---|
| Significant ecological adaptability.<br>Production of bioactive compounds (*e.g.,* zofielliamides A-D, zopfiellsasins A-D) with antibacterial and antifungal activity.<br>Formation of melanized mycelium, enhancing plant growth (*e.g.*, tomato) and drought tolerance (boost of the proline accumulation and antioxidant enzyme activities).<br>Generation of biopolymers and metabolites in association with *Bacillus*, enhancing plant growth and resistance to (a)biotic stresses. |
| Barlow *et al.,* 2023 ; Chen *et al.,* 2020 ; Futagawa *et al.,* 2002 ; Miranda *et al.,* 2023 ; Yi *et al.,* 2021 ; Zhang *et al.,* 2021b |
| **UFC 050** |
| Ecologically versatile fungus.<br>Production of secondary metabolites with antimicrobial and cytotoxic properties, with potential agricultural and pharmaceutical applications.<br>Synthesis of antibacterial compounds and unique aromatic sesquiterpenoids, showing ecological roles in microbial antagonism, herbivore deterrence, host signaling.<br>Phosphate solubilization enhancing plant growth (*e.g.*, rice). |
| Benerjee et al., 2017 ; Chen *et al.,* 2023 ; Dai *et al.,* 2021 ; Li *et al.,* 2024 ; Yin *et al.,* 2021 |
| **UFC 053** |
| Ecological adaptability.<br>Accumulation of HMs (*e.g.*, Cu).<br>Degradation of complex organic compounds (*e.g.*, polyurethane).<br>Biocontrol against pests (*e.g.*, cabbage maggot). |
| Carvajal *et al.,* 2023 ; Legonkova & Selitskaya, 2009 ; Razinger *et al.,* 2014 |
| **UFC 058** |
| IAA production.<br>Robust enzymatic activities (*e.g.*, chitinase, glucanases, protease, monooxygenase), contributing to OM decomposition and nutrient cycling.<br>Support beneficial microbial interactions. |
| Chatterton & Punja 2009 ; Han *et al.,* 2022; Zhang *et al.,* 2021 |
| **UFC 096** |
| Phosphate solubilization, enhancing plant growth and phosphorus uptake (*e.g.,* cotton, potato).<br>Secretion of enzymes (*e.g.,* chitinases and β-glucanases), essential for mycoparasitism and suppression of soil pathogens. |
| Chakraborty *et al.,* 2018 ; Duo-Chuan *et al.,* 2005 ; Madi *et al.,* 1997 ; Naraghi *et al.,* 2012 ; Stefanoni Rubio *et al.,* 2016 |
| **UFC 116** |
| Filamentous fungi, multifunctional.<br>Production of siderophore, mobilization of nutrients, IAA, gibberellins, promoting plant growth and nutrient uptake (*e.g.*, maize, pearl millet, lentils).<br>Enhancement of abiotic stress mitigation, drought tolerance (*e.g.,* wheat, maize).<br>Production of extracellular enzymes (*e.g.*, cellulases, xylanases, chitinases, lignin-modifying enzymes), that decompose complex OM, participate to nutrient cycling and carbon turnover.<br>Accumulation HMs, *e.g.,* Cd, Pb, Hg, Sb, Cu, Ni, Cr (cell wall adsorption, ion exchange and intracellular sequestration). |
| Altaf *et al.,* 2018 ; Gupta *et al.,* 2025 ; He *et al.,* 2023 ; Kaur & Saxena, 2023 ; Khan *et al.,* 2008 ; Khodja *et al.,* 2018 ; Leitão, 2009 ; Suraby *et al.,* 2023 ; Vujanovic *et al.,* 2019 |

*ABA = abscisic acid; ACC = 1-aminocyclopropan-1-carboxylic acid; Ag = silver ; BTEX = benzene, toluene, ethylbenzene, xylenes ; Cd = cadmium ; Co = cobalt ; Cr = chromium ; Cu = copper ; GA = gibberellic acid; Hg = mercury ; HM = heavy metals; IAA = indole-3-acetic acid; Mn = manganese ; Ni = nickel ; OM = organic matter; PAH = polyaromatic hydrocarbons; Pb = lead ; SOD = superoxide dismutase; Zn = Zinc.*

These microbial strains were used for the preparation of two different consortia (Consortium A and B) that are fully described in deliverable 3.5 (D3.5) of the BIOSYSMO project.

Table 1. Features of the selected strains, and of their genus (plant growth promoting properties, influence on plants, contaminant-related properties, others). Strains are given with the internal code of UMLP to prevent publication issues in the future.

# 3 Methodological approach

## 3.1 Soil sampling

Within the project, seven sites across Europe are being tested:

- **Carrière-sous-Poissy, France (CS1):** Vegetable field who used Parisians waste water to increase OM of the soil. Past spreading of the Parisians waste water caused a significant HM contamination of the soil.
- **Kozani, Greece (CS2):** Former lignin mining area, with a very high contamination with Ni (concentration 20 times the normal ranges).
- **Odiel basin, Spain (CS3):** Former mine presenting a high contamination with As, Cd, Pb, and Zn, together with acid mine drainage.
- **Silesian Voivodship, Poland (CS4):** A degraded area as a result of coal-mining activities, mainly contaminated by As, Pb, Zn and Cd with considerable contribution of Cu and Mn.
- **Galliera, Italy (CS5):** Industrial site where pesticides were made, which resulted in HM contamination (Cu and Zn), together with organic contaminants.
- **Vieux-Charmont, France (CS6):** Industrial wasteland where car industries let an important soil HM contamination due to an old and important accumulation of waste of a metallurgic site.
- **Lavrio, Greece (CS7):** Former mining and metallurgical site, highly contaminated with Pb and Zn.

The site full history and soil initial characterization (physical, chemical and microbial parameters) have been fully detailed in the project deliverables D2.1, which is publicly available.
At each site, soil was sampled at different points (≈ 30 kg) to obtain a representative sample of the area. After the sampling, each soil sample was dried at ambient temperature in a bright room. When the soil was completely dry, each sampling point was mixed together to ensure homogeneity.

## 3.2 Biological material

### 3.2.1 Preparation of poplar cuttings

Cuttings were prepared to contain only one bud before planting, in order to have all the same developmental stage (Figure 1A & B), with buds fully apparent but not yet elongated nor bloomed. The cuttings were around 5 to 10 cm length at least (Figure 1B). The initial branches were cut just above the bud, ensuring that a few centimeters could be buried in the soil while the bud remained exposed above ground (Figure 1C). After a few weeks the cuttings will appear as in Figure 1D.

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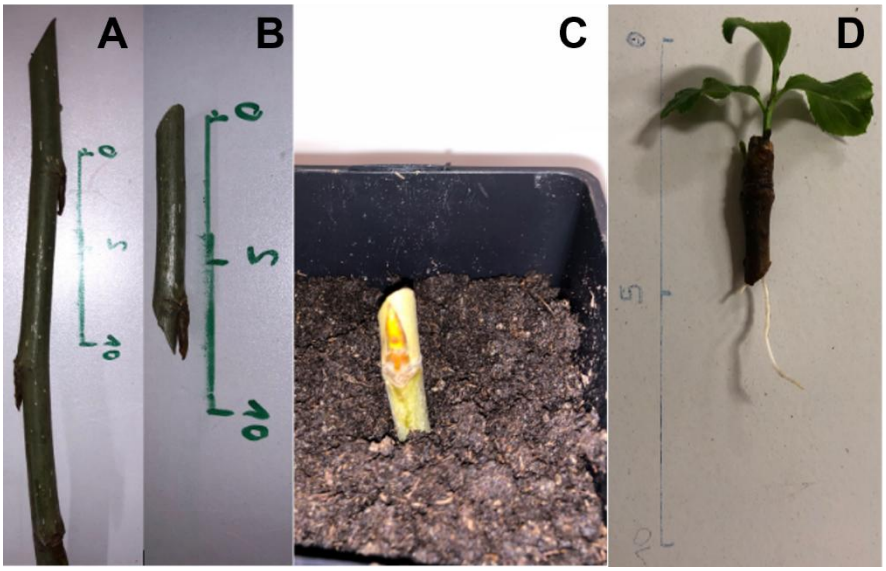


Figure 1. Cutting preparation. (A) Cutting before sizing step, (B) & (C) Cutting after sizing step; (D) Cutting after two months of growth in perlite.

### 3.2.2 Preparation of companion species

The project focused on three companion species, *i.e.*, *Brassica juncea*, *Lablab purpureus*, *Chrysopogon zizanoides*. For each species, commercial seeds/seedlings were acquired:

- *Brassica juncea*: For all partners, seeds of the "Sacal" variety were acquired from Seminart S.R.L seeds company.
- *Lablab purpureus*: For all partners, seeds of the "silver moon" variety were bought from Mullerseeds company. Before sowing, the seeds were pregerminated on humidified tissue for two days prior sowing.
- *Chrysopogon zizanoides*: Vetiver seedlings were acquired from "Vetiver Toscana". The transplanted seedlings were 2-month-old with at least 5 live tillers.

### 3.2.3 Microbial consortia

#### 3.2.3.1 Strain characterization

The study from BIOSYSMO project investigated the growth capabilities of nine fungal species: UFC 003, UFC 006, UFC 009, UFC 019, UFC 016, UFC 096, UFC 053, UFC 058, and UFC 050, on four different media, alongside their potential to promote plant growth. Each of the tested media offers a distinct nutrient profile and selective environment, allowing us to evaluate fungal growth under diverse conditions. Those strains were used in EDAPHOS WP3 and characterized.

Assessment of IAA, siderophore and exopolysaccharide (EPS) production, phosphate solubilisation, growth on lignin as sole carbon source and the capacity to grow under nitrogen-depleted conditions was carried out using liquid media in 96-well microplates. Three replicates per strain were performed, along with a negative control containing only culture medium without fungi. All the details about methodological protocols for IAA, siderophore and phospate solubilisation are described in D3.1 of BIOSYSMO project, which is publicly available. All the other methodologies used for the measurement of the other parameters are described in Table 2.

| Characteristic | Medium / Reactive solution | Incubation | Markers of positive result |
|---|---|---|---|
| Growth on lignin | Lignin 2 $g.L^{-1}$<br>$KH_2PO_4$ 1 $g.L^{-1}$<br>$MgSO_4$ $7H_2O$ 0.5 $g.L^{-1}$<br>pH 5.5-6.5 | 200 µL liquid medium<br>25 °C<br>1 week | Spore germination<br>Hyphal extension<br>Mycelial formation |
| Growth under N depletion | **Ashby liquid medium**:<br>$K_2HPO_4$ 0.2 $g.L^{-1}$<br>$MgSO_4 \cdot 7H_2O$ 0.2 $g.L^{-1}$<br>$CaCl_2$ 0.1 $g.L^{-1}$<br>NaCl 0.1 $g.L^{-1}$<br>$FeSO_4 \cdot 7H_2O$ 0.01 $g.L^{-1}$<br>D-glucose 10 $g.L^{-1}$ | 200 µL liquid medium | Germination<br>Hyphal expansion |
| EPS production | **Sabourand broth** | 7 days<br>Removal of fungal mycelium | |
| | Crystal violet 0.4 %<br>Congo red 0.2 % | 1 hour | Coloration to red or purple<br>Absorbance at 590 nm |
| Cellulolytic activity | Solid medium<br>Sodium carboxymethyl cellulose 1.5 %<br>Agar 1.5 %<br>Yeast extract 0.1 $g.L^{-1}$<br>$NH_4NO_3$ 0.7 $g.L^{-1}$<br>$K_2HPO_4$ 1.5 $g.L^{-1}$<br>$MgSO_4 \cdot 7H_2O$ 0.1 $g.L^{-1}$<br>KCl 0.5 $g.L^{-1}$<br>pH 6-7 | Growth until 2 cm diameter colony | |
| | Congo dye 0.1 % | 20 minutes | Presence of clear halo |

Table 2. Description of the methods used to characterize microbial strains.

### 3.2.3.2 Consortium elaboration

The consortia were elaborated based on their capacity to develop together, without causing any retardation in growth. For this, binary tests were performed. Details about the binary test for the preparation of the two consortia and main composition of the consortia (

) are reported in BIOSYSMO D3.5.

| Consortium A<br>"Fast growing specialists | Consortium B<br>"Diverse, non-native inspired community » |
|---|---|
| UFC 003 | UFC 020 |
| UFC 006 | UFC 035 |
| UFC 009 | UFC 038 |
| UFC 019 | UFC 116 |
| UFC 053 | UFC 013 |
| UFC 058 | UFC 008 |
| UFC 096 | UFC 113 |
| UFC 016 | UFC 017 |
| UFC 050 | |

Table 3. Consortium composition.

- **Consortium A** (Fast-Growing Specialists) comprises nine fast-growing, well-characterized fungal strains from the classes *Sordariomycetes* and *Eurotiomycetes* (phylum *Ascomycota*), selected for their rapid growth and well-documented functional traits relevant to phytomanagement. Together, these strains offer a multifunctional platform combining hormonal signalling, nutrient cycling, enzymatic degradation of OM, and biocontrol. While some strains might share similar traits, reflecting a degree of functional redundancy within a

limited phylogenetic range, this overlap enhances colonization reliability, as one strain can compensate for another in case of establishment failure. Their synergistic interactions are tailored to support rapid rhizosphere establishment and early-stage poplar growth in metal-contaminated soils.

- **Consortium B** (Diverse, Native-Inspired Community) is designed to emulate the native fungal diversity observed in contaminated soils. Consortium B incorporates broader phylogenetic and functional diversity than Consortium A. It includes representatives from multiple fungal classes and functional guilds, aiming to replicate ecological complexity and enhance community resilience, functional complementation, and synergistic interactions. Despite exhibiting comparatively slower growth dynamics, it has a reduced functional redundancy and a broader spectrum of ecological roles than Consortium A. This diversity enhances ecological compatibility and rhizosphere resilience, better emulating the complexity of native fungal communities. Such traits are essential for sustained phytomanagement under variable and complex environmental conditions, particularly in long-term restoration scenarios.

## 3.3 Experimental designs

### 3.3.1 Poplar hybrid selection

#### 3.3.1.1 Laboratory test

In addition to the contaminated soil sampled on each CS, a control soil, not contaminated, was tested. The non-contaminated soil was used to validate our greenhouse experiment, to provide information on plant growth (biomass) and to quantify the basal HM concentration in leaves when cultivated on a non-contaminated substrate. Each pot was filled with 2 L of soil and 1 cut of poplar per pot was planted. For the contaminated soil, ten replicates were prepared, while three were made for the control soil. The time and growing conditions for each CS are listed in Table 4. For CS3, due to the harsh soil conditions, *i.e.,* the very acidic pH, sugar lime amendment was added (30 $t.ha^{-1}$).

| CS | Time (days) | Day/Night (h) | T°C day | T°C night | Light intensity |
|---|---|---|---|---|---|
| **CS1** | 75 | 12 h / 12 h | 22 °C | 20 °C | 300 / 400 $\mu mol.m^{-2}.s^{-1}$ |
| **CS2** | 49 | 12 h / 12h | 24 °C | 20 °C | Natural light |
| **CS3** | 90 | 12 h /12 h | 24 °C | 15 °C | Natural light |
| **CS4** | | | | | |
| **CS5** | 68 | 12h/12h | 26 °C | 16 °C | Natural light |
| **CS6** | 75 | 12 h / 12 h | 22 °C | 20 °C | 300 / 400 $\mu mol.m^{-2}.s^{-1}$ |
| **CS7** | 42 | 12 h/ 12 h | 24 °C | 20 °C | Natural light |

Table 4. Growing conditions of the poplar hybrids.

The pots were distributed following a randomized block design (Figure 2). The pots were maintained at 70 % of water holding capacity for the entire time of the of experiments, through watering with tap water.

Figure 2. Experimental randomized block design.

### 3.3.1.2 Field test

Fifty cuttings, ten for each hybrid tested, were randomly planted on each plot in a randomized design (Figure 3) at the end of winter. Only five out of the seven CS were tested. Indeed, it was not possible for us to perform the preliminary experiment on CS1 and CS5 due to restricted access to the site. In fine, administrative issues prevented us from accessing the site of CS5 to perform the field experiment altogether. Due to those issues, we had to change the locality of the study site in Italy.

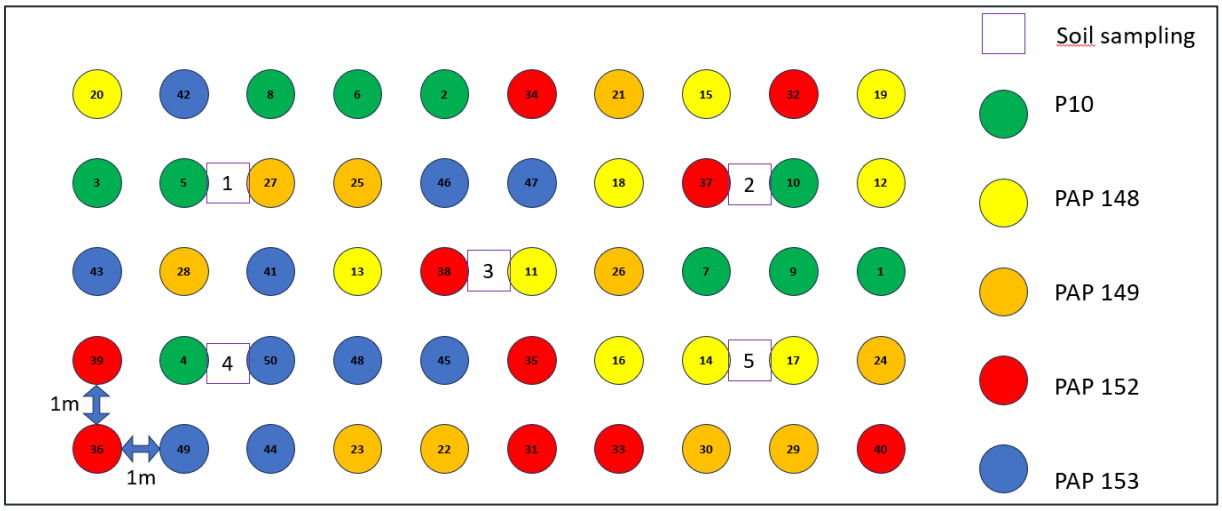


Figure 3. Conceptual diagram of the experimental contaminated and control plots.

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## 3.3.2 Optimization of poplar growth

### 3.3.2.1 Consortia inoculation

The two elaborated consortia (Consortium A & Consortium B,

) were applied to the two French CSs (CS1 & CS6), using the poplar parent line P10. Before the pot test, we evaluated several inoculation methods for their efficacy and field applicability, including root/tissue soaking to maximize initial colonization, soil drenching to establish fungi within the rhizosphere, and experimental foliar sprays to explore systemic colonization or surface protection. These delivery strategies prioritize scalable, practical approaches suited for phytomanagement applications. The soaking of pre-rooted cutting was selected.

The two soils were placed in 2 L pots (with 10 replicates per condition). Each pot was planted with one pre rooted cutting, which was prepared as described in section 3.2.1 and soaked for 30 minutes in a solution containing the appropriate consortium (prepared at a concentration of $10^5$ spores.mL$^{-1}$). Plants were grown for 68 days under controlled conditions: a photoperiod of 14 h of light and 10 h of dark, 70 % humidity, and a temperature of 23 °C during the light period and 20-21 °C during the dark period.

### 3.3.2.2 Amendment application

A laboratory experiment was performed in order to evaluate the influence of an organic amendment to improve the phytoextraction efficiency of the poplar. This experiment used the soils from the two French sites (CS1 & CS6) and an organic amendment, sugar beet vinasse, which is a waste from the sugar production from sugar beet. This amendment was selected from an incubation test which revealed its potential to mobilize Zn (data not shown).

For each soil, 30 pots were prepared. Ten pots were used as control, ten pots received one application of vinasse, and the last ten pots received two applications of vinasse. The vinasse was applied at 3.65 g.kg$^{-1}$ per application. The substrates were put in 2 L pots and one cutting (prepared as described in section 3.2.1) was placed in each pot. Plants were grown under controlled conditions (14 h light/10 h dark photoperiod, 22 °C / 18 °C day/night temperature, 300 µmol.m$^{-2}$.s$^{-1}$ light intensity, 70 % relative humidity) for 16 weeks.

## 3.3.3 Companion species

### 3.3.3.1 Influence of contamination

| CS | Time (days) | Day/Night (h) | T°C day | T°C night | Light intensity |
|---|---|---|---|---|---|
| CS1 | | | | | |
| CS2 | 63 | 12h/12h | 24 ºC | 20 ºC | Natural light |
| CS3 | 33 | 16 h / 8h | 24 ºC | 24 ºC | Natural light |
| CS4 | | | | | |
| CS5 | 65 | 12h/12h | 26 oC | 16oC | Natural light |
| CS6 | | | | | |
| CS7 | 63 | 12h/12h | 24ºC | 20ºC | Natural light |

Table 5. Growing conditions of *Brassica juncea*.

Similarly to the test with the poplars, in addition to the contaminated soil sampled on each CS, a control soil, not contaminated, was tested. Each pot was filled with 2 L of soil and seeds/seedlings of the companion species were sown. Ten replicates were made for the contaminated soil, three for the control soil. The time and growing conditions for each CS are listed in Table 5 for *Brassica juncea* and in Table 6 for *Lablab purpureus*. In addition, on CS5, vetiver (*Chrysopogon zizanoides*) was also tested

for 91 days, under the same conditions as *L. purpureus* and *B. juncea*. For CS3, as for the poplar test, sugar lime amendment was added (30 $t.ha^{-1}$).

Table 6. Growing conditions of *Lablab purpureus*.

| CS | Time (days) | Day/Night (h) | T°C day | T°C night | Light intensity |
|---|---|---|---|---|---|
| CS1 | | | | | |
| CS2 | 63 | 12h/12h | 24 ºC | 20 ºC | Natural light |
| CS3 | 34 | 16 h/ 8h | 24 ºC | 24 ºC | Natural light |
| CS4 | | | | | |
| CS5 | 63 | 12h/12h | 26 oC | 16oC | Natural light |
| CS6 | | | | | |
| CS7 | 63 | 12h/12h | 24 ºC | 20 ºC | Natural light |

### 3.3.3.2 Improvement with amendments

As for the poplar, laboratory tests were performed to evaluate the potential of different amendments to improve the extraction potential of *Brassica juncea* and *Lablab purpureus*, on the soils of the two French soils (CS1 & CS6).

For *Brassica juncea*, sulphur and sugar beet vinasse were tested. Sulphur was added at a dose of 20 $mM.kg^{-1}$ while vinasse was added at 1.25 $g.kg^{-1}$. Soils were put in 1 L, with ten replicates prepared per condition. Twelve seeds of *Brassica juncea* were sown per pot, reduced to three after germination.

For *Lablab purpureus*, sugar beet vinasse and activated carbon were used as amendments. Vinasse was applied at 1.25 $g.kg^{-1}$ and activated carbon at 5 $g.kg^{-1}$. Soils were put in 2 L pots. Five seeds of *Lablab purpureus* were sown per pot, reduced to two after germination.

Plants were grown under controlled conditions (14 h/10 h light/dark photoperiod; temperature 22 °C day / 18 °C night, 70 % relative humidity, and 300 $\mu mol.m^{-2}.s^{-1}$ light intensity) for two months for *Brassica juncea* and for three months for *Lablab purpureus*.

## 3.4 Analyses

At the end of the experiment, all the plants were harvested. For the poplars, leaves, stems and roots were separated; for the companion species, the aerial part was separated from the roots. Each tissue was cleaned in tap water followed by distilled water. After 48 h of drying (70 °C), dry biomass was recorded. Finally, element concentrations in the above-ground biomass were analysed as detailed in the deliverable D2.1. The indicators to be measured are as follows:

a. **Accumulation capacity** is calculated separately for leaves and stems as:

   Accumulation capacity (AC) = $(Conc_{leaf/stem} * DW_{leaf/stem}) / 1000$
   Where:
   $Conc_{leaf/stem}$ = HM concentration in leaf or stem (in $mg\ kg^{-1}$)
   $DW_{leaf/stem}$ = Dry leaf or stem biomass (in g)

b. **Phytoextraction potential** per plant is calculated as the total amount of HM accumulated in the aboveground biomass when both leaf and stem data were available.

   Phytoextraction potential (PP) = $((Conc_{leaf} * DW_{leaf})+(Conc_{stem} * DW_{stem})) / 1000$
   Where:
   $Conc_{leaf}$ / $Conc_{stem}$ = HM concentration in leaf / HM concentration in stem (in $mg\ kg^{-1}$)
   $DW_{leaf}$ / $DW_{stem}$ = Dry leaf or stem biomass (in g)

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c. **HM removal** is calculated according to the approach described by Vangronsveld et al. (2009) and will be applied for final field trial.

HM removal = ($Conc_{tot}$ * Biomass yield) / 1000
Where:
$Conc_{tot}$ = HM total concentration ($Conc_{leaf}$ + $Conc_{stem}$) expressed as mg $kg^{-1}$
Biomass yield was expressed as tonnes of DW per hectare (t/ha).
The final HM removal was expressed as kilograms of HM removed per hectare (kg/ha)

# 4 Results

## 4.1 Poplar hybrid selection

The WP3 aims to test different plant/microbe assemblages for the depollution of contaminated soils across Europe. The first step was to select the most efficient poplar hybrids, out of the five provided by the partner Phytowelt, in laboratory (Task 3.1) and field (Task 3.2) settings, based on their biomass production and HM accumulation.

### 4.1.1 Laboratory test

The task 3.1 focused on the evaluation of the five poplar hybrids under laboratory conditions. In this goal, all CS leaders performed a pot experiment using their contaminated soil(s) and a control soil, to evaluate the influence of HM pollution on biomass production and the efficiency of the different hybrids to accumulate HMs.

#### 4.1.1.1 Biomass production

The biomass production was evaluated after 42 to 90 days, depending on the CS, in both contaminated and control soils. This allowed for the comparison of the hybrids as well as the different soils.

All poplar hybrids were able to grow and produce biomass on the seven CS (Figure 4). On average, aerial (for CS1, CS2, CS5, CS6, CS7) or leaf biomass (CS3, CS4) productions on the control soils were 5.64 g (CS1), 3.39 g (CS2), 0.64 g (CS3), 1.24 g (CS4), 6.81 g (CS5), 5.64 g (CS6), and 3.39 g (CS7); while on the contaminated soils, dry weights were on average 5.31 g (CS1), 2.23 g (CS2), 0.29 g (CS3), 1.26 g (CS4), 8.02 g (CS5), 4.44 g (CS6) and 1.79 g (CS7). Overall, this shows that, when grown on contaminated soils, poplars lost on average 6 % of biomass on CS1, 34 % on CS2, 54 % on CS3, 21 % on CS6 and 47 % on CS7, compared to the control; while no loss of biomass production was observed on CS4 and a rise of 18 % of biomass production was measured on the contaminated soil of CS5 compared to the control.

When comparing the hybrids, we can see that the parent line P10 was the one performing best on the contaminated soils for the sites CS1, CS3, CS4, CS5, and CS6. On the other two sites, the hybrids PAP 153 performed best on CS2 and PAP 149 performed best on CS7. Apart from the parent P10, the hybrids producing the most biomass were PAP 152 on CS1, PAP 152 on CS3, PAP 148 on CS4, and PAP 148 on CS6.

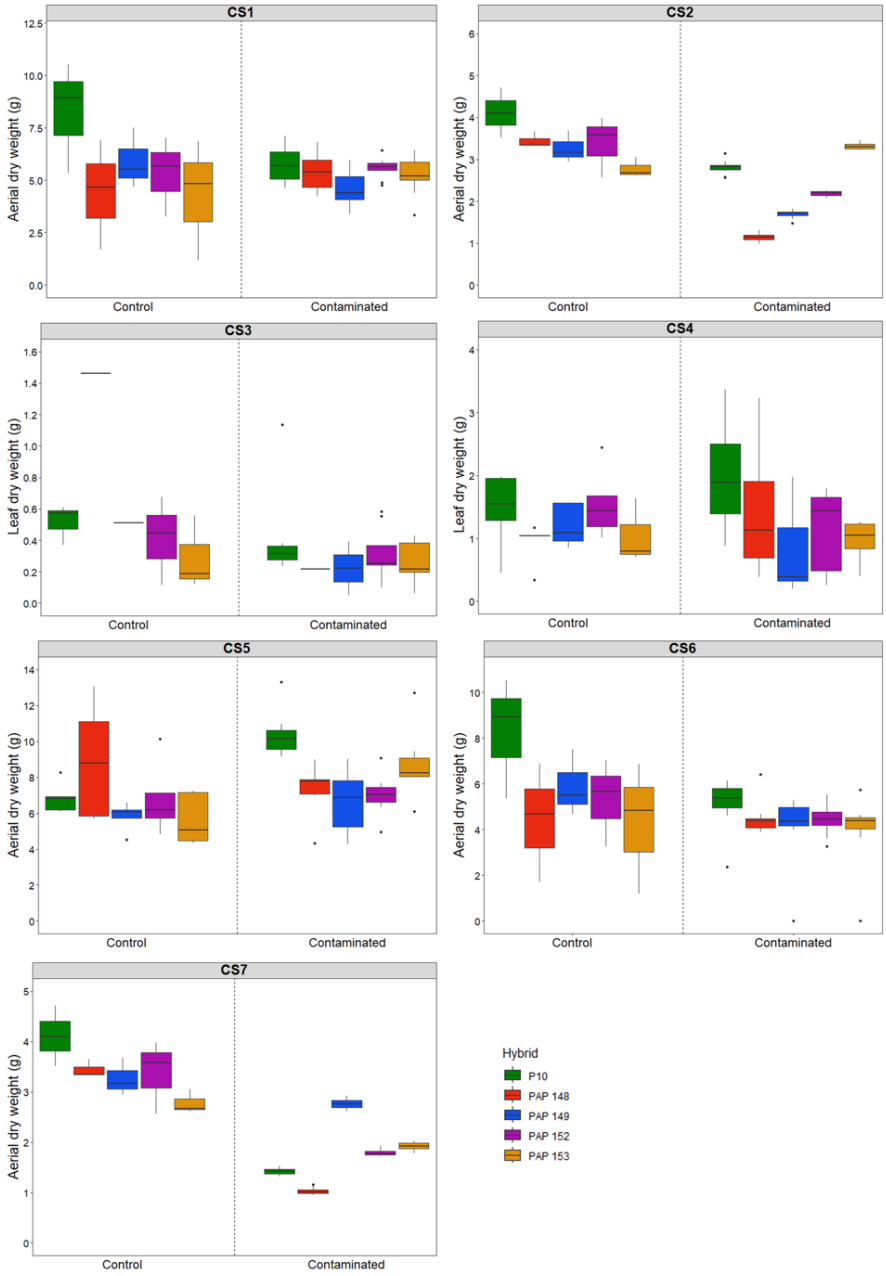


Figure 4. Aerial biomass production of the five tested poplar hybrids, grown on control and contaminated soils for each CS.

### 4.1.1.2 Metal accumulation

For each CS and hybrid, the HM concentration was measured in the leaf, stem, or total aerial tissues. Using biomass and concentration, the phytoextraction capacity was calculated, as an indicator of the efficiency of the poplar hybrids on a specific soil.

#### 4.1.1.2.1 CS1. Carrières-sous-Poissy (France)

On CS1, leaf concentrations were measured between 3.81 and 5.00 mg.kg$^{-1}$ Cd and between 174 and 206 mg.kg$^{-1}$ Zn. In both cases, P10 was the poplar accumulating the least metals, while PAP 149 presented the highest concentrations (Figure 5).

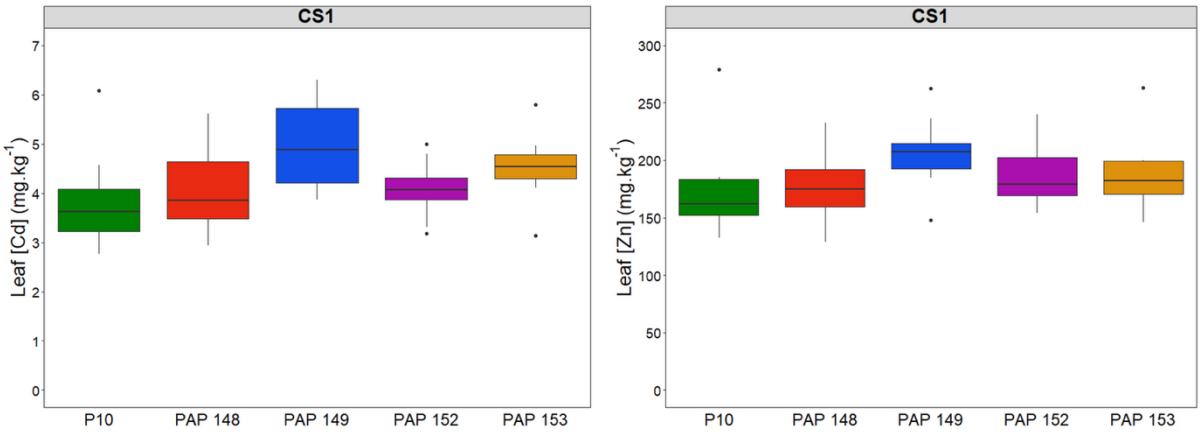


Figure 5. Cd and Zn concentration (mg.kg$^{-1}$) in the leaves of the five poplar hybrids grown on the contaminated soil of CS1.

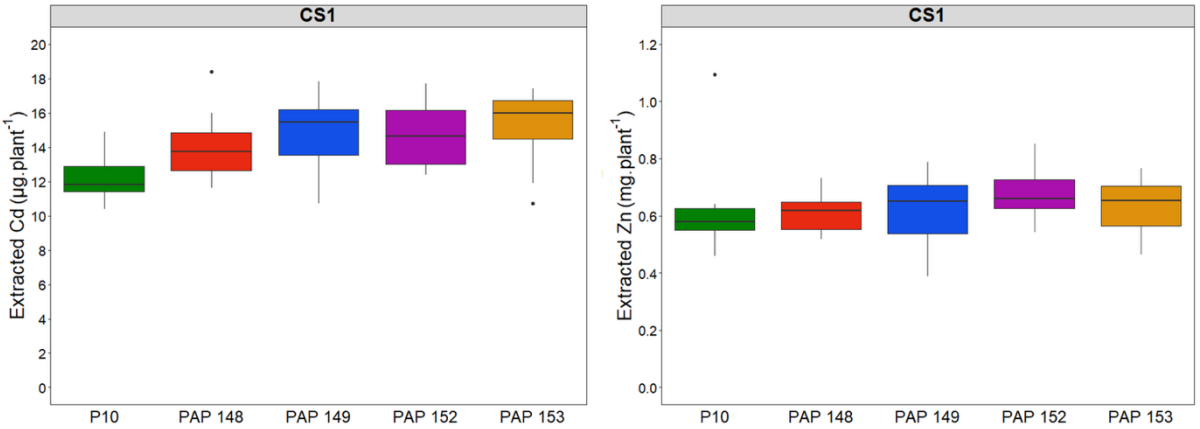


Figure 6. Quantity of Cd (µg.plant$^{-1}$) and Zn (mg.plant$^{-1}$) phytoextracted in the leaves of the five poplar hybrids grown on the contaminated soil of CS1.

In terms of phytoextraction potential, we measured that plants, through their leaves, could extract 13.40 to 15.22 µg Cd and 608 to 676 µg Zn (Figure 6). In this case, the poplar P10 was the one able to extract the least Cd, while PAP 148 was the hybrid with the least Zn extracted. The hybrids PAP 153 and PAP 152 were the ones with the highest extraction of Cd and Zn, respectively.

#### 4.1.1.2.2 CS2. Kosani (Greece)

On CS2, poplars were able to accumulate in their aerial tissues 1.52 to 4.41 mg.kg$^{-1}$ As and 50.85 to 126.81 mg.kg$^{-1}$ Ni (Figure 7). Again, in both cases, P10 was the poplar with the lowest As and Ni concentrations; while PAP 153 accumulated the most As and PAP 149 the most Ni.

Regarding HM phytoextraction, poplars could extract in their aerial biomass 2.3 to 14.7 µg As and 129 to 291 µg Ni (Figure 8). In this case, the lowest phytoextraction potential was measured in PAP 148, while the highest was determined in PAP 153.

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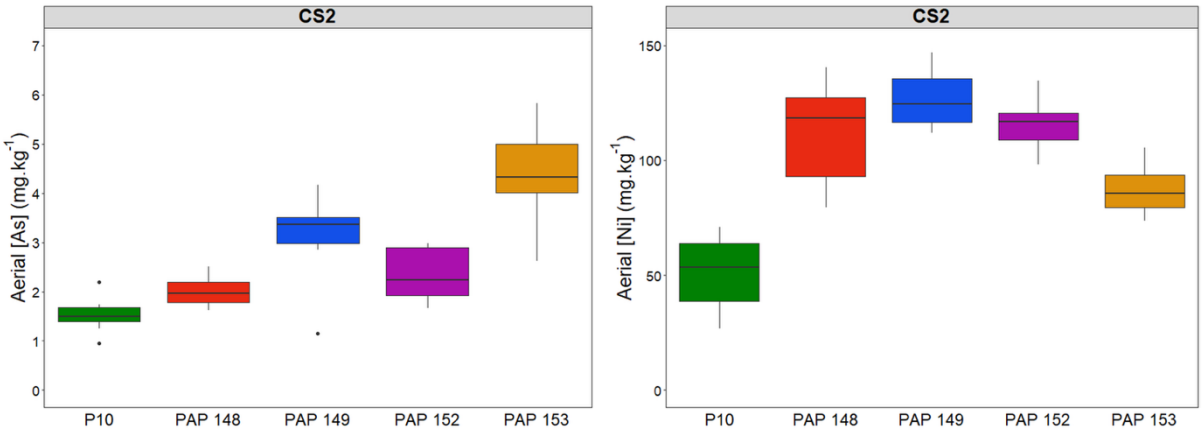


Figure 7. As and Ni concentration (mg.kg$^{-1}$) in the aerial tissues of the five poplar hybrids grown on the contaminated soil of CS2.

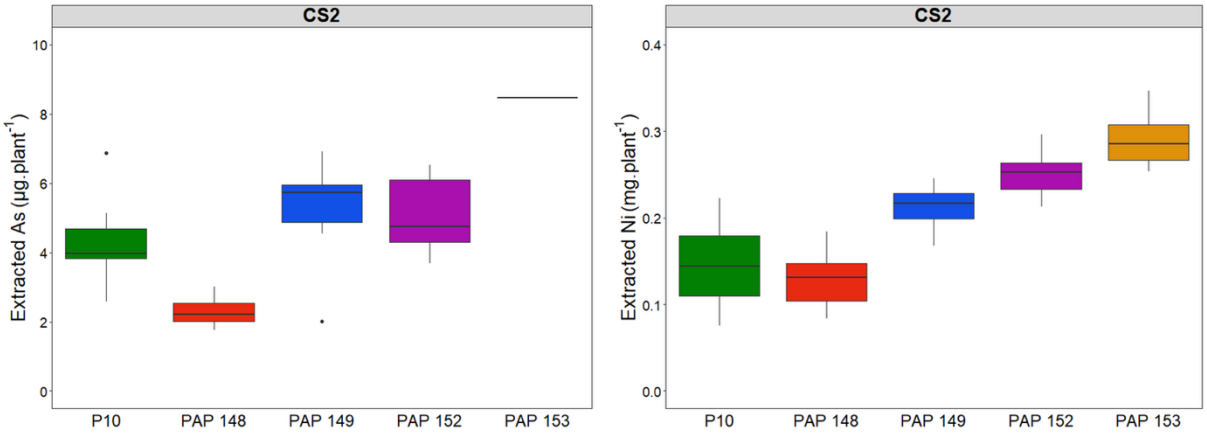


Figure 8. Quantity of As (µg.plant$^{-1}$) and Ni (mg.plant$^{-1}$) phytoextracted in the aerial tissues of the five poplar hybrids grown on the contaminated soil of CS2.

#### 4.1.1.2.3 CS3. Odiel basin Area (Spain)

On CS3, concentrations in the leaves were measured to be 3.36 to 7.25 mg.kg$^{-1}$ As, 0.75 to 2.05 mg.kg$^{-1}$, 46 to 117 mg.kg$^{-1}$, and 136 to 259 mg.kg$^{-1}$ Zn (Table 7). The highest and lowest concentration within the poplar hybrids differed between elements. More precisely, PAP 148 accumulated the least As and Pb, but the most Cd; P10 accumulated had the lowest Cd concentrations; and PAP 152 presented the highest concentrations in As, Pb and Zn.

Table 7. Metal concentration and accumulation capacity in the leaves of the five poplar hybrids grown on the contaminated soil of CS3.

| | Leaf concentration (mg.kg$^{-1}$) | | | | Leaf accumulation capacity (µg.plant$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| **Hybrid** | **As** | **Cd** | **Pb** | **Zn** | **As** | **Cd** | **Pb** | **Zn** |
| **P10** | 5.14 ± 3.01 | 0.75 ± 0.23 | 89 ± 52 | 164 ± 79 | 2.27 | 0.33 | 39.1 | 72.3 |
| **PAP 148** | 3.36 ± 0.00 | 2.05 ± 0.00 | 46 ± 0.00 | 233 ± 0 | 0.73 | 0.45 | 10.0 | 50.5 |
| **PAP 149** | 5.51 ± 0.00 | 0.95 ± 0.00 | 80 ± 0.00 | 136 ± 0 | 1.23 | 0.21 | 17.8 | 28.1 |
| **PAP 152** | 7.25 ± 2.76 | 1.61 ± 0.63 | 117 ± 47 | 259 ± 82 | 2.29 | 0.51 | 36.9 | 81.9 |
| **PAP 153** | 5.59 ± 1.57 | 1.79 ± 0.41 | 87 ± 20 | 262 ± 63 | 1.44 | 0.46 | 22.4 | 67.7 |

In terms of accumulation capacity of the leaves, values ranged between 0.73 µg and 2.29 for As, between 0.21 and 0.51 µg for Cd, between 10.0 and 39.1 µg for Pb, and between 28.1 and 81.9 µg for Zn (Table 7). Again, the highest and lowest leaf accumulation capacity of the poplars varied


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between elements. PAP 148 was the poplar with the lowest accumulation capacity of As, Pb and Zn, while PAP 149 extracted the least Cd; P10 had the highest accumulation capacity of Pb, while PAP 152 extracted the most As, Cd and Zn.

#### 4.1.1.2.4 CS4. Silesian Voivodship (Poland)

Unfortunately, only three out of the five hybrids could be analysed, due to technical issue (not enough biomass was sent to the company performing the analyses and starting over would have had too much cost). When comparing the three analysed hybrids, we found that PAP 153 was always the one with the lowest Cd, Pb and Zn concentrations, while PAP 148 presented the highest concentrations for Cd and Pb, and P10 for Zn (Table 8). However, as P10 produced the highest biomass, accumulation capacity was higher for P10, followed by PAP 148 while PAP 153 presented the lowest accumulation capacity in leaves (Table 8).

Table 8. Metal concentration and accumulation capacity in leaves of the five poplar hybrids grown on the contaminated soil of CS4 (n.a. = not available).

| | Leaf concentration (mg.kg$^{-1}$) | | | Leaf accumulation capacity | | |
|---|---|---|---|---|---|---|
| | Cd | Pb | Zn | Cd (µg.plant$^{-1}$) | Pb (µg.plant$^{-1}$) | Zn (mg.plant$^{-1}$) |
| **P10** | 62 | 89 | 973 | 120.3 | 172.7 | 1.89 |
| **PAP 148** | 70 | 90 | 860 | 102.9 | 132.3 | 1.26 |
| **PAP 149** | n.a | n.a | n.a | n.a | n.a | n.a |
| **PAP 152** | n.a | n.a | n.a | n.a | n.a | n.a |
| **PAP 153** | 43 | 74 | 642 | 41.7 | 71.8 | 0.62 |

#### 4.1.1.2.5 CS5. Galliera (Italy)

On the CS5, metal concentrations were measured in both leaves and stems separately. Copper concentrations were measured between 16.3 and 21.3 mg.kg$^{-1}$ in the leaves and between 7.2 and 8.5 mg.kg$^{-1}$ in the stems (Table 9). Total phytoextraction potential in the aerial biomass was between 0.07 and 0.11 µg Cu. In this case, PAP 149 presented the lowest concentration in the leaves but the highest in the stems, while PAP 152 had the least stem concentration and PAP 153 the highest leaf concentrations. Phytoextraction was calculated to be the lowest in PAP 148 and the highest in P10.

Table 9. Metal concentration and quantity phytoextracted in the leaves of the five poplar hybrids grown on the contaminated soil of CS5.

| | Leaf concentration (mg.kg$^{-1}$) | | Stem concentration (mg.kg$^{-1}$) | | Phytoextraction (mg.plant$^{-1}$) | |
|---|---|---|---|---|---|---|
| **Hybrid** | **Cu** | **Zn** | **Cu** | **Zn** | **Cu** | **Zn** |
| **P10** | 19.7 ± 5.5 | 129 ± 9 | 7.5 ± 1.0 | 61.7 ± 8.5 | 0.11 ± 0.02 | 0.82 ± 0.10 |
| **PAP 148** | 17.0 ± 1.0 | 159 ± 24 | 7.7 ± 0.6 | 85.0 ± 20.0 | 0.07 ± 0.01 | 0.70 ± 0.21 |
| **PAP 149** | 16.3 ± 2.1 | 121 ± 11 | 8.5 ± 0.9 | 71.3 ± 5.8 | 0.07 ± 0.01 | 0.56 ± 0.11 |
| **PAP 152** | 17.3 ± 2.5 | 142 ± 9 | 7.2 ± 1.7 | 75.0 ± 14.1 | 0.08 ± 0.01 | 0.72 ± 0.15 |
| **PAP 153** | 21.3 ± 3.1 | 167 ± 6 | 7.5 ± 1.4 | 70.7 ± 5.5 | 0.09 ± 0.01 | 0.80 ± 0.11 |

For Zn, leaf concentrations were between 121 and 167 mg.kg$^{-1}$, while stem concentrations ranged from 71 to 85 mg.kg$^{-1}$ (Table 9). Phytoextraction potential was calculated between 0.56 and 0.82 mg Zn. The rank of poplar hybrid was the same for leaf Zn concentration and for Zn phytoextraction as it was for Cu. In the stems, the lowest concentration was measured in P10 and the highest in PAP 148.

#### 4.1.1.2.6 CS 6. Vieux-Charmont (France)

On CS6, leaf concentrations ranged between 1.06 and 2.04 mg.kg$^{-1}$ Cd and between 580 and 946 mg.kg$^{-1}$ Zn (Figure 9). In this case, the lowest Cd concentration was measured in the parent line

P10, while the highest concentration was found in PAP 153. For Zn, the highest concentration was in PAP 153 and the highest in PAP 152.

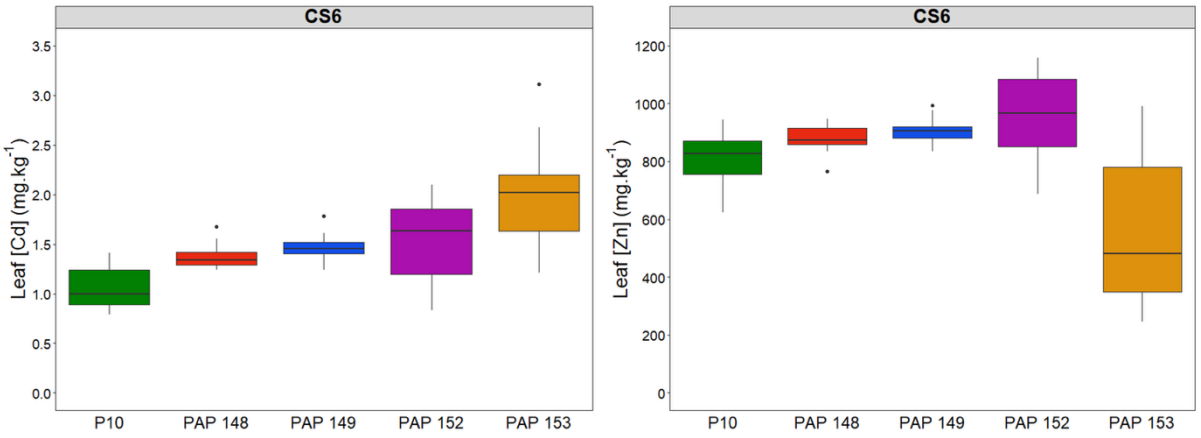


Figure 9. Cd and Zn concentration (mg.kg$^{-1}$) in the leaves of the five poplar hybrids grown on the contaminated soil of CS 6.

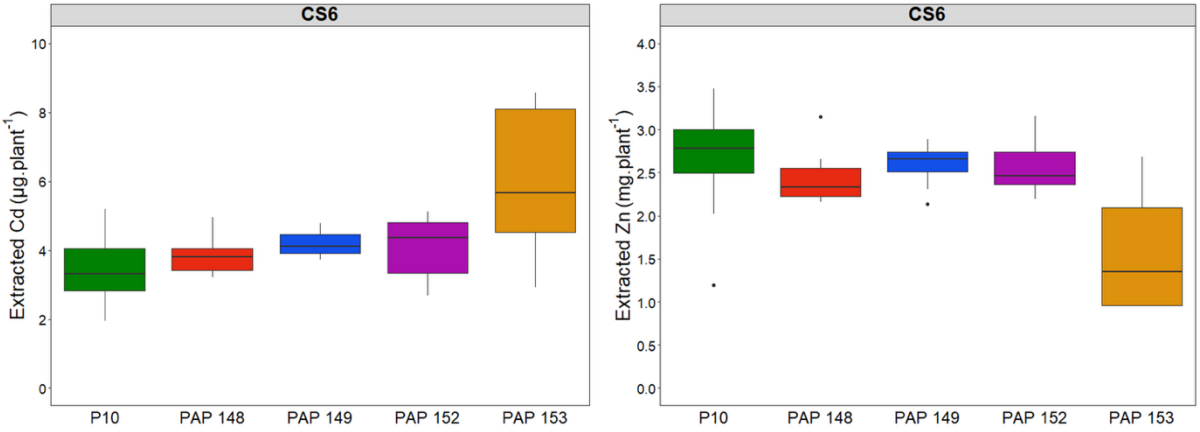


Figure 10. Quantity of Cd (µg.plant$^{-1}$) and Zn (mg.plant$^{-1}$) phytoextracted in the aerial tissues of the five poplar hybrids grown on the contaminated soil of CS 6.

Regarding the phytoextraction, poplars were able to extract through their leaves 3.4 to 5.9 µg Cd and 1.59 to 2.64 mg Zn (Figure 10). In this case, two poplar hybrids presented contrasted behaviours: P10 extracted the least Cd but the most Zn, while PAP 153 extracted the highest Cd and the least Zn.

#### 4.1.1.2.7 CS7. Lavrio (Greece)

When grown on CS7, poplars accumulated in their aerial tissues 1.19 to 14.32 mg.kg$^{-1}$ As, 2.10 to 12.67 mg.kg$^{-1}$ Ni and 13.87 to 258.06 mg.kg$^{-1}$ Pb (Figure 11). The lowest concentrations were measured in PAP 153 for As and Pb, and in PAP 152 for Ni, while PAP 149 was the hybrid with the highest concentrations of all three elements.

In terms of phytoextraction potential, poplars could extract from 2.3 to 39.7 µg As, 3.7 to 35.0 µg Ni, and 2.7 to 713 µg Pb (Figure 12). Similarly to the concentrations, the lowest extraction potential was calculated in PAP 153 for As and Pb and PAP 152 for Ni, while PAP 149 had the highest extraction potential for all three elements.

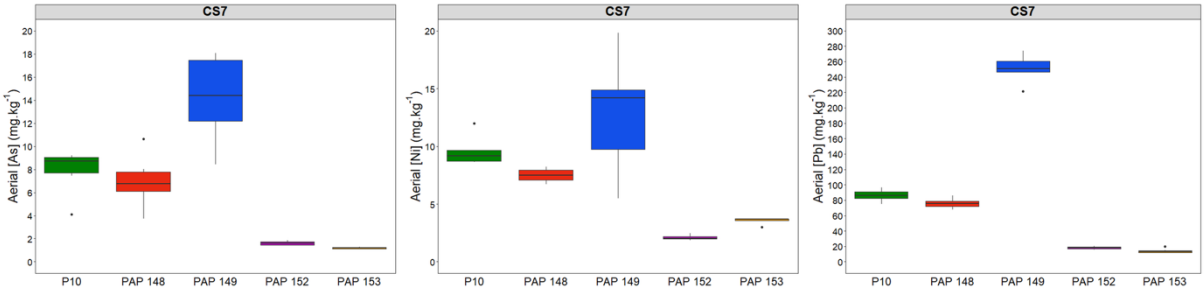


Figure 11. As, Ni, and Pb concentration (mg.kg$^{-1}$) in the leaves of the five poplar hybrids grown on the contaminated soil of CS7.

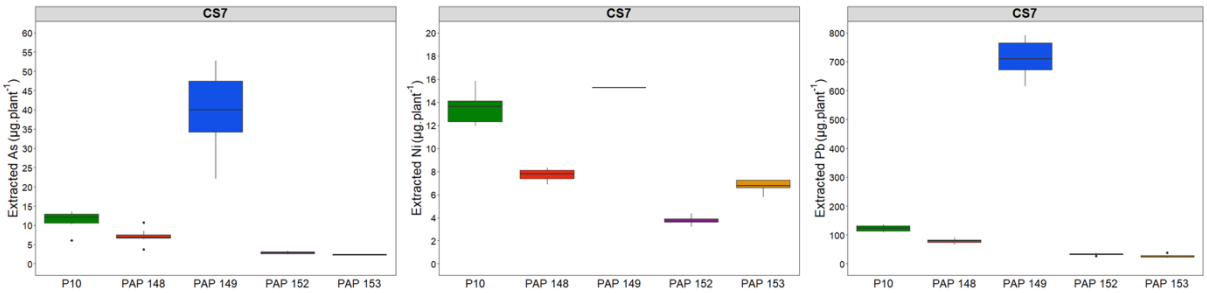


Figure 12. Quantity of As, Ni, and Pb (µg.plant$^{-1}$) phytoextracted in the aerial tissues of the five poplar hybrids grown on the contaminated soil of CS7.

#### 4.1.1.3 Conclusions

From the laboratory tests performed on all CS, we were able to show that all hybrids could grow on the contaminated soils, even if their biomass production was reduced compared to a non-contaminated control soil. Moreover, based on the data on biomass production and HM accumulation, we can see some hybrids, apart from the parent line P10, that performed best: PAP 152 on CS1, PAP 153 on CS2, PAP 152 on CS3, PAP 148 on CS4, PAP 153 on CS5, and PAP 149 on CS7. For CS6, it was more difficult to see one hybrid performing best on the different criteria.

### 4.1.2 Field test

Following the evaluation under greenhouse controlled conditions, a preliminary field test was performed (Task 3.2). This was performed to confirm the results of the pot test to select one hybrid, in addition to the parent line P10, to establish for the long test field experiment (Task 3.2), which will be monitored throughout the rest of the project (Task 3.2 & Task 3.3).

#### 4.1.2.1 Biomass production

On five out of the seven CSs, the five poplar hybrids were planted, monitored and harvested after several months of growing. The aerial biomass (leaves and stems) was collected and evaluated to biomass production, a key parameter of the success of phytoextraction.

The preliminary field experiment revealed the capacity of all the poplar hybrids to grow and develop on the contaminated soils (Figure 13). The lowest biomass production was measured in CS7, due to very harsh soil and climate conditions, followed by CS3, due to drastic soil conditions. On two out of the five CSs, *i.e.*, CS2 and CS3, all the PAP hybrids performed better than the parent line P10, while on CS4, only one performed better (PAP 149), on CS6, three performed better and on CS7 (PAP 148, PAP 149, PAP 152), none performed better.

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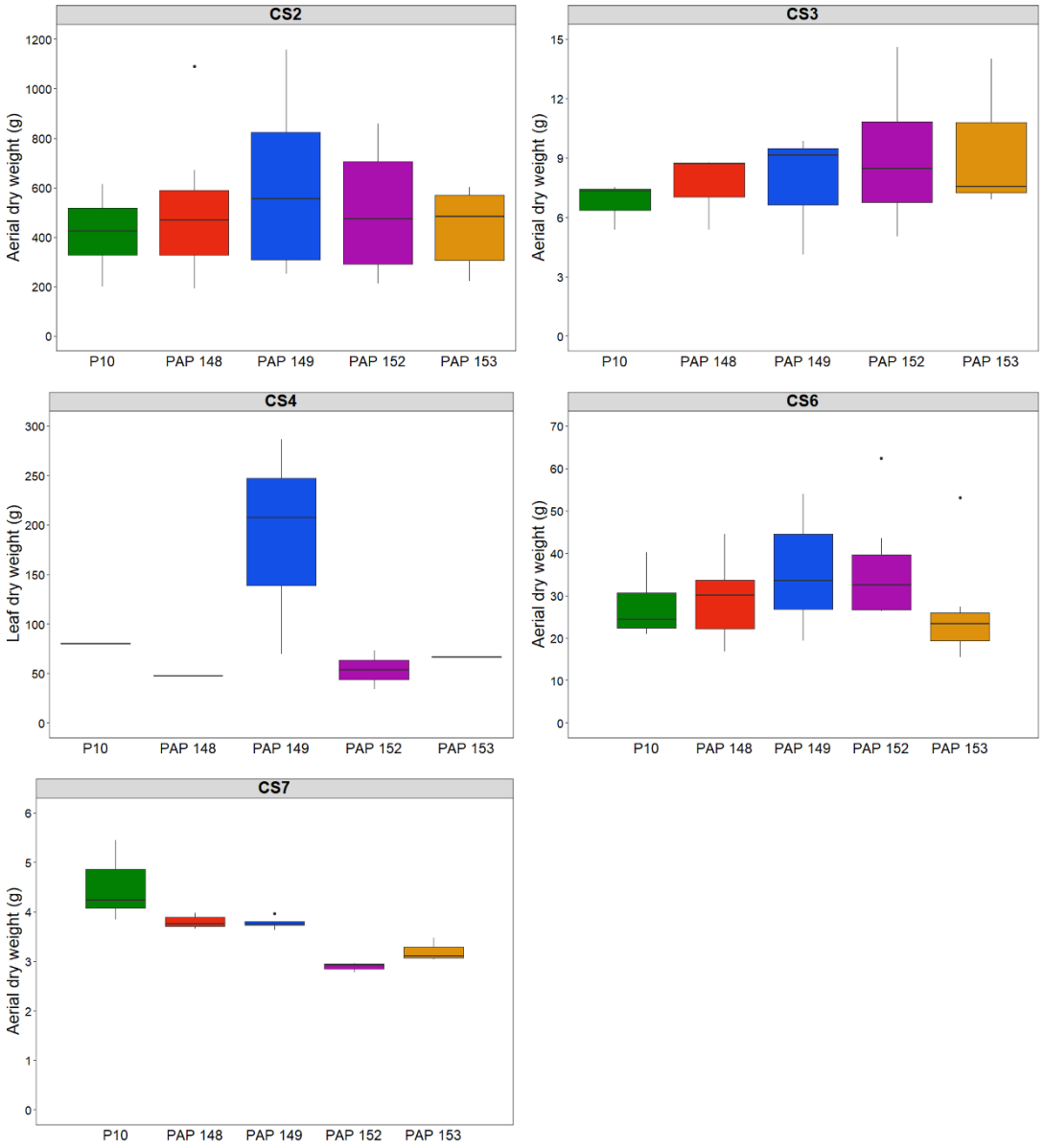


Figure 13. Aerial biomass production of the five tested poplar hybrids, grown on contaminated soils for each CS under real field conditions.

On CS2, aerial biomass production ranged from 418 g for P10 to 615 g for PAP 149. On CS3, the aerial biomass was recorded between 6.74 g for P10 and 9.49 g for PAP 153. On CS4, leaf biomass was measured between 47 g for PAP 148 and 188 g for PAP 149. On CS6, poplars produced between 39 g (P10) and 54 g (PAP 152) aerial biomass. Finally, on CS7, aerial biomass varied between 2.88 g (PAP 152) and 4.48 g (P10).

### 4.1.2.2 Metal accumulation

In addition to the biomass production, the concentration in the pollutant (specific to each CS) was measured in the leaves and/or stems. Those concentrations, and biomass, were used to calculate the quantity of each pollutant extracted by a specific hybrid, to evaluate their phytoextraction potential, and thus select the most efficient one.

#### 4.1.2.2.1 CS2. Kozani (Greece)

A high variability was observed within the hybrids in terms of HM accumulation (Figure 14). Concentration in As in the leaves ranged from 0.17 mg.kg$^{-1}$ to 0.73 mg.kg$^{-1}$, while concentration in the stems were measured between 0 and 0.24 mg.kg$^{-1}$. In both organs, P10 was the poplar with the lowest concentration, while PAP 152 presented the highest concentration in the leaves, and PAP 148 in the stems. Regarding Ni, leaf concentrations were found between 11.13 mg.kg$^{-1}$, while stem concentrations range from 5.01 mg.kg$^{-1}$ and 6.09 mg.kg$^{-1}$. In this case, PAP 152 was the hybrid with the lowest concentrations, whereas the highest leaf concentrations were measured in PAP 148 and PAP 149, and the highest stem concentration in P10.

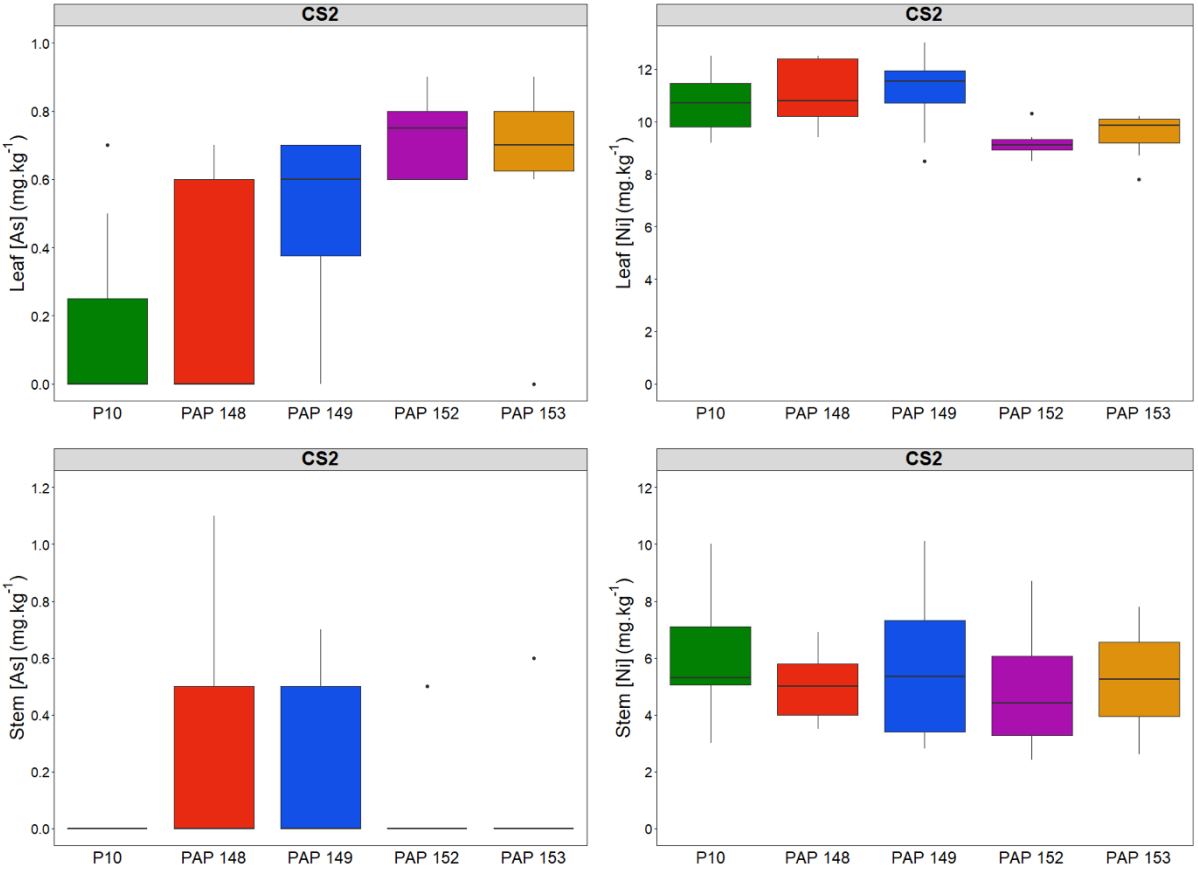


Figure 14. As and Ni concentration (mg.kg$^{-1}$) in the leaves and stems of the five poplar hybrids grown on the contaminated soil of CS2.

The total amount of As extracted in the aerial biomass of the poplar was highly different between the hybrids. The lowest amount extracted was by P10 (0.02 mg.plant$^{-1}$) while the highest amount was for PAP 149 (0.21 mg.plant$^{-1}$) (Figure 15). The phytoextraction of Ni was more homogeneous between hybrids, ranging from 2.81 mg.plant$^{-1}$ (PAP 153) to 3.46 mg.plant$^{-1}$ (PAP 149).

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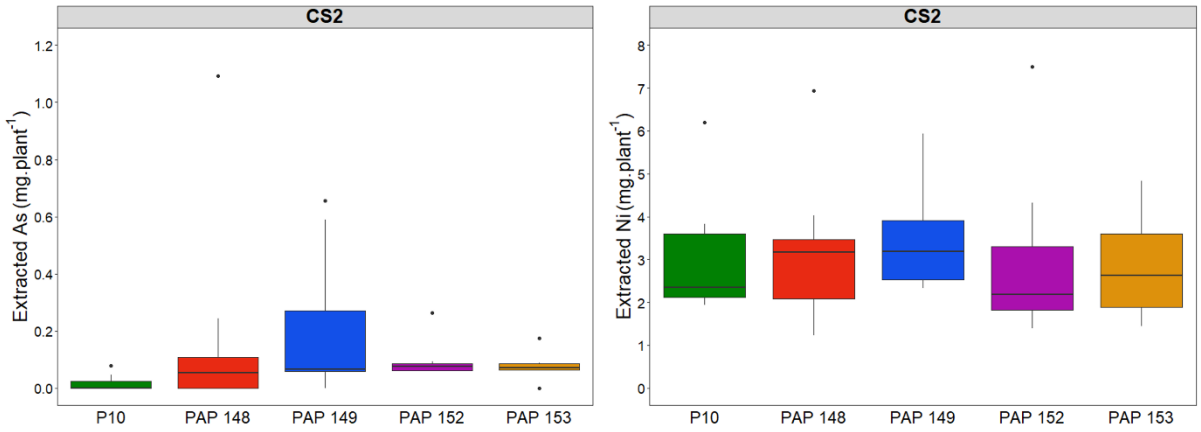


Figure 15. Quantity of As (mg.plant$^{-1}$) and Ni (mg.plant$^{-1}$) phytoextracted in the aerial tissues of the five poplar hybrids grown on the contaminated soil of CS2.

#### 4.1.2.2.2 CS3. Odiel basin Area (Spain)

Concentrations and phytoextraction potentials were measured for As, Cd, Pb, and Zn in plants grown on CS3. For As, concentrations in the leaves ranged from 18 to 55 mg.kg$^{-1}$, while concentrations in the stems were between 6.9 and 12.6 mg.kg$^{-1}$ (). The lowest concentrations were measured in the poplar hybrid PAP 149 for the leaves and PAP 152 for the stems, while the highest concentration was always found in the poplar parent line P10. However, P10 presented the lowest phytoextraction potential (78.1 µg), probably due to a lower biomass, while PAP 153 had the highest potential (118.1 µg) (Table 10).

For Cd, P10 had the lowest concentration in both leaves and stems (0.49 and 0.59 mg.kg$^{-1}$, respectively) (). The highest Cd concentration in the leaves was found in PAP 148 (0.63 mg.kg$^{-1}$), whereas in the stem it was in PAP 152 (0.73 mg.kg$^{-1}$). Overall, phytoextraction potential was calculated between 3.63 and 6.09 µg, with the lowest value for P10 and the highest for PAP 152 (Table 10).

Table 10. Metal concentration in the leaves and stems of the five poplar hybrids grown on the contaminated soil of CS3.

| | Concentration in leaves (mg.kg$^{-1}$) | | | | Concentration in stems (mg.kg$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| **Hybrid** | **As** | **Cd** | **Pb** | **Zn** | **As** | **Cd** | **Pb** | **Zn** |
| **P10** | 55 ± 7 | 0.49 ± 0.09 | 140 ± 14 | 127 ± 18 | 12.6 ± 4.7 | 0.59 ± 0.11 | 44 ± 14 | 106 ± 25 |
| **PAP 148** | 33 ± 18 | 0.63 ± 0.21 | 90 ± 47 | 154 ± 50 | 9.0 ± 3.3 | 0.69 ± 0.21 | 32 ± 13 | 129 ± 11 |
| **PAP 149** | 18 ± 2 | 0.62 ± 0.14 | 51 ± 2 | 162 ± 48 | 12.1 ± 3.0 | 0.72 ± 0.14 | 46 ± 11 | 132 ± 10 |
| **PAP 152** | 38 ± 8 | 0.57 ± 0.13 | 105 ± 27 | 155 ± 60 | 6.9 ± 2.9 | 0.73 ± 0.04 | 26 ± 11 | 108 ± 14 |
| **PAP 153** | 40 ± 16 | 0.59 ± 0.10 | 106 ± 43 | 154 ± 16 | 7.5 ± 2.0 | 0.62 ± 0.10 | 29 ± 9 | 106 ± 7 |

Table 11. Phytoextraction potential of the five poplar hybrids grown on the contaminated soil of CS3.

| | Phytoextraction (µg.plant$^{-1}$) | | | |
|---|---|---|---|---|
| **Hybrid** | **As** | **Cd** | **Pb** | **Zn** |
| **P10** | 78.1 | 3.63 | 269 | 650 |
| **PAP 148** | 77.0 | 4.71 | 256 | 901 |
| **PAP 149** | 87.8 | 4.88 | 320 | 931 |
| **PAP 152** | 81.6 | 6.09 | 277 | 948 |
| **PAP 153** | 118.1 | 5.62 | 381 | 1046 |

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Regarding Pb, concentrations in the leaves ranged from 51 to 140 mg.kg$^{-1}$; in stems, concentrations were found between 26 and 44 mg.kg$^{-1}$ (). In both organs, P10 presented the highest concentrations, whereas the lowest concentration was recorded in PAP 149 for the leaves and PAP 152 for the stems. In terms of phytoextraction potential, it was calculated to be between 256 and 381 µg (Table 10). The poplar with the lowest extraction potential was PAP 148, while the highest potential was found for PAP 153.

For Zn, P10 presented the lowest concentrations in both leaves (127 mg.kg$^{-1}$) and stems (106 mg.kg$^{-1}$) and PAP 149 had the highest concentrations (162 mg.kg$^{-1}$ in the leaves and 132 mg.kg$^{-1}$ in the stems) (). Being the poplar with the lowest biomass production and Zn concentrations, P10 was also the poplar with the lowest Zn phytoextraction potential (650 µg) (Table 10). Although it did not have the highest concentrations, PAP 153 presented the highest phytoextraction potential (1046 µg), thanks to its high biomass production.

#### 4.1.2.2.3 CS4. Silesian Voivodship (Poland)

The concentrations in Cd in the leaves ranged from 44 to 99 mg.kg$^{-1}$; in stems, concentrations were found between 20 and 39 mg.kg$^{-1}$ (Table 12). In both organs, PAP 148 presented the highest concentrations, whereas the lowest concentration was recorded in PAP 152 for the leaves and PAP 149 for the stems.

For Pb, PAP 152 presented the lowest concentrations in leaves (88 mg.kg$^{-1}$) and PAP 149 in stems (40 mg.kg$^{-1}$). PAP 148 had the highest concentrations (159 mg.kg$^{-1}$) in the leaves (Table 12). PAP 153 presented the highest concentrations in the stems (52 mg.kg$^{-1}$).

Table 12. Metal concentration in the leaves and stems of the five poplar hybrids grown on the contaminated soil of CS4.

| | Leaf concentration (mg.kg$^{-1}$) | | | Stem concentration (mg.kg$^{-1}$) | | |
|---|---|---|---|---|---|---|
| | Cd | Pb | Zn | Cd | Pb | Zn |
| **P10** | 82 | 124 | 1180 | 35 | 41 | 377 |
| **PAP 148** | 99 | 159 | 1590 | 39 | 41 | 362 |
| **PAP 149** | 62 | 141 | 1080 | 20 | 40 | 265 |
| **PAP 152** | 44 | 88 | 645 | 26 | 44 | 294 |
| **PAP 153** | 58 | 118 | 1040 | 35 | 52 | 361 |

For Zn, concentrations in the leaves ranged from 645 to 1590 mg.kg-1, while in the stems they were between 265 and 377 mg.kg-1 (Table 12). The lowest concentrations were measured in poplar PAP 152 (leaves) and PAP 149 (stems), whereas the highest concentrations were found in poplar PAP 148 and P10, respectively for the leaves and the stems.

#### 4.1.2.2.4 CS 6. Vieux-Charmont (France)

When grown on the contaminated soil of CS6, poplars were able to accumulate Cd and Zn in their leaves and stems. More precisely, Cd concentrations ranged from 1.78 to 2.28 mg.kg$^{-1}$ in the leaves and 0.85 to 1.05 mg.kg$^{-1}$ in the stems (Figure 16); and Zn concentrations were measured between 1434 and 1825 mg.kg$^{-1}$ in the leaves and between 287 and 389 mg.kg$^{-1}$ in the stems (Figure 16). In all cases, the lowest concentration was recorded for PAP 153 and the highest was found in P10.

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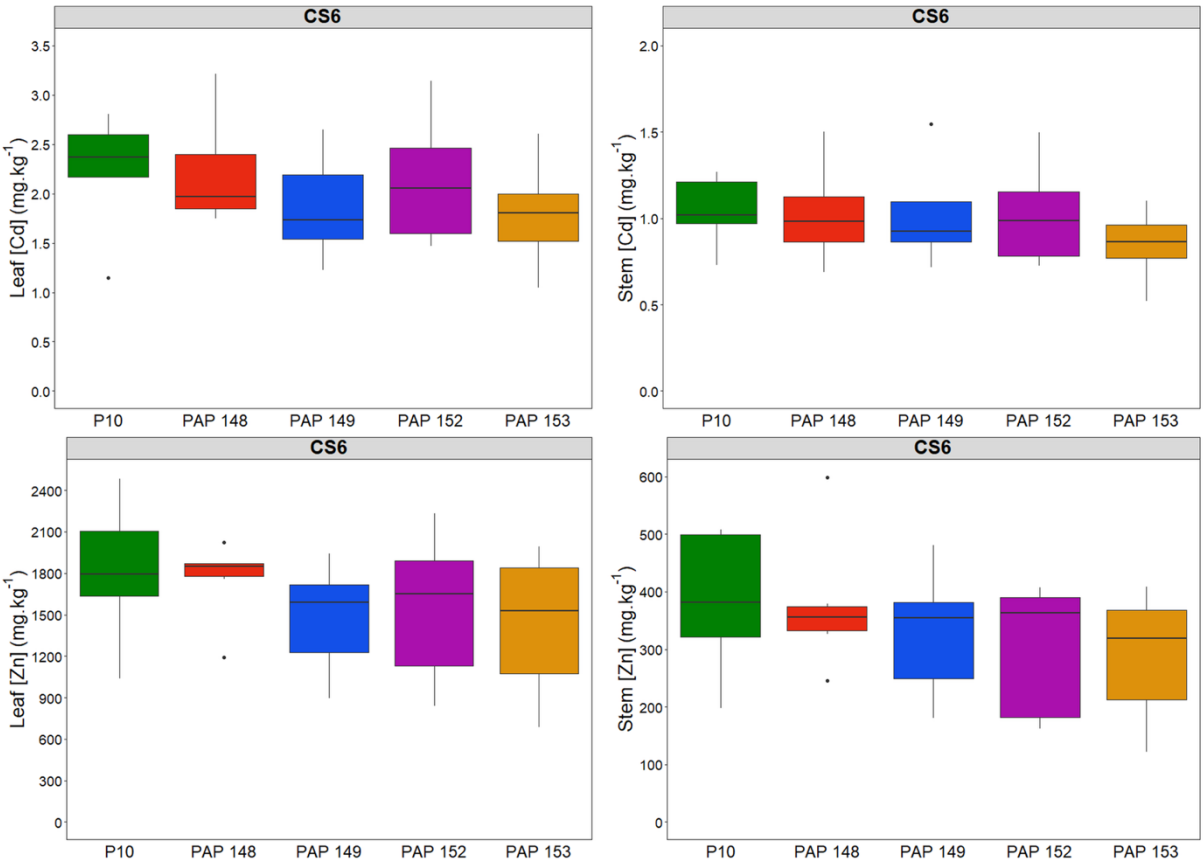


Figure 16. Cd and Zn concentration (mg.kg$^{-1}$) in the leaves and stems of the five poplar hybrids grown on the contaminated soil of CS 6.

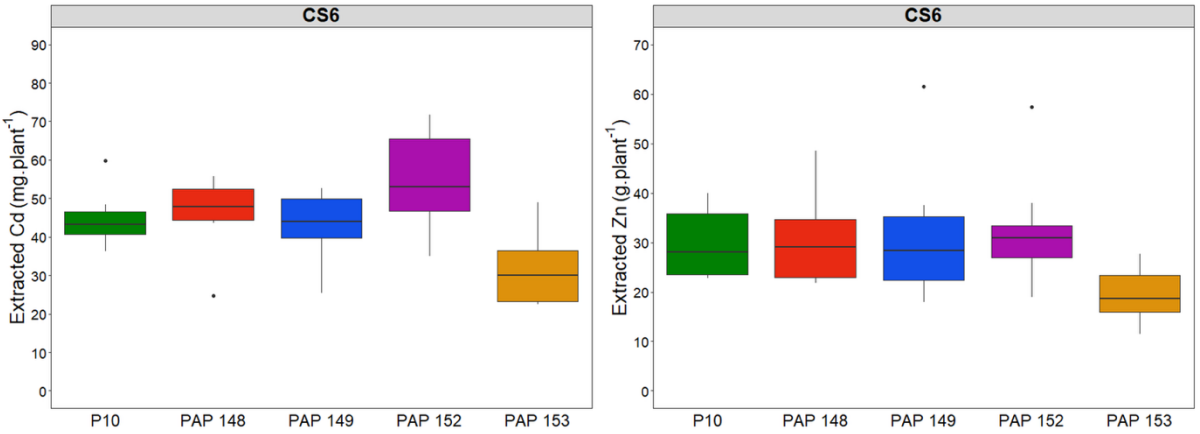


Figure 17. Quantity of Cd (mg.plant$^{-1}$) and Zn (g.plant$^{-1}$) phytoextracted in the aerial tissues of the five poplar hybrids grown on the contaminated soil of CS 6.

In terms of phytoextraction potential, PAP 153 presented the lowest values for both Cd (31.53 mg) and Zn (19.48 g), whereas PAP 152 was the hybrid with the highest phytoextractions potentials (54.38 mg Cd and 32.38 g Zn) (Figure 17).

#### 4.1.2.2.5 CS7. Lavrio (Greece)

On the site CS7, concentrations and phytoextraction potentials followed the same trend. In terms of As and Pb, the lower values were measured in PAP 153, with As concentration of 6.61 mg.kg$^{-}$

[1], As phytoextraction potential of 21.3 µg, Pb concentration of 58.60 mg.kg$^{-1}$ and Pb phytoextraction potential of 0.19 mg (Figure 18 and Figure 19). The lowest concentration and extraction for Ni were found in PAP 152 (10.93 mg.kg$^{-1}$ and 31.7 µg, respectively). For all cases, the highest accumulation values were observed in the hybrid PAP 149, with concentrations of 57.80 mg.kg$^{-1}$ As, 54.15 mg.kg$^{-1}$ Ni and 702.67 mg.kg$^{-1}$ Pb, and phytoextraction potential of 218.3 µg As, 205.0 µg Ni and 2.65 mg Pb.

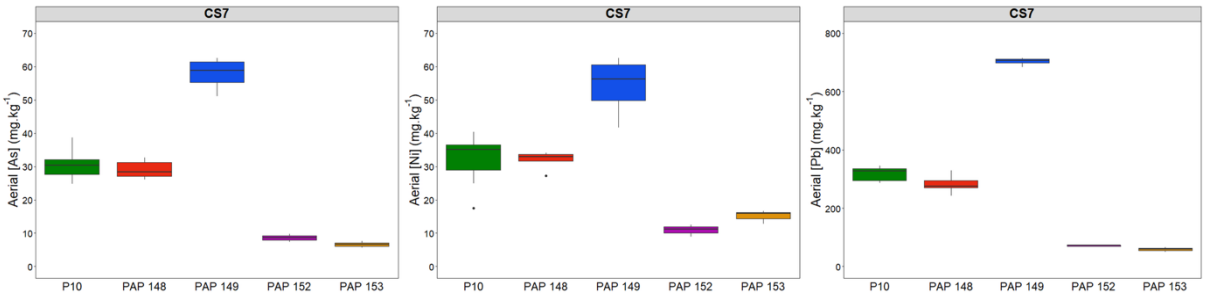


Figure 18. As, Ni, and Pb concentration (mg.kg$^{-1}$) in the aerial tissues of the five poplar hybrids grown on the contaminated soil of CS7.

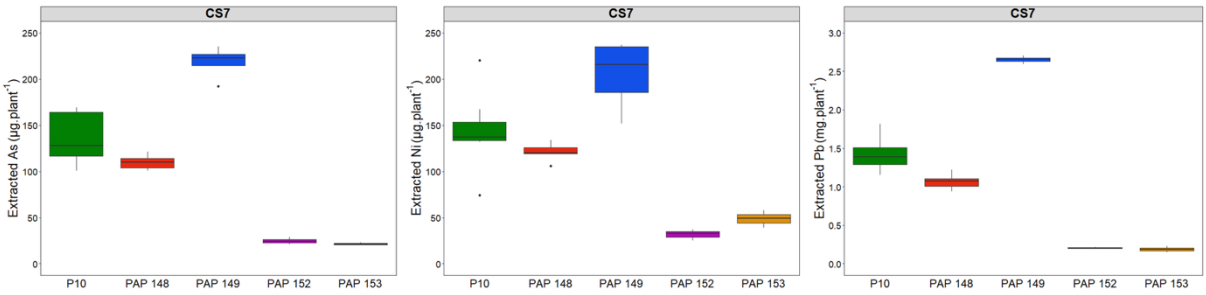


Figure 19. Quantity of As, Ni, and Pb (µg.plant$^{-1}$) phytoextracted in the aerial tissues of the five poplar hybrids grown on the contaminated soil of CS7.

#### 4.1.2.3 Conclusions

A preliminary field experiment was performed to confirm the results we obtained at laboratory scale. We confirmed that PAP 149 was the hybrid with the highest potential on CS2 and CS7 and PAP 148 on CS4. We could also determine PAP 152 to be best on CS6. However, on CS3, PAP 153 seemed to perform better on the field, compared to PAP 152 in the laboratory.

## 4.2 Optimization of the HM extraction efficiency by poplars

Phytoextraction efficiency is dependent on the plant biomass production and its HM accumulation. Another way to increase performance, in addition to plant selection, is to add amendments, chemical, organic and/or biological, that will act not only on plant growth but also on the HM availability. In the frame of Task 3.1, several laboratory tests were performed using the two French CSs to evaluate an organic and two biological amendments to improve the phytoextraction efficiency of poplars.

### 4.2.1 Consortium inoculation

Inoculating microbial strains can act on plant growth, through their PGP traits, and on the bioavailability of HMs, through their accumulation in the cells, binding or the secretion of molecules that will mobilize HMs. In EDAPHOS, as part of Task 3.1, we are evaluating two consortia (developed

in the frame of the BIOSYSMO project) on CS1 and CS6 for their influence on poplar phytoextraction performance.

#### 4.2.1.1 Microbial strain characterization

All nine fungal strains exhibited multiple PGP traits, including phosphate solubilisation, lignin utilisation as carbon source, growth on nitrogen-depleted conditions and the production of IAA and EPS (Table 13). Among them, six strains, *i.e.*, UFC 019, UFC 053, UFC 058, UFC 096, UFC 016, and UFC 050, demonstrated cellulolytic activity.

Most strains showed low to moderate apparent IAA production, as indicated by pale to moderately pink coloration in the Salkowski assay. UFC 058 was a notable exception, displaying a distinctly more intense pink colour compared to the other strains, suggesting a higher relative IAA production. In nitrogen-deficient medium, most strains exhibited limited growth, with the exception of UFC 009 and UFC 096, which showed moderate growth under these conditions. Both UFC 003 and UFC 009 showed mycelium formation on lignin as sole carbon source and produced comparatively high levels of siderophore and EPS compared to the other fungi. UFC 058 also displayed more intense dye binding in the EPS assay, indicating higher apparent EPS accumulation.

Siderophore production results for UFC 053 were inconclusive due to delayed colony development. In the case of UFC 096, red secondary metabolites interacted with the blue chromogenic siderophore assay medium, resulting in a purple colouration that shifted to orange after an additional three days of incubation.

Table 13. Plant growth promoting traits of the fungi consortium.

| Strains | Phosphate solubilisation | N depleted environment | Degradation | | Production | | |
|---|---|---|---|---|---|---|---|
| | | | Cellulose | Lignin | IAA | Siderophore | EPS |
| UFC 003 | ✔ | + | ✗ | +++ | + | +++ | +++ |
| UFC 006 | ✔ | + | ✗ | ++ | + | ++ | ++ |
| UFC 009 | ✔ | ++ | ✗ | +++ | + | +++ | +++ |
| UFC 019 | ✔ | + | ✔ | + | + | + | + |
| UFC 053 | ✔ | + | ✔ | ++ | + | - | ++ |
| UFC 058 | ✔ | + | ✔ | + | +++ | + | +++ |
| UFC 096 | ✔ | ++ | ✔ | + | + | + | ++ |
| UFC 016 | ✔ | + | ✔ | + | ++ | ++ | + |
| UFC 050 | ✔ | + | ✔ | + | + | ++ | ++ |

– inconclusive; + = low; ++ = moderate; +++ = high. ✓ = Presence of the trait. ✗ = Absence of the trait.

#### 4.2.1.2 Influence of consortium inoculation on plant performance

The monitoring of plant height throughout the experiment time showed that, on CS1, the inoculation of the consortium B helped the plants to grow faster than on the control, from the 28$^{th}$ day, whereas consortium A tended to have a negative influence within the first weeks (Figure 20). On CS6, the inoculation of the consortium A improved stem length during the first weeks of the experiment but did not have any effect later on (after 45 days), while the consortium B allowed for a higher stem height later on, from the 35$^{th}$ day and until the end of the experiment (Figure 20).

Contrary to the stem height, the consortia did not have a significant negative effect on the plant biomass production (Figure 21). On average, on the non-amended soils, aerial biomass production was 5.60 g on CS1 and 4.42 g on CS6. When consortium A was inoculated, biomass production was similar to the control (5.62 g for CS1 and 4.65 g for CS6), whereas when consortium B was inoculated, biomass production was similar on CS1 (5.68 g) and slightly reduced on CS6 (4.02 g).

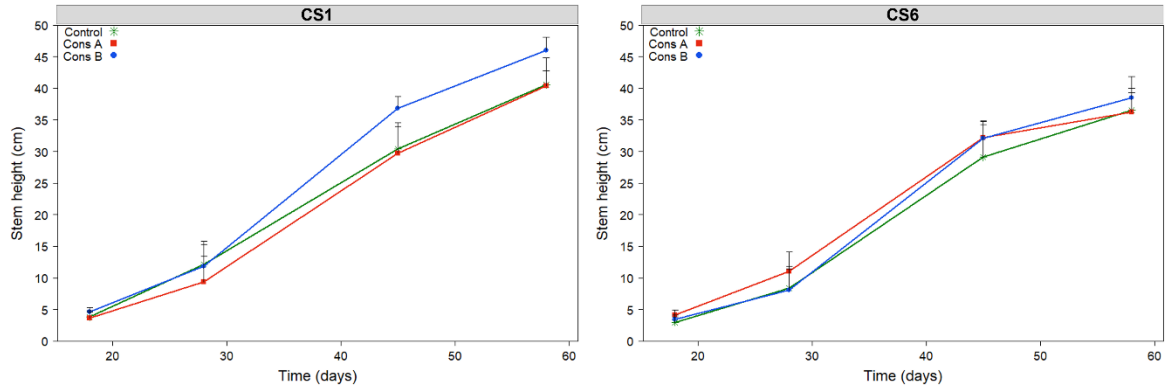


Figure 20. Stem height of poplar parent line P10, on CS1 and CS6, inoculated with either consortium A or consortium B.

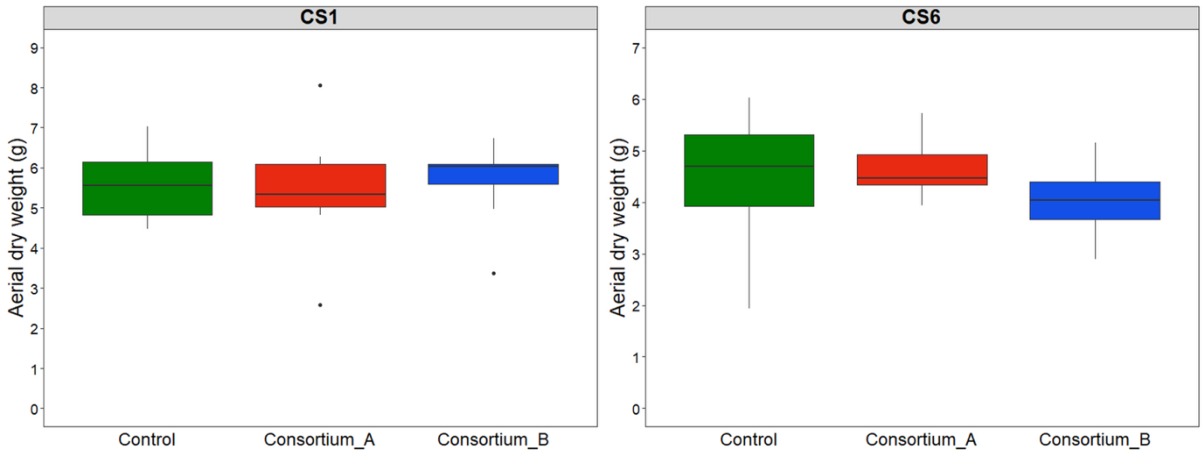


Figure 21. Aerial biomass production of the poplar parent line P10, on CS1 and CS6, inoculated with either consortium A or consortium B.

Table 14. Heavy metal concentrations and accumulation capacity in the leaves of the parent line P10 grown on CS1 and CS6, inoculated with either consortium A or B.

| | | Concentration ($mg.kg^{-1}$) | | Accumulation capacity ($\mu g.plant^{-1}$) | |
|---|---|---|---|---|---|
| | | Cd | Zn | Cd | Zn |
| CS1 | Control | 1.74 ± 0.23 | 67 ± 8 | 6.96 ± 0.46 | 267 ± 22 |
| | Cons A | 2.19 ± 0.36 | 76 ± 20 | 8.38 ± 1.39 | 285 ± 50 |
| | Cons B | 2.04 ± 0.67 | 70 ± 26 | 7.54 ± 2.16 | 262 ± 100 |
| CS6 | Control | 0.85 ± 0.25 | 335 ± 92 | 2.41 ± 0.68 | 934 ± 142 |
| | Cons A | 0.90 ± 0.53 | 270 ± 31 | 3.01 ± 1.82 | 893 ± 116 |
| | Cons B | 0.79 ± 0.40 | 287 ± 70 | 2.16 ± 1.06 | 791 ± 189 |

The analysis of HM concentration in the leaves revealed that consortium inoculation did not increase Cd and Zn concentration, for both CSs (Table 14). Similarly, accumulation capacity was not affected by consortium inoculation, whatever the consortium and whatever the soil.

### 4.2.2 Amendment application

Organic amendments are a way to not only improve soil fertility, through the addition of nutrients, and thus plant growth, but also to act on HM mobility and uptake, through the modification of the soil properties. Vinasse is a by-product, making it a sustainable amendment option, containing

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nutrients and able to act on soil pH (acidification) and thus HM mobility and availability. In EDAPHOS Task 3.1, this amendment was tested, at two dosages, on CS1 and CS6, in laboratory controlled conditions, to evaluate its capacity to improve the phytoextraction of HMs by poplar. Results are detailed in this section.

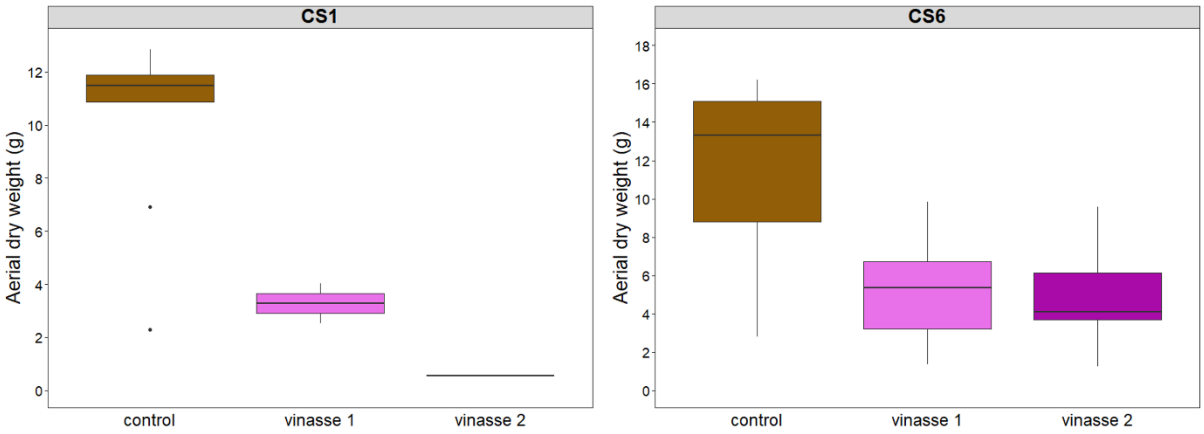


Figure 22. Aerial production of the poplar parent line P10, on CS1 and CS6, following the application of vinasse at 3.65 g.kg$^{-1}$ (vinasse 1) or two times 3.65 g.kg$^{-1}$ (vinasse 2).

The parent line P10 was able to grow on both soils. However, the application of the vinasse induced a high reduction of biomass production by 70 to 95 % on CS1, and from 55 to 60 % on CS6 (Figure 22). This showed that vinasse at this dosage was toxic to the plants, and that the toxicity was higher on CS1 than CS6, probably due to a difference in physico-chemical properties.

Table 15. Metal concentration in the leaves and stems and phytoextraction potential of P10 grown on CS1 and CS6, amended with vinasse (Control = no amendment; Vinasse 1 = one application of vinasse at 3.65 g.kg$^{-1}$; Vinasse 2 = two applications of vinasse at 3.65 g.kg$^{-1}$).

| | | Concentration | | | | Phytoextraction | |
|---|---|---|---|---|---|---|---|
| | | Leaf (mg.kg$^{-1}$) | | Stem (mg.kg$^{-1}$) | | Total (µg.plant$^{-1}$) | |
| | | Cd | Zn | Cd | Zn | Cd | Zn |
| CS1 | Control | 3.35 ± 0.61 | 105 ± 22 | 2.95 ± 0.43 | 36.1 ± 6.6 | 33.80 ± 12.65 | 850 ± 337 |
| | Vinasse 1 | 7.26 ± 2.21 | 175 ± 33 | 5.38 ± 0.47 | 60.9 ± 15.5 | 22.34 ± 11.94 | 447 ± 175 |
| | Vinasse 2 | 2.85 ± 0.00 | 66 ± 0 | 4.15 ± 0.00 | 51.5 ± 0.0 | 1.90 ± 0.00 | 33 ± 0 |
| CS6 | Control | 1.49 ± 0.63 | 429 ± 167 | 0.57 ± 0.33 | 63.0 ± 38.1 | 13.72 ± 4.58 | 3614 ± 1281 |
| | Vinasse 1 | 2.29 ± 0.38 | 499 ± 114 | 1.63 ± 0.31 | 146.3 ± 37.0 | 11.56 ± 6.71 | 2195 ± 1302 |
| | Vinasse 2 | 2.65 ± 0.72 | 598 ± 164 | 2.05 ± 0.47 | 200.3 ± 39.2 | 12.70 ± 7.10 | 2412 ± 1207 |

Plant analysis revealed that on CS1, Cd and Zn concentrations in the aerial tissues were highly increased by the application of vinasse, but only when it was applied once (Table 15). Cd concentrations were 2.2 times higher for the leaves and 1.8 times higher for the stems, while Zn concentrations increased by 1.7 times in both the leaves and stems. On CS6, concentrations also increased, but the second application of vinasse also increased concentration, more importantly than the single application (Table 15). One application of vinasse increased Cd concentration by 1.5 in the leaves and 2.8 in the stems, and Zn concentrations by 2.3 in the stems but not in the leaves. When vinasse was applied two times, increase fold changes were 1.8 (Cd in leaves), 1.4 (Zn in leaves), 3.6 (Cd in stems), and 3.2 (Zn in stems). However, due to the high toxicity of the vinasse, and thus lower biomass production, phytoextraction potential was reduced in the vinasse amended soils (Table 15).

### 4.2.3 Conclusions and next steps

Two greenhouse experiments were performed to test the potential of biological and organic amendments to improve phytoextraction.

- Fungal inoculation revealed to be beneficial during the first weeks of growth but failed to improve biomass production and HM extraction later on. This might be due to space limitation in the pots as the inoculated plants grew faster than the control ones. Further experiments in Reporting Period 2 in real conditions should help clarifying this point.
- The vinasse amendment had the expected effects, *i.e.,* i) decrease in soil pH and hence large increase in HM soil bioavailability, ii) high increase in plant HM concentrations which is the requested effect to improve HM capture by plants. However, the vinasse concentration used revealed to be toxic to the tested plants, and hence a poor growth was observed. Work still needs to be done to optimize the dosage of vinasse, which can also improve growth.

## 4.3 Companion species

Herbaceous species are great plants to be used in co-cropping systems. They can cover the ground surface of the soil, (hyper)accumulate HMs, and, for some, fix atmospheric nitrogen, making it available for plants such as poplars. One of the objectives of WP3 was to evaluate the potential of three species, *i.e., Brassica juncea, Lablab purpureus*, and *Chrysopogon zizanoides*, to accumulate and extract HMs on the different CSs. To answer this, as part of the work of Task 3.1, several pot experiments were set up under controlled conditions by the CS leaders.

### 4.3.1 Influence of soil contamination

The first step of Task 3.1 was to evaluate the influence of soil contamination on plant growth, by comparing biomass production between the contaminated soils and a control non-contaminated soil, and to assess the amount of HMs extracted by those species on the different soils. The data for CS4 are still under analysis and thus will not be presented in the Deliverable.

#### 4.3.1.1 Biomass production

All three species tested were able to grow on the different CSs (Figure 23, Figure 25, and Figure 24), except for *Brassica juncea* on CS3, for which an amendment was required.

On CS1, *Brassica juncea* did not show any reduction in aerial biomass production compared to the control (Figure 23), while *Lablab purpureus* showed a 21 % reduction (Figure 25). On CS3, even with the amendment, the aerial biomass production for both species was reduced by 65 % for *Brassica juncea* and by 56 % for *L. purpureus* (Figure 23 and Figure 25). On CS5, *B. juncea* grew better on the contaminated soil than the control soil, with a biomass production 31 % higher (Figure 23); whereas *L. purpureus* and *C. zizanoides* suffered from a 37-38 % reduction in biomass production (Figure 25 and Figure 24). Finally, on CS6, we measured low reduction in biomass production for both companion species, with a 7 % reduction for *B. juncea* and a 14 % reduction for *L. purpureus* (Figure 23 and Figure 25).

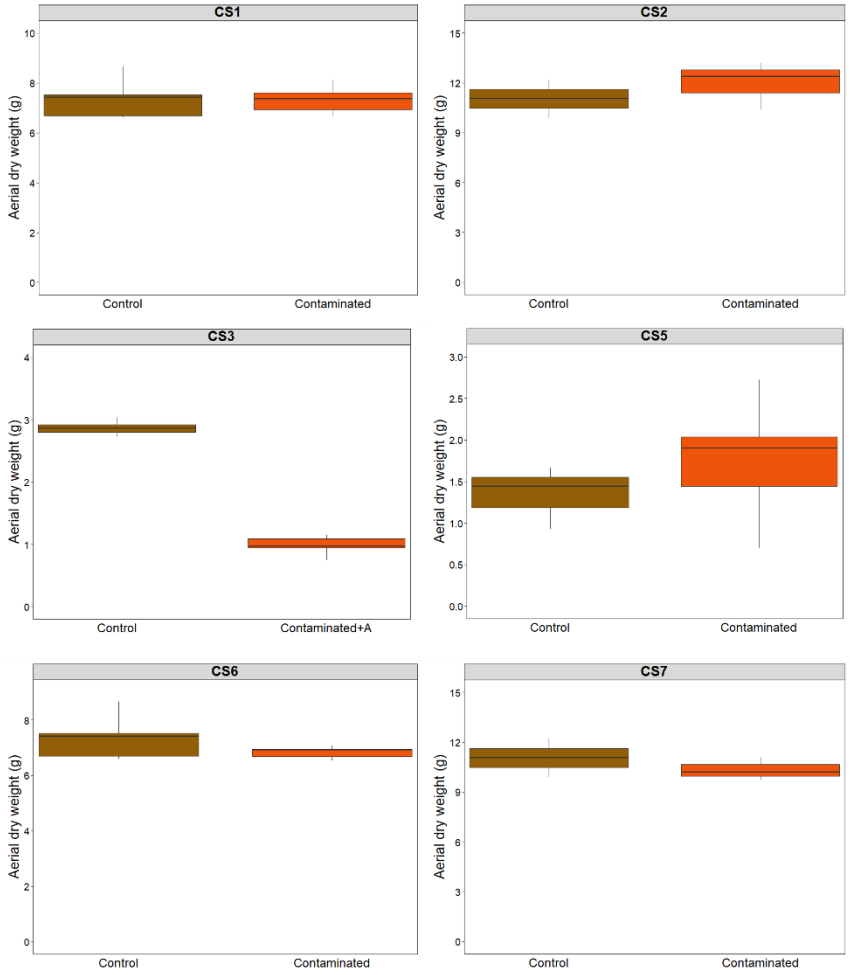


Figure 23. Aerial biomass production of *Brassica juncea* grown on control and contaminated soils for each CS. For CS3, the soil was amended with lime.

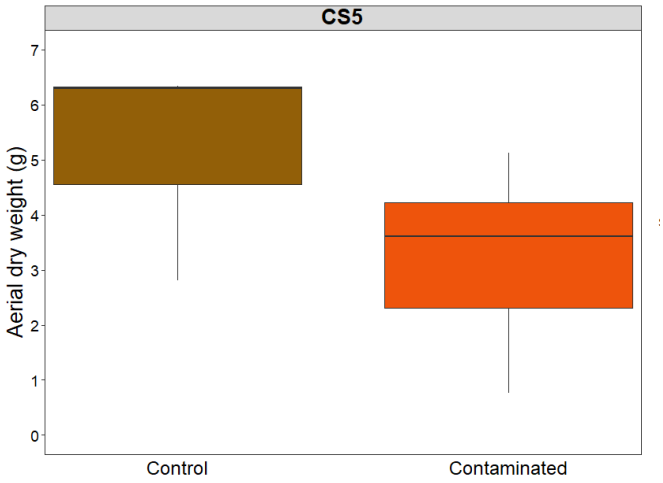


Figure 24. Aerial biomass production of *Chrysopogon zizanoides* grown on control and contaminated soils of CS5.

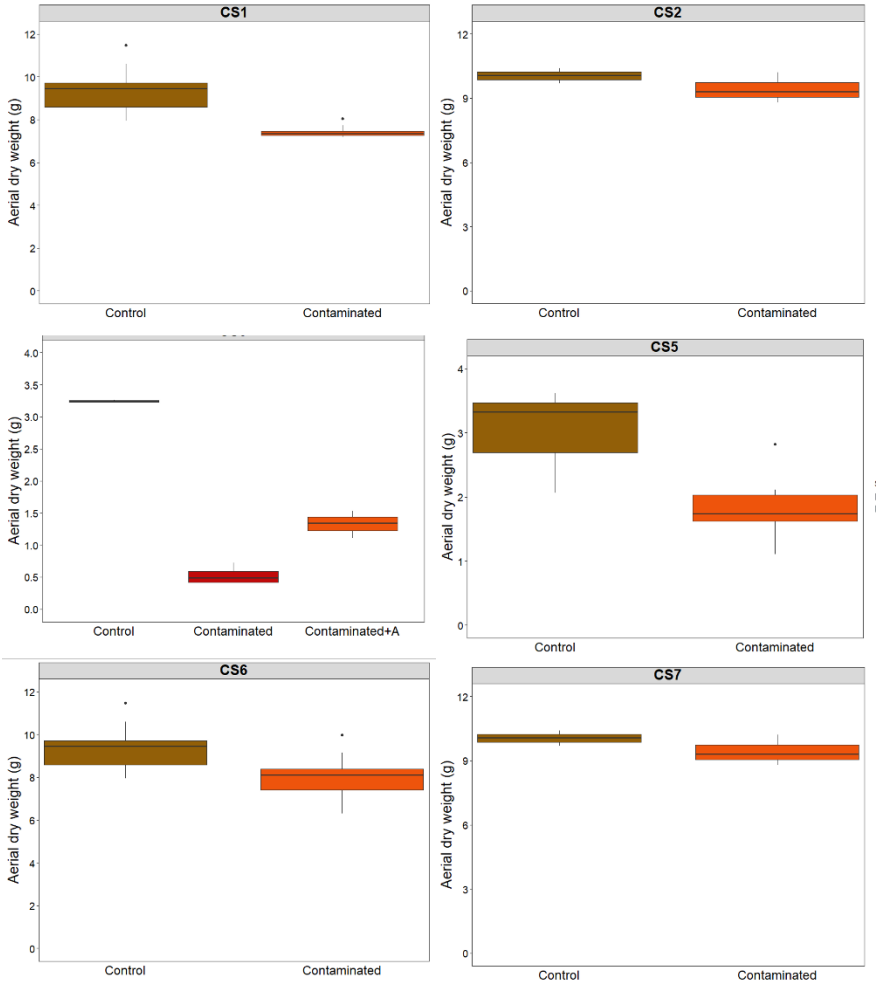


Figure 25. Aerial biomass production of *Lablab purpureus* grown on control and contaminated soils for each CS. For CS3, the soil was amended with lime.

### 4.3.1.2 Metal accumulation

#### 4.3.1.2.1 CS1. Carrières-sous-Poissy (France)

As expected, *B. juncea* accumulated more Cd and Zn than *L. purpureus*. On average, Cd concentrations were 1.89 mg.kg$^{-1}$ in *B. juncea* and 0.91 mg.kg$^{-1}$ in *L. purpureus* (Table 16), whereas Zn concentrations were 85 mg.kg$^{-1}$ and 63.6 mg.kg$^{-1}$, respectively. Similarly, phytoextraction potential was higher in *B. juncea* (13.76 mg Cd and 620 mg Zn) than *L. purpureus* (6.78 mg Cd and 472 mg Zn).

Table 16. Cd and Zn concentration and phytoextraction potential of the companion species (*Brassica juncea* and *Lablab purpureus*) grown on CS1.

| | Concentration (mg.kg$^{-1}$) | | Phytoextraction (mg.plant$^{-1}$) | |
|---|---|---|---|---|
| **Species** | **Cd** | **Zn** | **Cd** | **Zn** |
| ***Brassica juncea*** | 1.89 ± 0.28 | 85.0 ± 19.7 | 13.76 ± 1.66 | 620 ± 144 |
| ***Lablab purpureus*** | 0.91 ± 0.20 | 63.6 ± 8.6 | 6.78 ± 1.42 | 472 ± 64 |

#### 4.3.1.2.2 CS2. Kozani (Greece)

Unfortunately, technical issues, related to the available biomass, prevented us from making the analysis of the companion species for HM accumulation.

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#### 4.3.1.2.3 CS3. Odiel basin Area (Spain)

Again, accumulation was higher in *B. juncea* than *L. purpureus* (Table 17). *Brassica juncea* accumulated on average 17.6 mg.kg$^{-1}$ As, 0.6 mg.kg$^{-1}$ Cd, 177 mg.kg$^{-1}$ Pb and 335 mg.kg$^{-1}$ Zn. For *L. purpureus*, we can see that concentrations were lower (5.4 mg.kg$^{-1}$ As, 0.07 mg.kg$^{-1}$ Cd, 77 mg.kg$^{-1}$ Pb and 84 mg.kg$^{-1}$ Zn). In addition, we can observe that the application of the amendment reduced HM accumulation.

Table 17. As, Cd, Pb and Zn concentration and phytoextraction potential of the companion species (*Brassica juncea* and *Lablab purpureus*) grown on CS3.

| | | Concentration in leaves (mg.kg$^{-1}$) | | | | Phytoextraction (µg.plant$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Soil** | **As** | **Cd** | **Pb** | **Zn** | **As** | **Cd** | **Pb** | **Zn** |
| ***B. juncea*** | **Amended** | 17.6 ± 3.4 | 0.65 ± 0.08 | 177 ± 64 | 335 ± 18 | 17.44 | 0.64 | 174.8 | 332 |
| ***L. purpureus*** | **Contaminated** | 5.4 ± 1.2 | 0.07 ± 0.01 | 77 ± 17 | 84 ± 9 | 2.81 | 0.04 | 40.6 | 44 |
| | **Amended** | 0.3 ± 0.1 | 0.01 ± 0.00 | 2 ± 1 | 56 ± 2 | 0.43 | 0.01 | 2.8 | 74 |

#### 4.3.1.2.4 CS5. Galliera (Italy)

On CS5, the difference in concentration between *B. juncea* and *L. purpureus* was less pronounced ( However, *C. zizanoides* accumulated much less Cu and Zn. In terms of phytoextraction, due to its higher biomass production, *L. purpureus* was the companion species with the highest values.

Table 18). However, *C. zizanoides* accumulated much less Cu and Zn. In terms of phytoextraction, due to its higher biomass production, *L. purpureus* was the companion species with the highest values.

Table 18. Cu and Zn concentration and phytoextraction potential of the companion species (*Brassica juncea*, *Lablab purpureus* and *Chrysopogon zizanoides*) grown on CS5.

| | Concentration (mg.kg$^{-1}$) | | Phytoextraction (µg.plant$^{-1}$) | |
|---|---|---|---|---|
| **Species** | **Cu** | **Zn** | **Cu** | **Zn** |
| ***Brassica juncea*** | 15.7 ± 1.2 | 64.0 ± 6.1 | 40 ± 6 | 165 ± 23 |
| ***Lablab purpureus*** | 11.3 ± 2.4 | 60.7 ± 7.2 | 65 ± 17 | 360 ± 119 |
| ***Chrysopogon zizanoides*** | 5.2 ± 0.0 | 11.3 ± 2.9 | 33 ± 3 | 81 ± 31 |

#### 4.3.1.2.5 CS 6. Vieux-Charmont (France)

Table 19. Cd and Zn concentration and phytoextraction potential of the companion species (*Brassica juncea* and *Lablab purpureus*) grown on CS 6.

| | Concentration (mg.kg$^{-1}$) | | Phytoextraction (mg.plant$^{-1}$) | |
|---|---|---|---|---|
| **Species** | **Cd** | **Zn** | **Cd** | **Zn** |
| ***Brassica juncea*** | 0.61 ± 0.16 | 303 ± 70 | 4.18 ± 1.09 | 2074 ± 469 |
| ***Lablab purpureus*** | 0.39 ± 0.24 | 112 ± 50 | 3.01 ± 1.50 | 871 ± 265 |

On CS6, Cd and Zn were about two to three times higher in *B. juncea* than *L. purpureus* (

Table 19). For phytoextraction potential, *B. juncea* could extract 39 % more Cd and 2.4-times more Zn than *L. purpureus*.

#### 4.3.1.2.6 CS7. Lavrio (Greece)

Unfortunately, technical issues, related to the available biomass, prevented us from making the analysis of the companion species for HM accumulation.

#### 4.3.1.3 Conclusions

We showed that the tested companion species were able to grow on all CSs, except for CS3, for which amendment was required. When comparing HM concentrations, the HM-hyperaccumulator *Brassica juncea* presented the highest accumulation. However, its higher phytoextraction potential was only found in CS1, CS3, and CS6; while on CS5 *Lablab purpureus* was the best.

### 4.3.2 Amendment application

In addition to the first test, assessing the capacity of the companion species to grow on diverse contaminated soils and to accumulate HMs, additional tests were made in greenhouse conditions (Task 3.1) to evaluate the efficiency of several amendments (*e.g.,* sulphur and vinasse for *Brassica juncea*; activated carbon and vinasse for *Lablab purpureus*) to increase the phytoextraction of HMs by the companion species. Those tests were performed using the two French CSs and two companion species (*Brassica juncea* and *Lablab purpureus*).

#### *4.3.2.1 Brassica juncea*

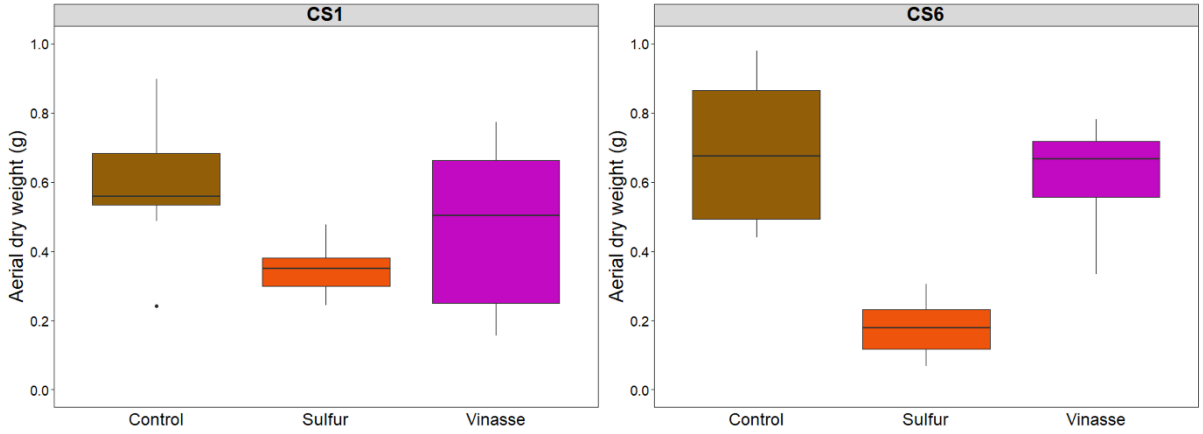


Figure 26. Aerial biomass production of *Brassica juncea* grown on CS1 and CS6, amended with sulphur (20 $mM.kg^{-1}$) or vinasse (1.25 $g.kg^{-1}$).

*Brassica juncea* was mainly affected by the application of sulphur, which induced a reduction in the biomass production by 40 % on CS1 and 70 % on CS6 (Figure 26). The application of the other sulphur-containing amendment, *i.e.,* vinasse, did not significantly affect the biomass production of *Brassica juncea*, on both soils.

Regarding the metal accumulation, Cd concentration was not affected by the application of the amendments, whereas its phytoextraction was reduced in the amended conditions, and in particular in the sulphur amended condition, due to the reduction in biomass production (Table 20). When looking at Zn, its concentration was reduced with the application of vinasse on both soils, while sulphur increased Zn aerial concentration on CS6 (Table 20). Again, phytoextraction was mainly reduced by the application of sulphur, due to the reduction in biomass production.

Table 20. Cd and Zn concentration and phytoextraction potential of *Brassica juncea* grown on CS1 and CS6, amended with sulphur (20 $mM.kg^{-1}$) or vinasse (1.25 $g.kg^{-1}$).

| | | Concentration ($mg.kg^{-1}$) | | Phytoextraction | |
|---|---|---|---|---|---|
| | | Cd | Zn | Cd ($\mu g.plant^{-1}$) | Zn ($mg.plant^{-1}$) |
| CS1 | Control | 0.39 ± 0.17 | 41.2 ± 31.4 | 211 ± 85 | 20.6 ± 8.2 |
| | Sulphur | 0.25 ± 0.09 | 43.0 ± 18.8 | 88 ± 35 | 15.2 ± 7.6 |

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| | | | | | |
|---|---|---|---|---|---|
| | Vinasse | 0.21 ± 0.22 | 35.0 ± 27.4 | 75 ± 84 | 14.6 ± 10.1 |
| CS6 | Control | 0.99 ± 0.30 | 2357 ± 659 | 699 ± 393 | 1670 ± 930 |
| | Sulphur | 1.00 ± 0.41 | 3935 ± 506 | 189 ± 103 | 680 ± 270 |
| | Vinasse | 0.82 ± 0.18 | 1988 ± 397 | 520 ± 177 | 1260 ± 400 |

#### *4.3.2.2 Lablab purpureus*

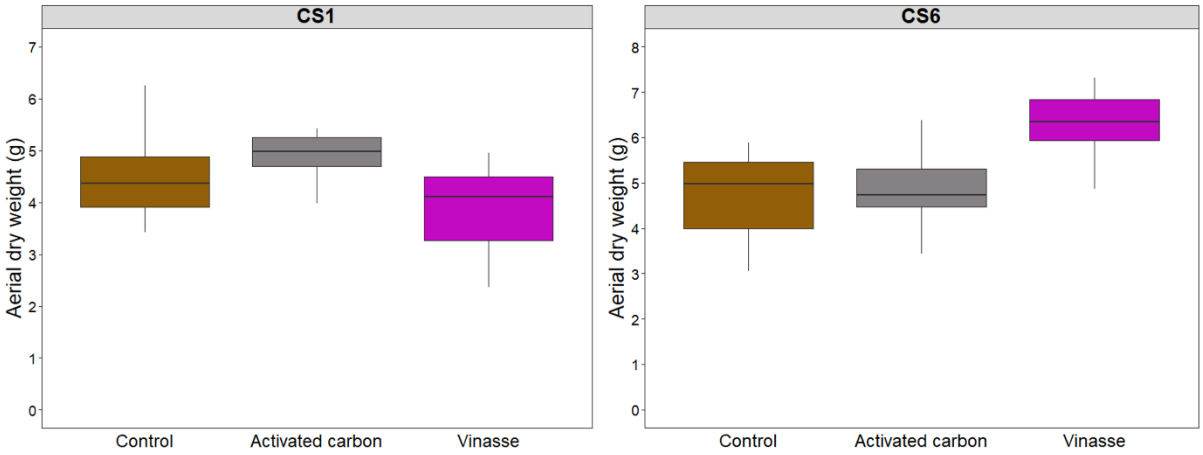


Figure 27. Aerial biomass production of *Lablab purpureus* grown on CS1 and CS6, amended with activated carbon (0.5 %, w/w) or vinasse (1.25 g.kg$^{-1}$).

When applied to CS1, the amendments had a small effect on biomass production, only vinasse induced a 16 % reduction in aerial dry weight (Figure 27). On the contrary, vinasse application on CS6 induced a 33 % increase in biomass production. This shows the influence of soil initial properties on amendment effects.

Table 21. Cd and Zn concentration and phytoextraction potential of *Lablab purpureus* grown on CS1 and CS6, amended with activated carbon (0.5 %, w/w) or vinasse (1.25 g.kg$^{-1}$).

| | | Concentration (mg.kg$^{-1}$) | | Phytoextraction (µg.plant$^{-1}$) | |
|---|---|---|---|---|---|
| | | Cd | Zn | Cd | Zn |
| CS1 | Control | 0.38 ± 0.08 | 28.89 ± 4.25 | 1.82 ± 0.35 | 139 ± 19 |
| | Activated carbon | 0.50 ± 0.07 | 34.28 ± 5.13 | 2.25 ± 0.47 | 154 ± 30 |
| | Vinasse | 0.42 ± 0.09 | 32.61 ± 4.05 | 1.60 ± 0.51 | 125 ± 28 |
| CS6 | Control | 0.25 ± 0.03 | 81.8 ± 12.3 | 1.21 ± 0.22 | 399 ± 102 |
| | Activated carbon | 0.21 ± 0.09 | 78.8 ± 14.1 | 0.95 ± 0.40 | 369 ± 78 |
| | Vinasse | 0.20 ± 0.03 | 58.6 ± 7.3 | 1.27 ± 0.23 | 370 ± 64 |

In terms of metal accumulation and phytoextraction, Cd and Zn concentrations were increased by the application of activated carbon on CS1, whereas their concentrations tended to decrease following the application of activated carbon or vinasse to CS6 (Table 21). The main influence of amendment application on metal phytoextraction was observed on CS1, with an increase by 24 % of the extraction of Cd by *L. purpureus* when activated carbon was applied.

### 4.3.3 Conclusions

The evaluation of the amendments was not conclusive. Although sulphur increased HM accumulation, it reduced plant growth and thus phytoextraction. Activated carbon had only a small effect on plant, whereas vinasse influence depended on soil and plant: (i) no effect on *Brassica juncea* (biomass and HM phytoextraction) on both soils; (ii) reduction of *Lablab purpureus* biomass on CS1,

without effect on HM phytoextraction; and (iii) improvement of *Lablab purpureus* growth on CS6, but no important effect on HM phytoextraction.

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# 5 General conclusions and links with other WPs

The work developed in Tasks 3.1 and 3.2 aims to test in greenhouse and field settings poplar hybrids provided by PHYTOWELT and companion herbaceous species to establish the best combination for each CS. Moreover, initially lab tests include the assemblage of crops and microbes and amendments.

The results presented in this deliverable (D 3.1) include an initial selection of poplar hybrid (one poplar parental (P10) and four poplar hybrids (PAP 148, PAP 149, PAP 152, and PAP 153) at greenhouse conditions (Task 3.1) in each CS soil. We were able to show that all hybrids could grow on the contaminated CS, even if their biomass production was reduced compared to a non-contaminated control soil. Moreover, based on the data on biomass production and HM accumulation, apart from the parent line P10, we can conclude that the following poplar hybrids performed best: PAP 152 on CS1, PAP 153 on CS2, PAP 152 on CS3, PAP 148 on CS4, PAP 153 on CS5, and PAP 149 on CS7. For CS6, it was more difficult to see one hybrid performing best on the different criteria. Task 3.1 includes also laboratory tests to determine the capacity of the proposed companion species (*B. juncea*, *L. purpureus,* and *C. zizanoides*) to perform well on the contaminated CS soils without presenting toxicity signs in terms of plant growth and development. The three companion species were able to develop on the different CSs, with a biomass of a few grams. All could accumulate HMs with a better potential of *Brassica juncea* on all CSs, except CS5 for which *Lablab purpureus* was best.

Moreover, in order to improve the poplar extraction efficiency three types of amendments, *i.e.,* biological (2 microbial consortia (Consortium A and B)), chemical (sulphur), and organic (vinasse and activated carbon), were tested and their influence on plant performance were determined at 2 CSs (CS1 and CS6). From the initial results, consortium inoculation was able to help grow faster on CS1 and CS6, at initial stage. However, the improvement of HM extraction was not straightforward. In the case of amendments, sulphur revealed to be toxic on both soils, also reducing phytoextraction while activated carbon did not have a major influence. Vinasse was toxic to plants (*Lablab purpureus* and poplar P10) when applied on CS1, whereas it was toxic or beneficial on CS6, depending on the plant.

In order to confirm the data obtained from greenhouse tests, a preliminary field experiment was performed (Task 3.2). We obtained the same results as the laboratory experiment for CS1, CS2, CS4, CS5, and CS7. We could also determine PAP 152 to be best on CS6. However, on CS3, PAP 153 seemed to perform better on the field, compared to PAP 152 in the laboratory.

All the results acquired from the greenhouse tests (Task 3.1) were used for the development of field scale tests (Task 3.2), which have been set-up in 2025 and will be monitored until the end of the project (Task 3.3).

From these initial greenhouse and field tests, we concluded that co-inoculation with beneficial microorganisms might offer complementary functions including nitrogen fixation, phosphorus uptake, pathogen suppression, and plant growth promotion, further enhancing phytomanagement outcomes. Although the consortium composition would need to be optimized for better outcomes, future research should also evaluate the effects of lower doses of organic amendment treatments on the plant development capacities and on the stability and efficiency of fungal consortia.

All the data obtained from greenhouse and field tests will be able to feed the WP1, WP2, WP4 and WP5 databases:

- **WP1**. The measured traits at leaf scale will be used in the WP1. For CS1, CS2 and CS6, spectral measurements at leaf scale covering the reflective domain were measured by a field spectrometer. These measurements will be correlated with HM concentrations and pigment


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contents in order to define an empirical model that will be applied at drone and airborne scales on multispectral and hyperspectral images. The work has been done for the CS6, is in progress for CS2 and will be done for CS1 in the next year (acquisitions were achieved in 2025 July and data preprocessing is in progress before starting prediction model specification)

- **WP2**. All the analyses performed at greenhouse scale, especially values of HM concentrations in soil and plant, were perused for filling the databases of WP2 and the assessment of the ERA included in the Task 2.2. Initial soil characterization was done with the TRIAD method (Task 2.3) and will serve to select the best indicators for ecotoxicological monitoring. The methodologies applied for these analyses and some results presented were exploited for the redaction of the D2.1 and D2.2.
- **WP4**. The homogenized results of D3.1 will be utilized in T4.1 for building and testing the multi-variate enviro-econometric model, relating biophysical growth metrics of the plants to soil decontamination efficiency. The first version and preliminary testing of the model will be implemented by September 2025. In addition, the results will contribute to the selection of studied ESS that are most representative of the 7 CSs for further monetary valuation. Field data of eco-toxicological results will be inputs in T4.3 as one of the 15 Product Environmental Footprint lifecycle analysis. Finally, the monitoring methodology and produced correlations will be a part of the Nature-Based Solutions Soil Restoration Observatory data collection branches and their communication to the European Union Soil Observatory for enriching its central soil health database.
- **WP5**. The results of D3.1 will contribute to T5.1 to develop the Decision Support Tool with 3-tiers; enriching the WebGIS risk assessment module with visualization of pollution (decontamination) intensities, the AI-enabled investment support module from correlating long-term time series, as well as the tailoring of BioL models by CS decontamination profile. T5.2 will utilize the D3.1 inputs used in T4.2 and T4.4 to elaborate the EDAPHOS Treasuries principles and ESS valuation validation process. T5.3 will utilize D3.1 results to build reliable performance risk profiles of the plants for the Green Bond instruments. T5.4 will incorporate D3.1 in the NBS Marketplace, while T5.5 will utilize them in the NBS Roadmap to highlight the challenges of the EDAPHOS experience across the upscaling to mainstream business and recommend solutions for future projects and applications.

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