



**BIORemediation systems exploiting SYnergies
for improved removal of Mixed pOllutants**

Deliverable D3.1

**Intermediate report on microbial consortia for enhanced
phytoremediation for each combination of target contaminants
and matrices**

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Executive Summary

Deliverable D3.1 – *Intermediate report on optimized bacterial consortia for enhanced phytoremediation for each combination of target contaminants and matrices* is the first deliverable of WP3. D3.1 is a result of *Task 3.1 – Characterization and enhancement of microorganisms for improved phytoremediation*. This document intends to be a compilation of functional data on relevant strains with remediation capabilities to be used to assemble the biosystems in WP4 and assist all future project tasks. The report is a document that describes the methodologies used and the results obtained on the functional characterization of relevant pollutant-degrading and metal tolerant microorganisms. These microbes were isolated from 6 case studies in France (UBFC), Spain (UBU) and Portugal (CIIMAR), either resulting from activities in T1.3 (UBFC and CIIMAR) as reported in D1.2, or from a wetland experiment with polluted water from Site 6 (UBU). The work developed in task T3.1 allowed the successful characterization of microbial isolates and consortia from three different matrices (contaminated soil, sediment and groundwater). These microorganisms and consortia will be further used in design and enhancement of bioremediation systems in WP4.

Table of Contents

Executive Summary	3
Table of Contents.....	4
List of Figures	7
List of Tables.....	8
Table of Abbreviations.....	9
1 Introduction.....	10
1.1 Concept and link with other WPs and related deliverables	10
1.2 Overall strategy	11
1.3 Summarized descriptions of the methodologies	13
2 Optimization of microbes and microbial consortia from rhizospheric soils	14
2.1 Methodology	14
2.1.1 Characterization of metal tolerance by soil microbes.....	14
2.1.2 Characterization of PGP traits.....	15
2.1.3 Selection of plant fungal assemblages	17
2.2 Results on the isolation of microbial communities at an agricultural soil contaminated with metals (Site 1).....	18
2.2.1 Metal tolerance of isolated microbes	18
2.2.2 Response of plant to microbial inoculation	19
2.2.3 Conclusions and planned activities	19
2.3 Results on the isolation of microbial communities at a chlor-alkali sediment disposal (site 2)..	19
2.3.1 Hg tolerance of isolated fungi.....	19
2.3.2 Hg tolerance of isolated bacteria.....	22
2.4 Results on the isolation of microbial communities at an industrial wasteland (site 10).....	25
2.4.1 Metal tolerance of isolated microbes	25
2.4.2 PGP traits of isolated microbes	26
2.4.3 Response of plant to microbial inoculation	27
2.4.4 Conclusions and planned activities	29

3	Optimization of microbes and microbial consortia from estuarine sediments	30
3.1	Methodology	31
3.1.1	Degradation of pharmaceuticals.....	31
3.1.2	Characterization of metal tolerance.....	33
3.1.3	Characterization of PGP traits in sediment bacteria	34
3.2	Results on the microbes and microbial consortia from the Douro Estuary (Site 4).....	34
3.2.1	Paroxetine removal by isolated bacteria and assembled consortia.....	35
3.2.2	Bezafibrate removal by isolated bacteria and assembled consortia.....	35
3.2.3	Ketoprofen and Cd removal by enriched microbial consortia.....	36
3.2.4	Conclusions and planned activities	37
3.3	Results on the microbes and microbial consortia from the Lima Estuary (Site 5).....	38
3.3.1	Paroxetine removal by enriched microbial consortium.....	39
3.3.2	Bezafibrate removal by enriched microbial consortium.....	39
3.3.3	Venlafaxine and Cu removal by enriched microbial consortium.....	40
3.3.4	Cu tolerance of isolated bacteria	40
3.3.5	PGP traits of isolated bacteria	40
3.3.6	Conclusion and planned activities	41
4	Optimization of microbes and microbial consortia from wastewater	41
4.1	Methodology	42
4.1.1	Isolation of epiphytic and endophytic bacteria from pilot experiment	42
4.1.2	Characterization of PGP traits for groundwater bacteria.....	43
4.1.3	Minimum Inhibitory Concentration (MIC) Assays.....	43
4.1.4	Isolates identification.....	44
4.1.5	Plant systems and optimization of plan-bacteria system	44
4.2	Results on the isolation of microbes from <i>P. australis</i> -contaminated groundwater experiment (Site 6)	45
4.2.1	Isolation of epiphytic and endophytic bacteria from pilot experiment	45
4.2.2	PGP traits of isolated microorganisms.....	46
4.2.3	MICs assays using contaminated groundwater	47

4.2.4	Identified isolates	48
4.2.5	Plant system and optimization of plan-bacteria system	49
4.2.6	Conclusions and planned activities	51
5	Synthesis and conclusions	52
6	References	54

List of Figures

Figure 1. Fungal biomass developed after 2 weeks incubation at 25°C as a function of the Hg concentration of the culture medium. *Error bars correspond to the standard deviation calculated from 4 replicates*..... 20

Figure 2. PGP traits for fungal strains. (A) Ratio of the quantity of IAA produced (in mM), (B) ratio of the quantity of total siderophores produced (in µM), and (C) dissolved phosphate ratio (in mg/L) to the fungal biomass developed after 2 weeks of incubation..... 21

Figure 3. OD at 600 nm of bacterial cultures grown after 5 days incubation at room temperature as a function of Hg concentration in the culture medium. *Error bars correspond to the standard deviation calculated from 4 replicates*..... 22

Figure 4. PGP traits for bacterial strains. (A) Ratio of the quantity of IAA produced (in mM), (B) ratio of the quantity of total siderophores produced (in µM), and (C) dissolved phosphate ratio (in mg/L) to the OD at 600 nm of bacterial cultures grown after 5 days of incubation. 24

Figure 5. Dry biomass produced by willow trees after 36 days of growth. The trees were inoculated with fungal strains. Letters in common indicate statistically similar groups at $p < 0.05$ according to mean comparison tests. “Control” corresponds to trees that were not inoculated..... 27

Figure 6. Height increase of willow trees after 15 (D15) and 36 (D36) days of growth. The trees were inoculated with fungal (top graph) or bacterial (bottom graph) strains. Letters in common indicate statistically similar groups at $p < 0.05$ according to mean comparison tests. “Control” corresponds to trees that were not inoculated 28

Figure 7. Biomass produced by willow trees after 2 months of growth. The trees were inoculated with fungal strains (UFC010 to UFC018 and UFC036). Significant differences are indicated with *, a, b, c, d, e and f. “CON” corresponds to the control (trees that were not inoculated). 29

Figure 8. Percentage of Kpf removal by the enriched consortium during nine cycles (T1-T9) of the enrichment experiment using Cd and Kpf. Values are represented for enriched cultures (w/MOs), sediments adsorption controls (Seds. Ads.) and abiotic controls (Abiotic) for microcosms with Kpf or with Cd and Kpf. *Error bars correspond to the standard deviation calculated from 3 replicates*..... 37

Figure 9. Percentage of Cd removal by the enriched consortium during nine cycles (T1-T9) of the enrichment experiment using Cd and Kpf. Values are represented for enriched cultures (w/MOs), sediments adsorption controls (Seds. Ads.) and abiotic controls (Abiotic) for microcosms with Cd or with Cd and Kpf. *Error bars correspond to the standard deviation calculated from 3 replicates*. 37

Figure 10. Percentage of Cu removal by the enriched consortium during eight cycles (T5-T12) of the enrichment experiment using Cu and Vfx. Values are represented for enriched cultures (w/MOs), sediments adsorption controls (Seds. Ads.) and abiotic controls (Abiotic) for microcosms with Cu or with Cu and Vfx. *Error bars correspond to the standard deviation calculated from 3 replicates*. 40

Figure 11. Representation of bucket used for plot experiment with *P. australis* 42

List of Tables

Table 1. Sites and groups of microbes investigated in T3.1.....	12
Table 2. Summary of the methodologies used for measuring functional traits in isolated microbes ...	13
Table 3. Pb and Zn maximal concentrations (MC in mM) tolerated by microorganisms isolated from the rhizospheric soil of site 1.	18
Table 4. Cd, Pb and Zn MIC ₅₀ values (mM) and standard errors for the microorganisms isolated from the rhizospheric soil of site 10.	25
Table 5. Characterisation of the production of total siderophores, IAA and phosphate solubilisation capacity of the bacteria isolated from site 10.	26
Table 6. Removal efficiency of Prx for each bacterial strain, for the paroxetine degrading consortium (DPS) and for the abiotic control, over an experimental period of four weeks.	35
Table 7. Removal efficiency of Bzf for each bacterial strain, for the bezafibrate degrading consortium (DBS) and for the abiotic control, over an experimental period of four weeks.	36
Table 8. Prx Removal efficiency performed by Prx enriched culture, after 21 cycles of 15 days.	39
Table 9. Bzf removal efficiency performed by Bzf enriched culture, after 21 cycles of 15 days.	39
Table 10. Bacteria isolated from the epiphytic, endophytic, and total communities of <i>P. australis</i> roots exposed to three different treatments.	46
Table 11. PGP traits of bacteria isolated from the epiphytic community of <i>P. australis</i> roots under control, acute or chronic exposure. TEP, Tap EPiphytic; AEP, Acute EPiphytic; CEP, Chronic EPiphytic.	46
Table 12. PGPR traits of bacteria isolated from the endophytic community of <i>P. australis</i> roots under control, acute or chronic exposure. TEN, Tap ENDophytic; AEN, Acute ENDophytic; CEN, Chronic ENDophytic.	47
Table 13. MIC assays of bacteria isolated from the epiphytic and endophytic communities of <i>P. australis</i> roots exposed to three different treatments.	48
Table 14. Germination percentages of <i>P. australis</i> seeds concerning their disinfection protocol (SP), growth medium and whether they underwent stratification or not.	49
Table 15. Phenotypic measurements of <i>P. australis</i> inoculated with the PGPR bacteria <i>P. fluorescens</i> F113, according to the inoculation method employed: seed inoculation, soil inoculation or root inoculation.	50

Table of Abbreviations

Abbreviation	Definition
ACC	aminocyclopropane-1-carboxylate
Bzf	Bezafibrate
DFOM	DeFerOxamineMesylate
IAA	Indole acetic acid
Kpf	Ketoprofen
LC-MS	Liquid Chromatography with a Mass spectrometry
ME	Malt extract
MIC	Minimal inhibitory concentration
MS	Murashige and Skoog medium
OD	Optical density
Prx	Paroxetine
Vfx	Venlafaxine
WGS	Whole genome sequencing

1 Introduction

1.1 Concept and link with other WPs and related deliverables

The industrial revolution and modern agricultural practices have caused widespread pollution over the last few decades, from soil to water and atmosphere. Phytoremediation, the use of plants to remove contaminants from the environment, has become an important approach in ecological engineering. Microorganisms can actively participate in these phytoremediation processes and extend the application of phytoremediation. Indeed, they are responsible for many key ecosystem functions. They are not only capable of decomposing organic matter, improving fertility, promoting energy flow and nutrient cycling, helping plants obtain nutrients and resist pests and diseases, but also contribute to degrading organic pollutants and reducing the toxicity of heavy metals (Teste et al., 2020). It is extensively demonstrated that the combination of plant with microorganisms might enhance plant resistance to contamination and improve biomass production. Hence, abundant and diverse microorganisms are the basis of healthy environments.

In the past, microorganisms were usually investigated at a taxonomic level, with a focus on microbial community structure (Yang, 2021), which cannot reflect the ecology function directly. Recently, researchers have started to pay attention to the importance of microbial functional traits, as they are directly related to the adaptability and properties of microorganisms in specific environments, and it has been shown that microbial functional traits are more sensitive to environmental perturbations than community structure. Once they have accumulated in soil or sediments, contaminants will not only affect soil microbial community structure, but also influence key microbial functions. The use of functional traits in microbial ecology holds great promise for improving our ability to develop biogeochemical models and predict ecosystem responses to environmental pollution. Functional traits, such as plant growth promoting (PGP) traits, tolerance to and degradation of contaminants, are measurable characteristics that affect an organism's fitness under certain environmental conditions and can serve as potential tools for monitoring environmental changes.

Plant growth promoting microbes (PGPM) promote bioremediation of soils, sediments and water by direct or indirect mechanisms when in association with plants. Such mechanisms developed by PGPM include metal-protein complex formation, biotransformation, methylation, and demethylation, the production of the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACC-deaminase) that hydrolyses ACC into α -ketobutyrate and ammonia. This reaction induces the decrease of endogenous level of ethylene in plants, often provoked by metal excess. PGPM could also boost plant growth and development by the secretion of a variety of metabolites and hormones, by the increase of nutrient - bioavailability through mineral solubilisation, by nitrogen fixation, by the production of biofilms, and by protection against pathogens.

In BIOSYSMO, we first viewed the microorganisms isolated from the various contaminated matrices through a taxonomic lens, primarily resolving taxonomic microbial diversity, using biomarkers of the 16S rRNA gene, 18S rRNA gene, or nuclear ribosomal internal transcribed spacer (ITS), as reported in D1.2 by CIIMAR and UBFC. Additionally, UBU has been focused on the isolation and characterization of endophytic and epiphytic microorganisms in the roots of *Phragmites australis* growing in metal(loid)s polluted (Site 6) and control water. In Task 3.1, CIIMAR, UBFC and UBU focused on a set of functional

traits (PGP traits, tolerance to metals, degradation of organic pollutants) that are usually recognized as key indicators in polluted environments, as they govern the interactions between plants and microbes under stressful environments.

The objective of D3.1 is to report functional characteristics of these microbes and consortia isolated from polluted sites (Task 1.3-D1.2) and from a pilot experiment using polluted water from Site 6 i) to tolerate the target pollutants; ii) degrade the target organic contaminants; and iii) promote plant growth.

- Task 3.1 will further generate relevant data that will allow the development of the GEM models foreseen by IDE in WP2.
- Consortia from Task 3.1 will be used in Task 3.2 to develop strategies for consortia aggregation and immobilization for improved efficiencies of biodegradation of pollutants in collaboration with JSI and LEITAT.
- The set of functional traits studied in Task 3.1 will allow for selecting the best performing microbes to engineer the experimental tree model *P. tremula x alba* INRA clone 717-1B4130, as deciphered in task T3.3 in collaboration with UPM.
- Overall, microbial consortia selected through task 3.1 will be further used in WP4 as constructed biosystems that will be applied in different bioremediation strategies and to validate them with polluted samples in the lab.

The overall strategy developed on the sites investigated by CIIMAR, UBFC and UBU are summarized in section 1.2 below and the methodologies are summarized in section 1.3 below.

1.2 Overall strategy

Six sites have been investigated, as depicted in Table 1. The full characterization and soil analysis are provided in D1.1.

- Contamination with metals :
 - 3 rhizospheric soils (sites 1, 2 & 10, FR)
 - 1 groundwater polluted site (site 6, BE) : *P. australis* plot system using Site 6 water.
- Contamination with organic compounds (diffuse pollution)
 - 2 rhizo-sediments (sites 4 & 5, PO)

Table 1. Sites and groups of microbes investigated in T3.1

Type of matrix	Site	Partner	Major contaminants	Main group of microbes	Provenance of microbial isolates
Rhizospheric soils	1	UBFC	Zn, Pb and Cd	Bacteria & fungi	WP1 (D1.2)
	2	UBFC	Hg	Bacteria & fungi	WP1 (D1.2)
	10	UBFC	Zn, Pb and Cd	Bacteria & fungi	WP1 (D1.2)
Estuarine sediments	4	CIIMAR	Prx, Kpf, Bzf, Cd	Bacteria	WP1 (D1.2)
	5	CIIMAR	Bzf, Prx, Vfx, Cu	Bacteria	WP1 (D1.2)
Contaminated groundwater from industrial area	6	UBU	Cu, Ni, Zn	Bacteria & fungi	Plot experiment with <i>Phragmites australis</i> exposure to polluted water from Site 6. WP3, Task 3.1

1.3 Summarized descriptions of the methodologies

Table 2 provides to the readers a summarized description of the methodologies used. Full description of the methodologies is provided in the following sections.

Table 2. Summary of the methodologies used for measuring functional traits in isolated microbes

Trait	Summarized methodology	Equipment
Metal tolerance for soil microbes	Microbial cultures exposed to increasing concentration of metals in solid and liquid media	TECAN, ICP-MS or ICP-AES
Removal capacities of organic + metal pollutants for sediment microbes	<ul style="list-style-type: none"> • Paroxetine degradation • Bezafibrate degradation • Ketoprofen degradation • Venlafaxine degradation • Copper removal • Cadmium removal <p>Cultures exposed to pharmaceuticals and metals and removal capacities are measured by High-performance Liquid Chromatography (HPLC) with Diode Array Detector (DAD) and Liquid Chromatography with Mass Spectroscopy (LC-MS) for pharmaceuticals; and Atomic Absorption Spectroscopy, with flame atomisation (FAAS) and with electrothermal atomisation (ETAAS) for metals</p>	Enriched culture method, HPLC, LC-MS, FAAS, ETAAS
Plant growth promoting traits	<ul style="list-style-type: none"> • Siderophore production • Nitrogen fixation • Phosphate solubilization • IAA production • Biofilm production • ACC deaminase activity <p>Cultures are exposed to metals and PGP traits are measured by colorimetric method or bacterial growth</p>	TECAN, colorimetric techniques, Bacterial growth
Selection of plant microbe assemblages	Model plants / microbe assemblage are co-cultivated in pot experiments while exposed to contaminants. Growth traits are measured.	Greenhouse

2 Optimization of microbes and microbial consortia from rhizospheric soils

A relatively new area of soil phytoremediation is phytomanagement in which non-food high biomass yielding crops such as poplar are used to reduce and control risks arising from pollution, while making a profitable and sustainable use of resources possible by extracting contaminants and valorising marketable biomass. Considering the increasing demand of biomass and resulting potential land-use conflicts, the cultivation of industrial crops in contaminated soils offers great environmental benefits and new social and economic opportunities for primary producers, broader society and the entire bio-based value system. Salicaceae trees, particularly poplar, have been widely studied for their ability to create diverse microbial associations, including rhizospheric and endophytic interactions with microorganisms (Ciadamidaro et al., 2022). In BIOSYSMO, poplar has been selected as a target plant for the remediation of polluted soils as this one of the only woody species which can be transformed, which utility in phytomanagement has been widely proven (Ciadamidaro et al., 2022). The presence of microorganisms, such as ectomycorrhizal fungi and endophytes, could be extremely important in the amelioration of tree performance in a very stressful environment, such as soils contaminated with metals. In task T3.1, UBFC aimed at selecting soil microorganisms that are both tolerant to metals and able to interact with woody species. We therefore measured PGP traits using well-adopted biochemical protocols, and tolerance to metals as evaluated by MIC measurements. The microbes that showed the most relevant traits were further inoculated to model woody plants to evaluate their impact on tree growth and development.

2.1 Methodology

2.1.1 Characterization of metal tolerance by soil microbes

Fungal isolates

In order to determine the Minimum Inhibitory Concentration (MIC) for the fungal isolates, growth tests in 12-well plates were carried out for different concentrations of trace metal elements: 0, 50, 100, 200, 400 and 600 μM for Hg, 0, 1, 2, 3.5, 5, 7.5, 10 mM for Cd, 0, 1, 2, 4, 6, 12, 25, 50, 100 mM for Pb and 0, 5, 10, 20, 40, 80, 100 and 120 mM for Zn. The fungal strains were grown on Malt Extract (ME) agar for 2 weeks at 25°C and these cultures were used to inoculate the wells of 12-well plates containing 2 mL of liquid ME medium at the concentration tested, using a plug (4 mm in diameter). Four replicates were carried out for each concentration tested and for each strain. The inoculated 12-well plates were then incubated for 2 weeks at 25°C.

- As a first pre-screening test when the number of strains is high, the data consist in simply providing the maximal concentrations (MC) at which isolates are growing, as mentioned for site 1.
- Once the first screening test is validated, a second round of test using a lower number of strains is performed to obtain more accurate and quantitative data as MIC₅₀, which defines the concentration of metal required to inhibit 50% of growth, is observed. At the

end of this incubation period, for a given well, the mycelium that had developed was freeze-dried and then weighed, to determine the final dry biomass.

Bacterial isolates

In order to determine the MIC for the bacterial isolates, growth tests in 96-well plates were carried out for different concentrations of trace metal elements: 0, 10, 25, 50, 75, 100 and 200 μM for Hg, 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6 mM for Cd and Pb and 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 mM for Zn (Becerra-Castro et al., 2012). The bacterial strains were pre-cultured in 3 mL of 284 medium for 48 h at room temperature (around 20°C) under agitation (250 rpm) and these cultures were used to inoculate 2 μL of pre-culture into each well containing 200 μL of 284 medium at the concentration tested. Four replicates were performed for each concentration tested and for each strain. The inoculated 96-well plates were then incubated for 5 days at room temperature (around 20°C) with agitation (250 rpm). At the end of this incubation period, the optical density at 600 nm (OD_{600}) was measured in each well using the Spark® microplate reader (Tecan) to assess the bacterial biomass that had developed.

2.1.2 Characterization of PGP traits

2.1.2.1 Siderophore production

Fungal isolates

To determine the siderophore production of fungal isolates, tests were carried out in 12-well plates (Schwyn & Neilands, 1987). The fungal strains were grown on ME agar for 2 weeks at 25°C and these cultures were used to inoculate the wells of 12-well plates containing 2 mL of minimum CAA medium (Casamino acid 5 g/L; K_2HPO_4 1.18 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 g/L; pH 7.0) with a plug (4 mm in diameter). Four replicates were performed for each strain. The inoculated 12-well plates were then incubated for 2 weeks at 25°C. At the end of this incubation period, for a given well, the mycelium that had developed was freeze-dried and then weighed, to determine the final dry biomass. In a 96-well plate, 15 μL of the previous culture medium is taken and diluted in 135 μL of ultrapure water, then 150 μL of CAS reagent is added. The CAS reagent is prepared as follows: for 100 mL of reagent, 6 mL of 10 mM CTAB (hexadecyltrimethylammonium bromide) and 10 mL of ultrapure water are mixed in a 100 mL volumetric flask; a solution of 1.5 mL Fe (III) (1mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; 10 mM HCl) and 7.5 mL of the 2 mM CAS solution is prepared and added slowly to the previous mixture; a buffer solution at pH 5.6 composed of 4.307 g of anhydrous piperazine in 20 mL of distilled water and 12.5 mL of 6N HCl was then added to the mixture; the CAS reagent was obtained by adding 0.1 g of 5-sulphosalicylic acid and then adjusting the final volume to 100 mL with ultrapure water (the CAS reagent is photosensitive and cannot be stored for more than a few hours). After two hours incubation in the dark at 25°C, the OD at 630 nm (OD_{630}) was measured. In parallel, the same reaction was carried out with a range of DeFerOxamineMesylate (DFOM), which is also an iron chelator equivalent to siderophores: this range includes concentrated solutions at 0, 2.5, 5, 7.5, 10 and 15 μM . In this way, a standard curve can be plotted and siderophore production can be quantitatively estimated for each strain, based on the biomass grown.

Bacterial isolates

To determine the siderophore production of the bacterial isolates, tests were carried out in 96-well plates (Schwyn & Neilands, 1987). The bacterial strains were pre-cultured in 3 mL of 284 medium for

48 h at room temperature (around 20°C) with agitation (250 rpm). After incubation, the OD₆₀₀ of the cultures was measured and adjusted to 1 in a 9 g/L KCl solution. Cultures with an OD₆₀₀ adjusted to 1 were then used to inoculate 25 µL of pre-culture into each well containing 225 µL of CAA minimum medium (see above). Four replicates were performed for each strain. The inoculated 96-well plates were then incubated for 3 days at room temperature (around 20°C) with agitation (250 rpm). At the end of this incubation period, the OD₆₀₀ was measured in each well to assess the bacterial biomass that had developed. The plates were then centrifuged for 20 min at 2000 rpm. In a new 96-well plate, for each replicate, 150 µL of supernatant was taken to perform an OD measurement at 405 nm, to quantify the presence of pyoverdine ($\epsilon = 19600 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$). In a new series of wells, 15 µL of the previous supernatant was taken and diluted in 135 µL of ultrapure water, then 150 µL of CAS reagent was added. After two hours incubation in the dark at 25°C, the OD₆₃₀ was measured (the presence of iron chelating siderophores caused the blue CAS-Fe complex to disappear, replaced by the orange free form). In parallel, the same reaction was carried out with a range of DFOM (see above). In this way, a standard curve can be plotted and the production of siderophores can be estimated quantitatively for each strain, in relation to the biomass that has grown.

2.1.2.2 Phosphate solubilization

Fungal isolates

To determine the phosphate-solubilizing capacity of fungal isolates, tests were carried out in 12-well plates (Braud et al., 2015). The fungal strains were grown on ME agar for 2 weeks at 25°C and these cultures were used to inoculate the wells of 12-well plates containing 2 mL of NBRIY medium ((NH₄)₂SO₄ 0.5 g/L ; NaCl 0.2 g/L; MgSO₄ 7H₂O 0.1 g/L; KCl 0.2 g/L; MnSO₄ H₂O 0.002 g/L; FeSO₄ 7H₂O 0.002 g/L; Ca₃(PO₄)₂ 5 g/L; pH 7.0) supplemented with 10 g/L glucose. Four replicates were performed for each strain. The inoculated 12-well plates were then incubated for 2 weeks at 25°C. At the end of this incubation period, for a given well, the mycelium that had developed was freeze-dried and then weighed, to determine the final dry biomass. The 12-well plates were then centrifuged for 20 min at 2000 rpm to pellet the undissolved phosphate. In a 96-well plate, for each replicate, 150 µL of supernatant was added to 150 µL of nitro-vanadomolybdic reagent. After one hour's incubation in the dark at room temperature, the OD₄₅₀ was measured. In parallel, the same reaction was carried out with a range of K₂HPO₄, which corresponds to a range of dissolved phosphate: this range includes solutions concentrated to 0, 25, 50, 100, 200 and 400 µg/mL. In this way, a standard curve can be plotted and the production of phosphate can be estimated quantitatively for each strain, in relation to the biomass that has grown.

Bacterial isolates

To determine the phosphate solubilization capacity of the bacterial isolates, tests were carried out in 96-well plates (Braud et al., 2015). The bacterial strains were pre-cultured in 3 mL of 284 medium for 48 h at room temperature (around 20°C) with agitation (250 rpm). After incubation, the OD₆₀₀ of the cultures was measured and adjusted to 1 in a 9 g/L KCl solution. Cultures with an OD₆₀₀ adjusted to 1 were then used to inoculate 25 µL of pre-culture into each well containing 225 µL of NBRIY medium (see above). Four replicates were performed for each strain. The inoculated 96-well plates were then incubated for 3 days at room temperature (around 20°C) under agitation (250 rpm). At the end of this incubation period, the plates were centrifuged for 20 min at 2000 rpm. In a new 96-well plate, for each

replicate, 150 μL of supernatant was added to 150 μL of nitro-vanadomolybdic reagent. After one hour's incubation in the dark at room temperature, the OD_{450} was measured. In parallel, the same reaction was carried out with a range of K_2HPO_4 (see above). In this way, a standard curve can be plotted and siderophore production can be estimated quantitatively for each strain, based on the biomass grown. The pellets from the centrifuged 96-well plates were resuspended with 150 μL of 6N HCl to dissolve all the phosphate that had not been dissolved by the bacteria. In this way, the OD_{600} could be measured in each well where the bacteria had been resuspended to assess the bacterial biomass that had developed. Results are expressed as the quantity of product (μM) related to growth (OD_{600}) and per day.

2.1.2.3 IAA production by bacterial strains

To determine IAA production for the bacterial isolates from soils, tests were carried out in 96-well plates (Zhao et al., 2023). The bacterial strains were pre-cultured in 3 mL of 284 medium for 48 h at room temperature (around 20°C) with agitation (250 rpm). After incubation, the OD_{600} of the cultures was measured and adjusted to 1 in a 9 g/L KCl solution. Cultures with an OD_{600} adjusted to 1 were then used to inoculate 25 μL of pre-culture into each well containing 25 μL of 5 g/L L-tryptophan solution and 200 μL of DF minimal medium (see above). Four replicates were performed for each strain. The inoculated 96-well plates were then incubated for 3 days at room temperature (around 20°C) under agitation (250 rpm). At the end of this incubation period, the OD_{600} was measured in each well to assess the bacterial biomass that had developed. The plates were then centrifuged for 20 min at 2000 rpm. In a new 96-well plate, 50 μL of supernatant was added to 200 μL of Salkowski reagent. After one hour's incubation in the dark at room temperature, the OD at 541 nm was measured (a red coloured complex was formed between the iron present in the Salkowski reagent and the IAA). In parallel, the same reaction was carried out with a range of IAA (see above). In this way, a standard curve can be plotted and the production of IAA can be estimated quantitatively for each strain, in relation to the biomass that has grown.

2.1.3 Selection of plant fungal assemblages

The fungal inocula were prepared in 500 ml glass jars in which 250 ml of perlite were mixed with 250 ml of MEA medium. After sterilization, 6 fungal agar implants (0.8 cm diameter) were mixed with the perlite. The jars were incubated in the dark for three to four weeks at 24°C and were shaken every seven days to ensure homogeneous development of the inoculum. The inoculated perlite was mixed (1:10) with the TE-contaminated soil that was previously sterilized by three successive autoclaving steps (120 °C for 20 min, 2-day intervals between each step). Two hundred twenty-five grams of sterilized soil was added in 300-ml pots together with 25 g of fungal inoculum. A 5 cm *Salix aquatica grandis* cutting was planted in each pot (n=10 per fungal species). Plants were placed in a growth chamber for 8 weeks (24°C, 16 h light per day, 90% moisture).

The plants were harvested and successively washed with three baths (tap water and distilled water). Several plant parameters were measured, such as plant biomass, leaf area, or plant TE concentrations. For the determination of the fresh biomass, the roots were separately weighed from the aerial part of the plant. The leaves have been scanned and their area was determined using the software ImageJ. Then, the leaves and the stems were placed in a forced-air oven at 70°C for at least 48 hr and grounded using

a stainless-steel mill. Samples were then mineralized in HNO₃ (68%) and H₂O₂ (30%), filtered and analysed by ICP-AES.

2.2 Results on the isolation of microbial communities at an agricultural soil contaminated with metals (Site 1)

Site 1 is an agricultural soil with high levels of lead (Pb), cadmium (Cd) and zinc (Zn) due to wastewater deposition (Ciadamidaro et al., 2019). However, the effect of these contaminants on the rhizospheric microbial communities that likely drive plant productivity is unknown. It is thus important to characterize the microbial communities from the site, as it may help in understanding the revegetation processes of contaminated areas. At this site, one single sampling campaign was done in May 2023, due to restricted access to the site until Spring 2023. At this stage, 32 fungal isolates from Site 1 have been retained after a first selection screening, as reported in D1.2.

2.2.1 Metal tolerance of isolated microbes

UBFC performed a pre-screening test to sort out the best fungal strains from site 1, as detailed in section 2.1.1. Some isolates showed strikingly high metal tolerance capacities (Table 3), such as some *Penicillium* isolates, which are still growing at the maximum concentrations used (e.g. 100 mM Pb).

Table 3. Pb and Zn maximal concentrations (MC in mM) tolerated by microorganisms isolated from the rhizospheric soil of site 1.

Isolate	MC Zn (mM)	MC Pb (mM)
UFC036	10	50
UFC037	1	25
UFC038	1	100
UFC039	1	50
UFC034	5	100
UFC040	0	50
UFC041	5	25
UFC042	20	75
UFC043	10	0
UFC025	20	100
UFC044	20	100
UFC045	1	25
UFC046	10	0
UFC047	0	0
UFC048	1	0
UFC049	10	75
UFC050	10	100
UFC051	20	0
UFC052	1	0
UFC053	20	100
UFC054	10	25
UFC055	20	0
UFC056	0	0
UFC057	10	0
UFC058	20	100
UFC059	20	12,5
UFC060	20	0

UFC061	20	0
UFC062	10	0
UFC063	na	0
UFC064	na	100
UFC065	na	0

2.2.2 Response of plant to microbial inoculation

This activity started in January 2024 and will be reported in the next deliverable.

2.2.3 Conclusions and planned activities

The fungal stains tested in this first round of screening tests showed unequal tolerance to Pb and Zn. A set of approx. 6 strains will be further selected and tested for a full screening test, for whole genome sequencing (WGS) and for further development of a microbial consortium for use in T3.3.

2.3 Results on the isolation of microbial communities at a chlor-alkali sediment disposal (site 2)

Here, we aim to improve our knowledge of the molecular and cellular mechanisms involved in the accumulation and tolerance of Hg by soil microorganisms to better understand their role in the biotransformation and transfer of this contaminant. The work was performed at a Hg-contaminated chlor-alkali site (site 2) where Hg speciation has been fully characterized (D1.1). The Hg-tolerant bacteria and fungi were isolated by culture-dependent approaches, as reported in D1.2.

2.3.1 Hg tolerance of isolated fungi

The concentration of Hg in the soil at the study site varies between 2.9 and 9.1 mg/kg of soil, with an average of 5.6 ± 1.6 mg/kg and remains homogeneous in the first 30 cm of depth (Maillard et al., 2016). This is equivalent to approximately 28 $\mu\text{mol/kg}$ of soil. For this reason, the Hg concentration in the isolation media was set at 10 μM , to maintain a selection pressure like the one present in situ, although the bioavailability of Hg varies in liquid media compared with that of soil in situ.

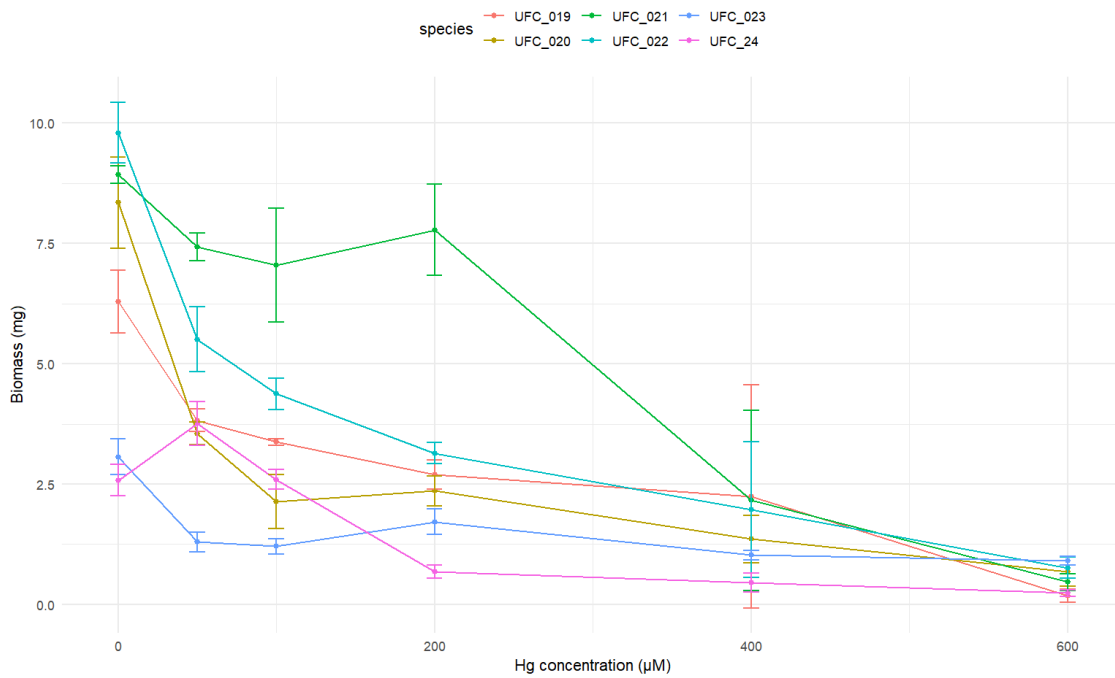
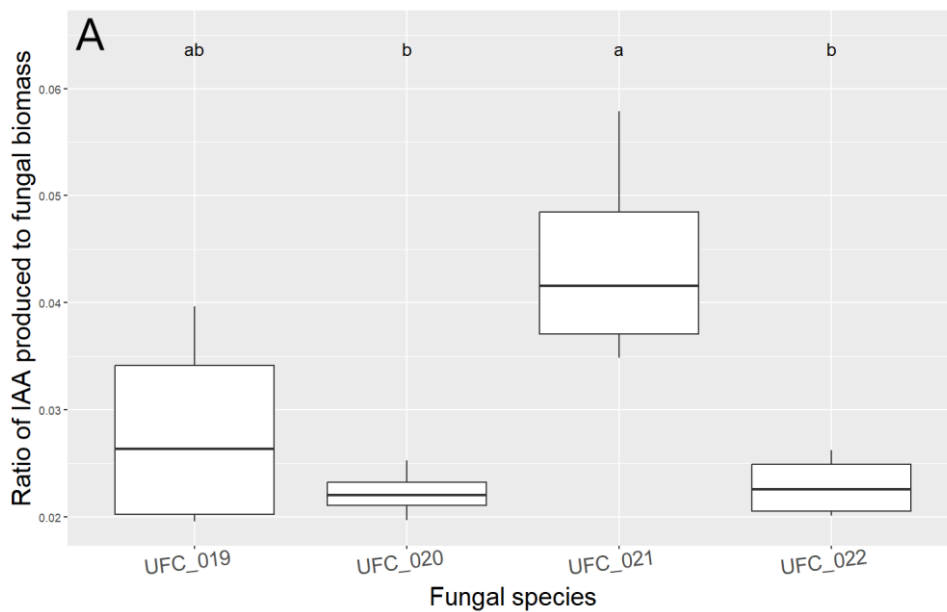


Figure 1. Fungal biomass developed after 2 weeks incubation at 25°C as a function of the Hg concentration of the culture medium. Error bars correspond to the standard deviation calculated from 4 replicates.

The results of MIC tests on fungi are shown in Figure 1: the fungal strains all grew at Hg concentrations above 100 µM, and the most resistant (UFC_019, UFC_020, UFC_021 and UFC_022) grew above 400 µM.



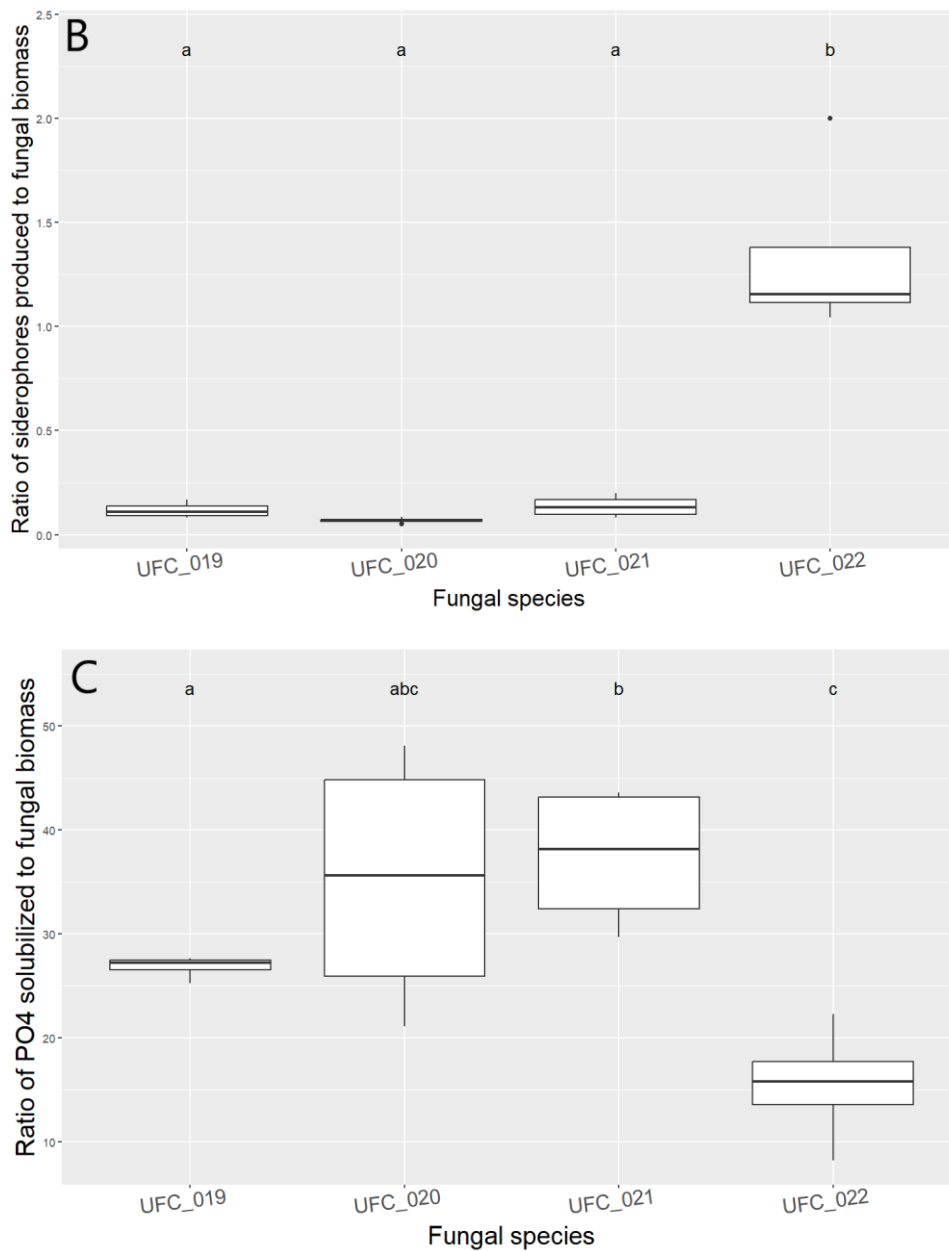


Figure 2. PGP traits for fungal strains. (A) Ratio of the quantity of IAA produced (in mM), (B) ratio of the quantity of total siderophores produced (in μM), and (C) dissolved phosphate ratio (in mg/L) to the fungal biomass developed after 2 weeks of incubation.

As shown in Figure 2, regarding indole acetic acid (IAA) production, the UFC_021 strain stands out from the other isolates even if they seem to produce some. Conversely, only UFC_022 appears to produce siderophores. Finally, all strains solubilize phosphate at various levels.

2.3.2 Hg tolerance of isolated bacteria

In the same way as the fungal strains, the bacterial strains were isolated on an isolation media containing Hg 10 μM . However, as resistance mechanisms differ in bacteria and fungi, similar MIC results should not be necessarily expected.

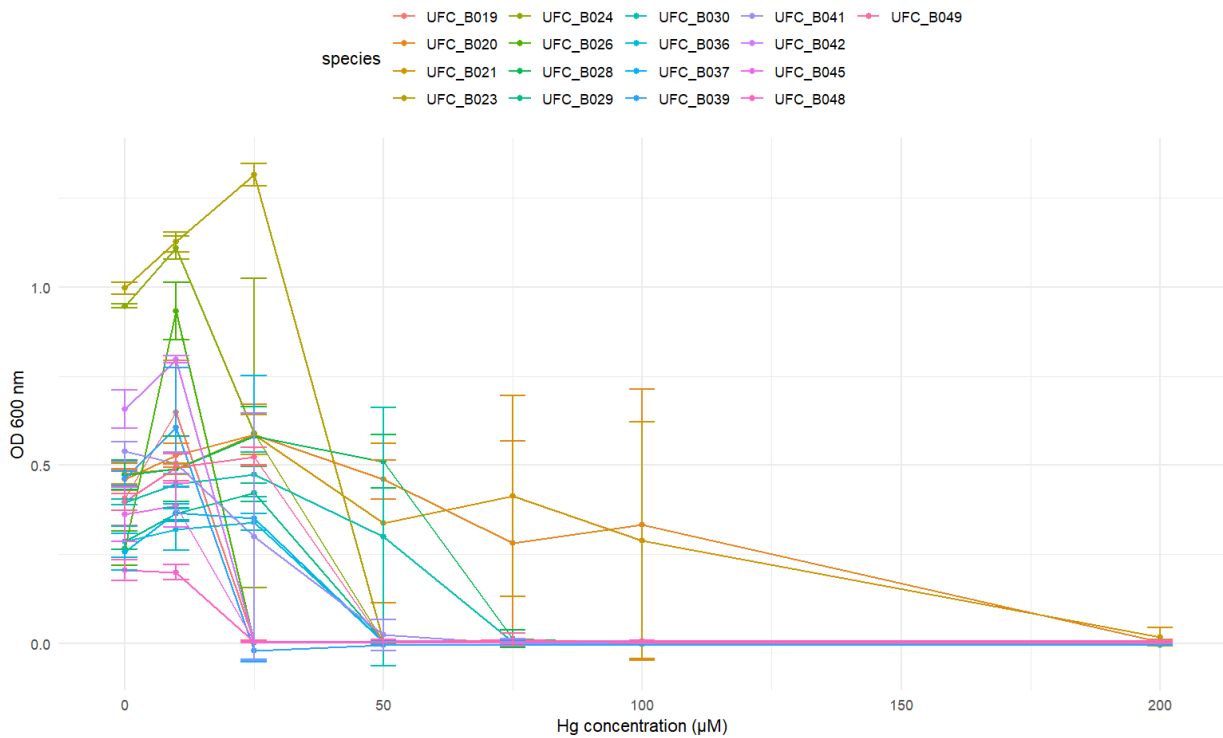
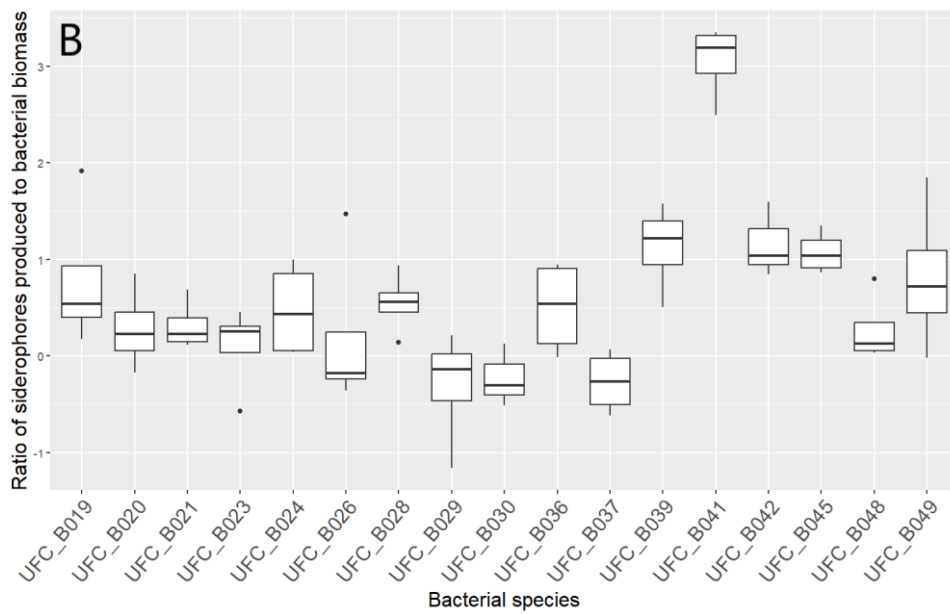
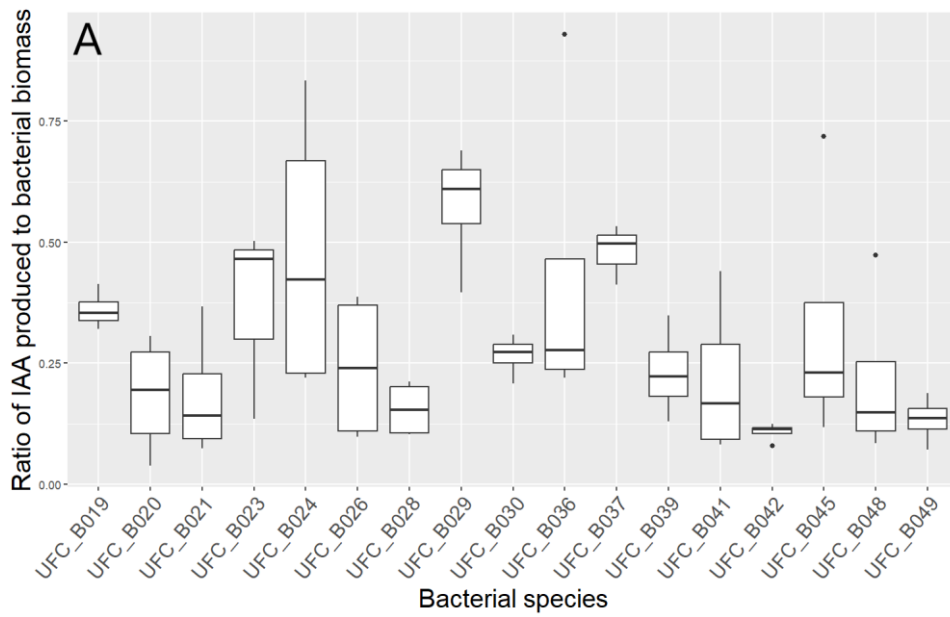


Figure 3. OD at 600 nm of bacterial cultures grown after 5 days incubation at room temperature as a function of Hg concentration in the culture medium. Error bars correspond to the standard deviation calculated from 4 replicates.

The results of the bacterial MIC tests are shown in Figure 3. The bacterial strains isolated appear to be much less resistant to Hg than the fungal strains, since no strain develops above 200 μM , and only 2 strains show growth above 100 μM . Almost all the strains also showed greater growth at low Hg concentrations (10 μM) compared with mercury-free cultures. One hypothesis is that the stress induced by the presence of low levels of Hg, which is tolerable for most strains, leads to an increase in biomass, perhaps as a survival strategy.



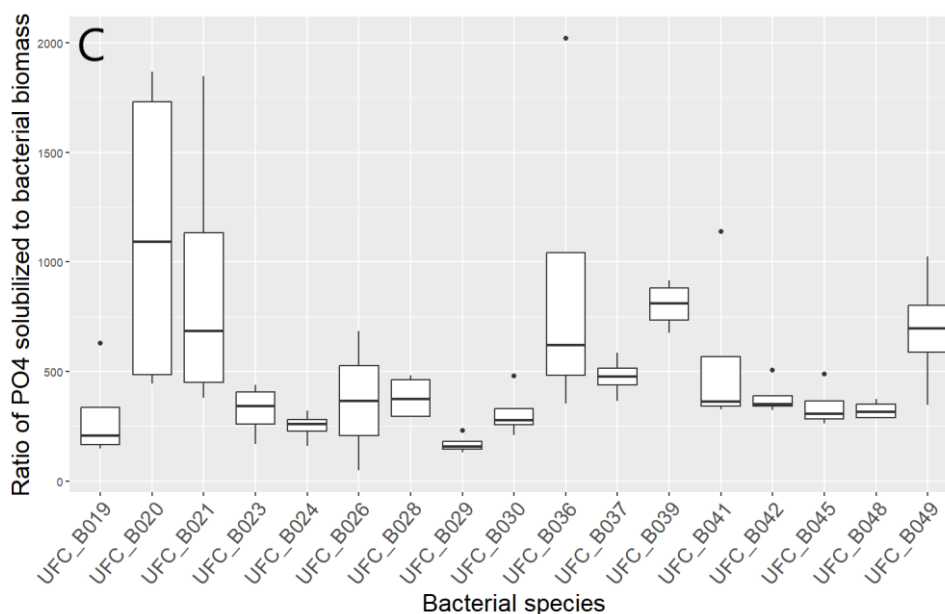


Figure 4. PGP traits for bacterial strains. (A) Ratio of the quantity of IAA produced (in mM), (B) ratio of the quantity of total siderophores produced (in μM), and (C) dissolved phosphate ratio (in mg/L) to the OD at 600 nm of bacterial cultures grown after 5 days of incubation.

In contrast to fungi, according to Figure 4, all bacterial strains produce IAA and siderophores, and are capable of solubilizing phosphate, sometimes in large quantities.

2.3.2.1 Response of plant to microbial inoculation

This activity started in January 2024 and will be reported in the next periodic report.

2.3.2.2 Conclusions and planned activities

The Hg-resistant microbial strains isolated from Site 2 were characterised for their functional traits. The results above indicate that some of these microorganisms have relevant traits for the implementation of bioremediation strategies at Hg contaminated sites.

- As an on-going activity, the genome of the isolated microbes (D1.2) will be thoroughly investigated to apprehend the physiological processes behind these functional traits, as a collaborative work between UBFC and IDENER in WP2 (Tasks 2.1 and 2.2). WGS of 4 fungal isolates is ongoing. Based on the genomic information, further RNAseq experiments will be set-up to evaluate the contribution of various genes in Hg resistance and methylation processes.
- It is also planned to investigate whether certain functional traits are enhanced by combining several strains, in the form of microbial consortia. The innovation will rely on the use of mixed consortia, which include bacteria and fungi.
- In the coming weeks, the most relevant candidates will be inoculated as consortia into the rhizosphere of the model plant (poplar 717 1 B4) developed in WP3 (Task 3.3), in cooperation with UPM, to validate their use as bioremediation tools.

2.4 Results on the isolation of microbial communities at an industrial wasteland (site 10)

The polluted wasteland at Site 10 is located on the edge of the urban centre, in the immediate vicinity of the Belfort-Montbéliard Greenway and exhibits high risks for human health as recently demonstrated, due to the presence of As, Pb and Zn in excess (Collot et al. 2023). The microbiome from this highly contaminated soil has been described in D1.2. Here we aim to improve our knowledge on bacteria and fungi isolated from this site, and to determine the PGP potential of the isolated strains.

2.4.1 Metal tolerance of isolated microbes

The results of MIC₅₀ tests on bacteria and fungi are shown in Table 4:

- For bacteria, the Zn MIC₅₀ varied from 0.18 to 2.08 mM with highest values observed for UFCB010 (2.08 mM), UFCB014 (1.59 mM) and UFCB013 (0.88 mM). The Pb MIC₅₀ varied from 0.18 to 1.62 mM with highest values observed for UFC B012 (1.62 mM), UFCB002 (1.37 mM) and UFCB011 (1.18 mM). The Cd MIC₅₀ varied from 0.13 to 2.32 mM with highest values observed for UFCB002 (2.32 mM), UFCB007 (1.28 mM) and UFCB004 (0.67 mM).
- For fungi, the Zn MIC₅₀ varied from 0.00 to 110 mM with highest values observed for UFC018 (110 mM), UFC016 (100 mM) and UFC003 (72.33 mM). The Pb MIC₅₀ varied from 0.00 to 20.00 mM with highest values observed for UFC011 (20.00 mM), UFC007 (19.60 mM) and UFC003 (17.65 mM). The Cd MIC₅₀ varied from 0.00 to 6.58 mM with highest values observed for UFC009 (6.58 mM), UFC004 (4.43 mM) and UFC003 (3.11 mM).

Table 4. Cd, Pb and Zn MIC₅₀ values (mM) and standard errors for the microorganisms isolated from the rhizospheric soil of site 10.

	MIC ₅₀ Zn (mM)	MIC ₅₀ Pb (mM)	MIC ₅₀ Cd (mM)
Bacterial isolates			
<i>UFCB001</i>	0.40 ± 0.07	0.18 ± 0.01	0.13 ± 0.00
<i>UFCB002</i>	0.70 ± 0.13	1.37 ± 0.49	2.32 ± 0.17
<i>UFCB003</i>	0.59 ± 0.04	0.20 ± 0.00	0.15 ± 0.01
<i>UFCB004</i>	0.29 ± 0.05	0.20 ± 0.00	0.67 ± 0.38
<i>UFCB005</i>	0.18 ± 0.00	0.47 ± 0.04	0.20 ± 0.03
<i>UFCB006</i>	0.57 ± 0.09	0.27 ± 0.00	0.14 ± 0.00
<i>UFCB007</i>	0.34 ± 0.06	0.43 ± 0.02	1.28 ± 0.57
<i>UFCB008</i>	0.73 ± 0.40	0.64 ± 0.02	0.13 ± 0.00
<i>UFCB009</i>	0.34 ± 0.05	0.25 ± 0.05	0.16 ± 0.01
<i>UFCB010</i>	2.08 ± 0.77	0.60 ± 0.00	0.24 ± 0.04
<i>UFCB011</i>	0.46 ± 0.27	1.18 ± 0.03	0.23 ± 0.05
<i>UFCB012</i>	0.87 ± 0.09	1.62 ± 0.21	0.13 ± 0.00
<i>UFCB013</i>	0.88 ± 0.24	1.06 ± 0.14	0.13 ± 0.00
<i>UFCB014</i>	1.59 ± 0.64	0.99 ± 0.16	0.16 ± 0.01
	MIC ₅₀ Zn (mM)	MIC ₅₀ Pb (mM)	MIC ₅₀ Cd (mM)

Fungal isolates			
UFC003	72.33 ± 20.28	17.65 ± 2.91	3.11 ± 0.82
UFC004	38.36 ± 11.01	5.31 ± 0.77	4.43 ± 1.17
UFC006	1.41 ± 1.41	0.00 ± 0.00	0.00 ± 0.00
UFC007	56.70 ± 9.00	19.60 ± 5.37	0.87 ± 0.44
UFC008	0.00 ± 0.00	0.89 ± 0.47	1.64 ± 0.73
UFC009	49.68 ± 18.84	3.21 ± 0.80	6.58 ± 1.19
UFC010	2.50 ± 0.45	2.75 ± 0.69	ongoing
UFC011	5.10 ± 1.23	20.00 ± 1.85	ongoing
UFC013	2.50 ± 0.89	1.25 ± 0.23	ongoing
UFC014	32.00 ± 5.58	3.50 ± 0.45	ongoing
UFC015	6.00 ± 2.45	1.50 ± 0.18	ongoing
UFC016	100.00 ± 19.25	16.00 ± 2.85	ongoing
UFC017	20.00 ± 5.12	2.00 ± 0.46	ongoing
UFC018	110.00 ± 12.45	16.00 ± 2.05	ongoing

2.4.2 PGP traits of isolated microbes

The results of PGP tests on bacteria are shown in Table 5.

- All isolates showed a production of siderophores with values that varied from 92.75 to 323.95 $\mu\text{M}/\text{optical density}/\text{day}$ with highest values observed for UFCB006 (323.95 $\mu\text{M}/\text{OD}_{600}/\text{d}$), UFCB003 (287.78 $\mu\text{M}/\text{OD}_{600}/\text{d}$) and UFCB008 (251.83 $\mu\text{M}/\text{OD}_{600}/\text{d}$).
- Only the isolate UFCB012 showed no phosphate solubilisation, however, other isolates showed values that varied from 15.05 to 119.52 mg/l with highest values observed for UFCB008 (119.52 mg/l), UFCB002 (98.08 mg/l) and UFCB009 (94.63 mg/l).
- The isolates UFCB008 and UFCB013 showed no production of IAA, however, other isolates had values that varied from 1.20 to 213.05 mM with highest values observed for UFCB012 (213.05 $\mu\text{M}/\text{OD}_{600}/\text{d}$), UFCB007 (141.87 $\mu\text{M}/\text{OD}_{600}/\text{d}$) and UFCB004 (109.10 $\mu\text{M}/\text{OD}_{600}/\text{d}$).

Table 5. Characterisation of the production of total siderophores, IAA and phosphate solubilisation capacity of the bacteria isolated from site 10.

Isolate	Total Siderophore ($\mu\text{M}/\text{OD}_{600}/\text{d}$)	Phosphate solubilisation (mg/l)	IAA production ($\mu\text{M}/\text{OD}_{600}/\text{d}$)
UFC B001	92.75 ± 46.42	36.63 ± 5.38	96.91 ± 2.92
UFC B002	215.97 ± 16.73	98.08 ± 32.67	58.46 ± 12.47
UFC B003	287.78 ± 19.12	47.08 ± 16.13	39.72 ± 1.37
UFC B004	231.44 ± 33.49	73.50 ± 38.79	109.10 ± 42.82
UFC B005	188.92 ± 43.23	47.41 ± 18.36	27.32 ± 12.39
UFC B006	323.95 ± 59.68	19.08 ± 4.50	88.65 ± 63.38
UFC B007	129.65 ± 5.62	24.27 ± 12.57	141.87 ± 14.91
UFC B008	251.83 ± 59.36	119.52 ± 14.36	0.00 ± 0.00
UFC B009	247.12 ± 19.56	94.63 ± 15.86	1.20 ± 1.20
UFC B010	147.86 ± 74.07	15.05 ± 8.98	2.96 ± 2.52

UFC B011	167.20 ± 21.32	40.19 ± 9.09	29.76 ± 7.16
UFC B012	211.92 ± 16.12	0.00 ± 0.00	213.05 ± 19.62
UFC B013	239.99 ± 79.02	51.75 ± 10.54	0.00 ± 0.00
UFC B014	203.36 ± 26.51	78.63 ± 42.45	25.03 ± 6.27

2.4.3 Response of plant to microbial inoculation

The impact of microbial inoculation on plant growth was measured in controlled conditions, under greenhouse conditions, using the model plant willow.

A first experiment was set up with 6 strains from the first harvesting campaign at site 10 (D1.2) in perlite system to confirm the PGP traits of the isolated microbes. Figure 5 highlights that willow leaf biomass was strongly increased when inoculated with UFC003 (0.64 g) and UFC009 (0.69 g) in comparison with control (0.24 g). The same trend is observed for stem biomass when inoculated with UFC003 (0.27 g), UFC009 (0.30 g) and UFC007 (0.23 g) in comparison with control (0.08 g). No variations were observed for root biomass for all strains tested in comparison with control. Concerning bacterial strains (data not shown), no variation in leaf, stem and root biomass was observed for any of the strains tested.

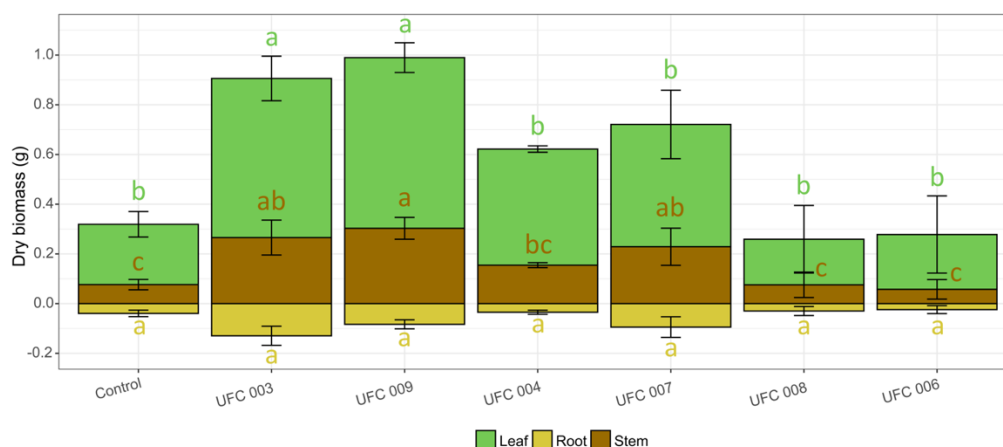


Figure 5. Dry biomass produced by willow trees after 36 days of growth. The trees were inoculated with fungal strains. Letters in common indicate statistically similar groups at $p < 0.05$ according to mean comparison tests. “Control” corresponds to trees that were not inoculated.

Figure 6 shows that the height of willows after 36 days of culture has been more than doubled when inoculated with UFC003 (19.1 cm) and UFC008 (20.0 cm) in comparison with control (8.7 cm). However, no significant differences were observed for all the other isolates after 15 and 36 days of culture. For bacterial strains, few augmentations were observed after 15 days of culture for willows inoculated with UFCB002 (1.17 cm), UFCB014 (0.47 cm), UFCB009 (1.13 cm) and UFCB010 (0.80 cm) in comparison with control that did not start to grow after 15 days. However, no significant differences were observed after 36 days of growth between control and all the inoculated willows.

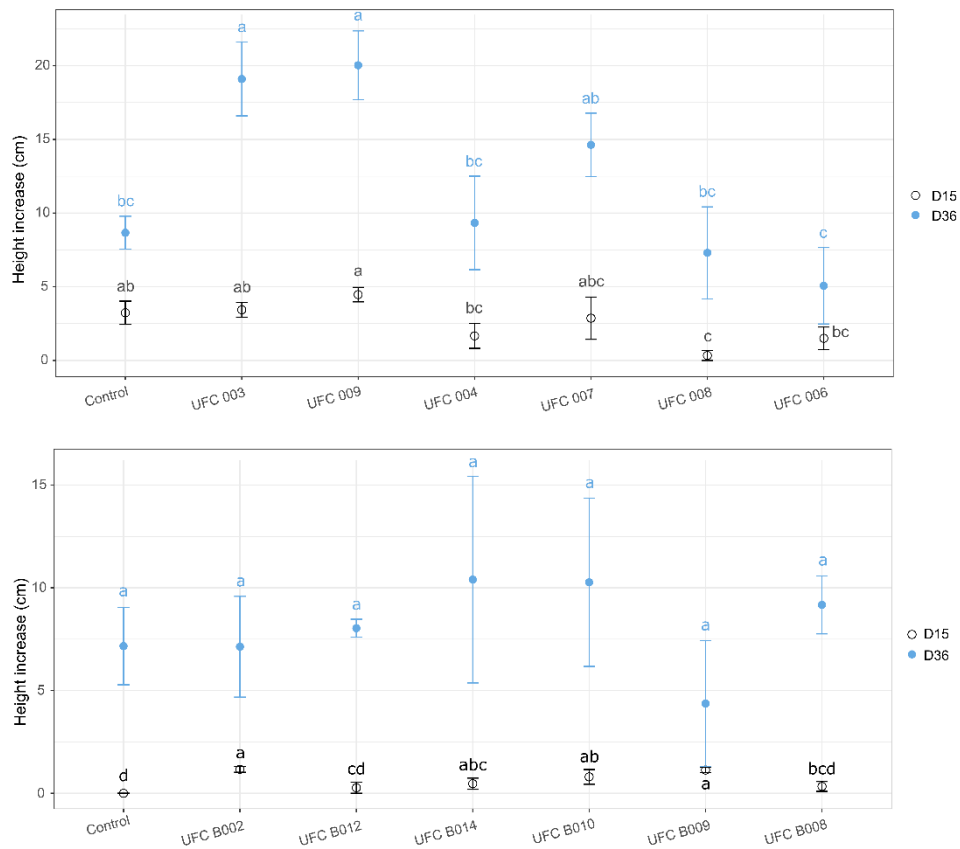


Figure 6. Height increase of willow trees after 15 (D15) and 36 (D36) days of growth. The trees were inoculated with fungal (top graph) or bacterial (bottom graph) strains. Letters in common indicate statistically similar groups at $p < 0.05$ according to mean comparison tests. “Control” corresponds to trees that were not inoculated

A second experiment was set up with 11 strains from the second harvesting campaign at site 10 (D1.2), with the model tree willow in soil conditions. At harvest after 2 months of growth, willow trees inoculated with UFC018 (11.2 g), UFC017 (8.8 g) and UFC014 (9.41 g) produced significantly more shoot and root biomass than the control treatment. The UFC010, UFC011, UFC012, UFC015 and UFC036 are not significantly different from the control.

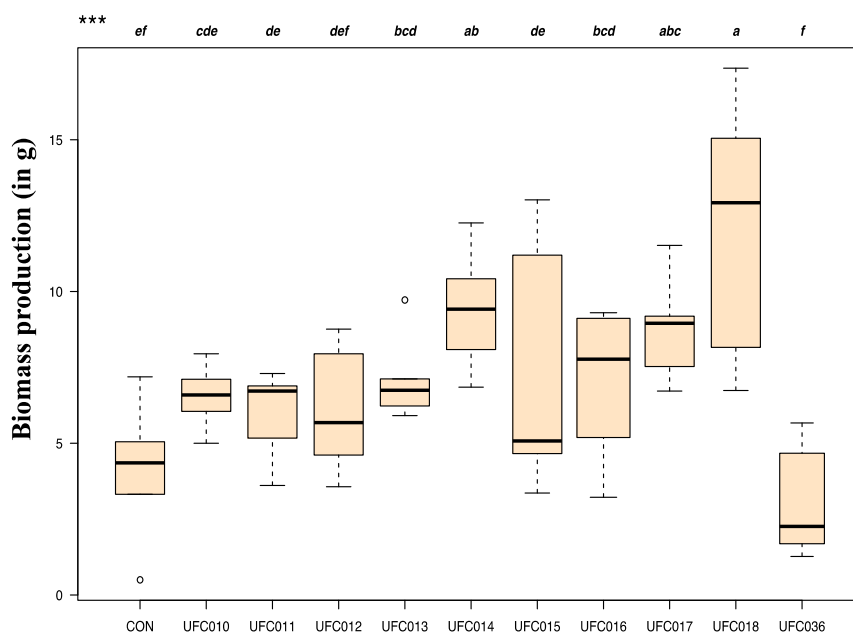


Figure 7. Biomass produced by willow trees after 2 months of growth. The trees were inoculated with fungal strains (UFC010 to UFC018 and UFC036). Significant differences are indicated with *, a, b, c, d, e and f. “CON” corresponds to the control (trees that were not inoculated).

2.4.4 Conclusions and planned activities

- Some of the isolated microbes from site 10 showed very high capacities to tolerate metals stress, which is due to the very high concentrations of metals at the isolation area (D1.2). This is linked to the elevated production of siderophores, which allows microbes to scavenge metals. Indeed, metal sequestration is known as a major mechanism for metal tolerance in microbes.
 - As a future activity, the genome of isolated microbes (D1.2) will be thoroughly investigated to apprehend the physiological processes behind these functional traits, as a collaborative work between UBFC and IDENER in WP2 (Tasks 2.1 and 2.2). Based on the genomic information, further RNAseq experiments will be set-up to evaluate the contribution of various genes in metal tolerance. These genes will be evaluated for their performance as a selectable marker of metal tolerance.
 - The potential of microbes to scavenge metals using active mechanisms (e.g. release of siderophores, active chelation into internal bodies such as vacuoles) will be distinguished from purely physically based mechanisms (unspecific adsorption onto mycelium) by using adequate controls e.g. the use of dead fungal mycelia (the so-called necromass) or the use of biochemical inhibitor (fungicide or bactericide) in dedicated experiments.
- The usefulness of isolated microbes and their PGP traits were further demonstrated by confrontation with plants in simple growth systems, using the model tree willow. Some of the isolated strains showed significant and high performance in terms of growth improvement.

- Based on the genomic information (WP2), further RNAseq experiments will be planned to evaluate the contribution of various genes in PGP properties.
- Further confrontations between plant and microbial consortia will be done with the model plant developed in WP3 (poplar 717 1 B4), in cooperation with UPM.

3 Optimization of microbes and microbial consortia from estuarine sediments

Estuaries are highly productive ecosystems that have a crucial role in biogeochemical cycles and unique physical-chemical conditions that support a large diversity and a multitude of ecosystem services. However, they are subjected to high anthropogenic pressures, and they are considered sinks of organic and inorganic contaminants which can affect their ecological functions. Contamination of estuarine ecosystems by different contaminants, including metals and pharmaceuticals, has been widely reported (e.g. Cunha et al. 2024, Fernandes et al. 2021). Thus, new tools and sustainable alternatives are in need to remediate and recover these coastal ecosystems. The development of bioaugmented phytoremediation strategies as nature-based solutions for recovery of estuarine environments exposed to mixed contamination arises as a sustainable alternative, as saltmarsh plants and their associated microbial communities can have an important role in the removal of organic and inorganic contaminants from estuarine sediments. One of the main challenges for the effective implementation of phytoremediation strategies for recovery of estuarine environments exposed to mixed contamination is the selection of a combination of salt marsh plants and microbial consortia that can be efficient in the removal of both organic and inorganic contaminants. Despite previous studies have shown the role of salt marsh plants and associated microbial communities for the removal of organic and inorganic pollutants, in BIOSYSMO we intent to implement plant-bacteria systems that can be effectively used as a nature-based solution for the removal of mixed pollution from estuarine sediments. For this, the microbial communities associated to salt marsh plants and sediments are being explored for their diversity and function, and relevant bacterial strains are being isolated, characterized for their bioremediation potential, and combined in optimized consortia for enhanced phytoremediation.

The two estuaries selected as cases studies for in BIOSYSMO are Douro estuary (site 4), an urban estuary receiving diffuse pollution, and Lima River estuary (site 5), an urbanized-industrialized estuarine environment affected by different anthropogenic activities. Contamination by metals and pharmaceuticals has been already reported for both estuaries, so these were the contaminants selected for the enrichment cultures for the isolation of bacterial strains in task 1.3 (D1.2), using estuarine sediments and rhizosediments associated to the plants *Phragmites australis* and *Juncus maritimus*. The isolated bacteria, described in D1.2, as well as the enriched microbial consortia from where the bacteria were isolated, and the consortia assembled by combining several of these bacteria, are being tested for their capacity to degrade the pharmaceuticals (Paroxetin, Benzafibrate, Ketoprofen or Venlofaxine), in the absence and/or in the presence of metals (Cu or Cd). In addition, bacterial isolates are being characterized in terms of metal tolerance, both by the trace metal resistance assay and the minimum inhibitory concentration assay. Finally, bacterial isolates are being characterized for their plant

growth promoting traits, including indole acetic acid and siderophore production, and phosphate solubilization.

3.1 Methodology

3.1.1 Degradation of pharmaceuticals

3.1.1.1 Paroxetine degradation

Bacterial isolates and assembled consortia

Bacterial strains, previously isolated from an enriched culture, using Douro estuarine sediment as source of inoculum and exposed to paroxetine (Prx) (Fernandes et al., 2020a), were tested individually and in consortium to assess their potential to degrade Prx. For that, biodegradation experiments were assembled, in which each strain was inoculated, in triplicate in MM medium and were spiked with 1 mg/L of Prx and fed with 500 mg/L of sodium acetate. Bacterial consortium was also assembled in MM and exposed to the same concentration of Prx to compare the removal performance among the strains and the consortium in the same experimental conditions. Abiotic controls (with no cells) were assembled with sodium acetate to assess abiotic degradation of Prx (namely removal by chemical degradation). All cultures were incubated in the dark and at room temperature (21°C), under static conditions, for 4 weeks. Bacterial cultures were transferred to new sterilized flasks once a week, to avoid oxygen depletion, and fed with 500 mg/L of sodium acetate. The removal efficiency for all isolates and consortia was assessed by HPLC (High performance liquid chromatography) each week.

Enriched cultures

Enriched cultures were assembled (in triplicate) using the protocol described in Duarte et al. (2019). For that, homogenised rhizosediment (from rhizosediments from *Phragmites australis* from Lima Estuary) was placed in sterilized flasks containing MM medium. The cultures were supplemented with sodium acetate (500 mg/L) and doped with 1 mg/L of Prx. Then, the cultures were incubated at room temperature (20 °C), in the dark and in static conditions. Experiments were carried out during 21 cycles of 2 weeks. During each cycle, microcosms were fed with sodium acetate (500 mg/L) twice a week. To ensure the presence of oxygen in the microcosms, each microbial culture was transferred to a new sterilized flask once a week. At the end of each cycle, half of each microbial culture was transferred to a new flask containing 100 mL of MM, the flasks were doped with 1 mg/L of Prx and supplemented with sodium acetate (500 mg/L). The removal efficiency in each cycle was assessed by HPLC. In the end of the 10th cycle, isolation of culturable bacterial was performed following the protocol described in Fernandes et al. (2020).

3.1.1.2 Bezafibrate degradation

Bacterial isolates and assembled consortia

Bacterial strains, previously isolated from an enriched culture, using Douro estuarine sediment as source of inoculum and exposed to Bezafibrate (Bzf) (Fernandes et al., 2020a), were tested individually and in consortium to assess their potential to degrade Bzf. The strains *Dyadobacter* DBS 3, *Leucobacter* DBS5 and *Microbacterium oxydans* DBS10 were fed with 500 mg/L of yeast extract, as second carbon source and the remaining strains were supplemented with 500 mg/L of sodium acetate. Bacterial

consortium was also assembled in MM and exposed to the same concentration of Bzf to compare the removal performance among the strains and the consortium in the same experimental conditions. Two different bacterial consortia were assembled: one was fed with 500 mg/L of sodium acetate and the other with 500 mg/L of yeast extract. Abiotic controls (with no cells) were assembled with sodium acetate and with yeast extract to assess abiotic degradation of Bzf (namely removal by chemical degradation). All cultures were incubated in the dark and at room temperature (21°C), under static conditions, for 4 weeks. Bacterial cultures were transferred to new sterilized flasks once a week, to avoid oxygen depletion, and fed with 500 mg/L of sodium acetate or yeast extract twice a week. The removal efficiency for all isolates and consortia was assessed by HPLC each week.

Enriched cultures

Enriched cultures were assembled (in triplicate), using the protocol described in Duarte et al. (2019). For that, homogenized rhizosediment (from Lima Estuary) was placed in sterilized flasks containing MM medium. The cultures were supplemented with sodium acetate (500 mg/L) and doped with 1 mg/L of Bzf. Experiments were carried out during 21 cycles of 2 weeks. The removal efficiency in each cycle was assessed by HPLC. In the end of the 10th cycle, isolation of culturable bacterial was performed following the protocol described in Fernandes et al. (2020).

3.1.1.3 Ketoprofen degradation

To analyse the ability of an enriched culture, acquired from an enrichment with Cd and Ketoprofen (Kpf), using rhizosediments from *Juncus maritimus* from site 4 enriched cultures were assembled (in triplicate) using the protocol described in Duarte et al. (2019). For that, homogenised sediment (from rhizosediments from *Juncus maritimus* from Douro River estuary) was placed in sterilized flasks containing MM medium with 1% NaCl. Abiotic controls (with no cells) were assembled with sodium acetate to assess abiotic degradation of Kpf (namely removal by chemical degradation). The cultures were supplemented with sodium acetate (500 mg/L) and doped with 1 mg/L of Kpf, Cd or Kpf and Cd. Then, the cultures were incubated at room temperature (22-26 °C), in the dark and in static conditions. Experiments were carried during 9 cycles of 2 weeks. During each cycle, microcosms were fed with sodium acetate (500 mg/L) twice a week. To ensure the presence of oxygen in the microcosms, each microbial culture was transferred to a new sterilized flask once a week. At the end of each cycle, half of each microbial culture was transferred to a new flask containing 20 mL of MM, the flasks were doped with 1 mg/L of the corresponding contaminant and supplemented with sodium acetate (500 mg/L). The removal efficiency of pharmaceutical in each cycle was assessed by Liquid Chromatography with a Mass spectrometry (LC-MS). In the end of the 9th cycle, isolation of culturable bacteria was performed following the protocol described in Fernandes et al. (2020).

3.1.1.4 Venlafaxine (Vfx) degradation

To analyse the ability of an enriched culture, acquired from an enrichment with Copper (Cu) and Venlafaxine (Vfx), using rhizosediments from *Phragmites australis* from site 5 enriched cultures were assembled (in triplicate) using the protocol described in Duarte et al. (2019). For that, homogenised sediment (from rhizosediments from *Phragmites australis* from Douro River Lima estuary) was placed in sterilized flasks containing MM medium with 1% NaCl. Abiotic controls (with no cells) were assembled with sodium acetate to assess abiotic degradation of Vfx (namely removal by chemical degradation). The cultures were supplemented with sodium acetate (500 mg/L) and doped with 1 mg/L of Kpf, Cd or Kpf and Cd. Then, the cultures were incubated at room temperature (22-26 °C), in the dark and in static

conditions. Experiments were carried during 12 cycles of 2 weeks. During each cycle, microcosms were fed with sodium acetate (500 mg/L) twice a week. To ensure the presence of oxygen in the microcosms, each microbial culture was transferred to a new sterilized flask once a week. At the end of each cycle, half of each microbial culture was transferred to a new flask containing 20 mL of MM, the flasks were doped with 1 mg/L of the corresponding contaminant and supplemented with sodium acetate (500 mg/L). The removal efficiency of pharmaceutical in each cycle was assessed by High-performance Liquid Chromatography with a Diode-Array Detector (HPLC-DAD). In the end of the 12th cycle, isolation of culturable bacteria was performed following the protocol described in Fernandes et al. (2020).

3.1.2 Characterization of metal tolerance

To characterize the metal tolerance of the different obtained isolates, two methodologies were applied – Trace Metal Resistance (TM's) Assays (TM's) and Minimum Inhibitory Concentration (MIC) Assays. For both, all isolates were activated from cryopreservation in PCA (Plate Count Agar - 5.0 g of Tryptone, 2.5g of Yeast Extract, 1g of glucose, 15g of agar p/ 1 L of deionized water) media to ensure proper growth and purity. The metal tolerance traits are being assessed using the bacterial strains isolated from enriched cultures exposed to Vfx or Cu or Cu/Vfx.

3.1.2.1 Trace Metal Resistance (TM's) Assays

The TMs resistance assays were performed using an adapted Disk Diffusion method. The isolates were suspended in PC broth liquid medium (5.0 g of Tryptone, 2.5g of Yeast Extract, 1g of glucose), and remained incubated at 28°C until reaching an optical density (OD) between 0.8-1.2 at 600nm. The inoculum was spread using cotton swabs in PCA plates. Sterile blank discs (5 mm diameter) were distributed in parallel along the plate to serve as duplicate for three tested concentrations of each metal tested (Cu, Cd, Zn and Cr). In each disk the solution of each metal (prepared with different metal salts) at the different concentrations were added to the discs. In each plate a negative control was added. The plates were incubated at 28°C, during 48h and the inhibition halos were measured for each condition.

3.1.2.2 Minimum Inhibitory Concentration (MIC) Assays

To test the susceptibility of isolates to heavy metals the MICs were determined in a microdilution assay in a 96-well microplate (Sütterlin et al., 2018). The heavy metals stock solution was prepared using Cu (II) Chloride (Cheng-Han Liu et al., 2023). The isolates were suspended in PC broth liquid medium (5.0 g of Tryptone, 2.5g of Yeast Extract, 1g of glucose), and remained incubated at 28°C until reaching an optical density of 0.4-0.6 at 600nm (Cheng-Han Liu et al., 2023). The MIC's assays were prepared as follows (Ćirković et al., 2023): 100 µL of PC broth medium were added to each well of the microplate, followed by the addition of 100 µL of metal stock solution to the first column; Along the columns of the microplate 9 two-fold dilutions were performed and then 10 µL of suspension of cell culture of the microorganisms was added into each well of the different columns; Each plate includes a positive growth control that contains medium and the isolate, but no heavy metals; one sterile control that only contains medium, no heavy metal and no microorganism; and one negative control consisting of medium and heavy metal solution and no isolate; The plates were incubated at 28° C for 48h and the results were read based on the turbidity of each well. The turbidity was analysed by measuring the absorbance at 600 nm in a microplate reader.

3.1.3 Characterization of PGP traits in sediment bacteria

3.1.3.1 IAA production

The amount of indole acetic acid (IAA) produced by the bacterial isolates from sediment samples were measured using a colorimetric technique (Paredes-páliz et al., 2016). The selected strains were used to inoculate test tubes containing 4 mL of liquid PCA supplemented with L-tryptophan (0.5 mg/mL). The inoculated tubes were incubated during 48-hour t 28 °C with continuous shaking at 200 rpm. Then, 1 mL of each the bacterial culture was centrifugated for 2 minutes at 14,000 rpm, and the resulting supernatant was transferred to a new test tube. Then, 4 mL of Salkowski reagent (Patten & Glick, 2002) was added, and the tubes left to incubate for 20 minutes at room temperature. The optical density at OD530 was determined using a spectrophotometer and compared with a standard curve of pure IAA. The appearance of a pink colour indicates IAA-production.

3.1.3.2 Siderophore production

Siderophore production was detected in deferrated PCA, by adding 50 µM 2,2'-dipyridil (Balado et al., 2015). The colonies were incubated for 72 h at a temperature of 28 °C. Following incubation, plates showing bacterial growth was overlaid with Chrome Azurol S agar (Lankford, 1994) and incubated for 2 hours. The change in colour of the overlay from blue to orange, yellow, or purple indicated the detection of siderophore production. The test had been conducted in triplicate.

3.1.3.3 Phosphate solubilization

Phosphate-solubilizing bacteria were identified using the phosphate growth medium from (Ejikeme & Uzoma, 2013): Agar (15 g L⁻¹), Glucose (10 g L⁻¹), Ca₃(PO₄)₂ (5 g L⁻¹), MgCl₂·6H₂O (5 g L⁻¹), MgSO₄·7H₂O (0.25 g L⁻¹), KCl (0.2 g L⁻¹), (NH₄)₂SO₄ (0.1 g L⁻¹). Two additional media were created by replacing glucose with tryptone and yeast extract in the same quantities. After inoculating the medium, the cultures were incubated at 28 °C for one week. Growth is being assessed after 72 hours and after one week. Positive results stand for the presence of a clear zone surrounding the inoculum, indicating phosphate solubilization. "Negative results with growth" were recorded when the colony grew without P-solubilization (non-appearance of a transparent halo).

3.2 Results on the microbes and microbial consortia from the Douro Estuary (Site 4)

The presence of pharmaceuticals in aquatic ecosystems, particularly in estuarine environments, has emerged as a growing concern due to their potential ecological impact and implications for aquatic health (Fernandes et al., 2021). Among pharmaceutical compounds, paroxetine, ketoprofen and bezafibrate have garnered attention for their widespread occurrence and persistence in estuarine habitats. To tackle this issue, previous works clue the application of autochthonous bacteria to biodegrade pharmaceuticals in water (Duarte et al., 2019). In the present project, CIIMAR aims to test the pharmaceutical biodegradation activity of a microbial consortium isolated from Douro River estuary. The Douro River (site 4) estuary represents a site contaminated with diffuse pollution, where different pharmaceutical compounds have been detected in its waters, including psychiatric drugs, as well as different metals (Fernandes et al., 2020; Iglesias et al., 2020). For this reason, bacterial consortium and bacterial strains recovered from Douro-river estuarine sediment were tested, in laboratory scale assays,

for paroxetine, ketoprofen and bezafibrate degradation ability, as well as for their capacity to resist to metals, and remove metals from solution. The identification of autochthonous bacteria and consortium able to degrade pharmaceuticals and resist/remove metals may facilitate the bioremediation of polluted estuarine environments.

3.2.1 Paroxetine removal by isolated bacteria and assembled consortia

For Prx, the removal efficiency displayed by the 10 bacterial strains isolated from estuarine sediments (isolated in Task 1.3 and described in D1.2) are presented in Table 6. Results show that 4 strains displayed removal efficiency higher than 80 % after 4 weeks. Moreover, 6 bacterial strains displayed removal percentages between 68-79 %. The bacterial consortium, which includes the 10 bacterial strains, showed removal efficiency up to 83 %. Prx abiotic removal was 38 % after 4 weeks. In addition, defluorination was measured for bacterial strains and the bacterial consortium, since Prx is a fluorinated compound. Results showed that the best 4 bacterial strains presented defluorination percentages higher than 70%, highlighting the potential of this bacterial strain to biodegrade Prx (Table 6). Moreover, the bacterial consortium showed defluorination potential of 79%. In addition, no defluorination was observed in the abiotic controls, showing that defluorination only occurred in the presence of the bacterial strains, corroborating the biodegradation observed.

Table 6. Removal efficiency of Prx for each bacterial strain, for the paroxetine degrading consortium (DPS) and for the abiotic control, over an experimental period of four weeks.

Bacterial strain	Removal efficiency	Defluorination
<i>DPS1</i>	36 % - 99 %	17% - 73%
<i>DPS2</i>	21 % - 72 %	0% - 67%
<i>DPS3</i>	42 % - 72 %	9% - 17%
<i>DPS4</i>	63 % - 81 %	16% - 74%
<i>DPS5</i>	58 % - 65 %	15% - 65%
<i>DPS6</i>	42 % - 69 %	14% - 62%
<i>DPS7</i>	41 % - 74 %	5% - 63%
<i>DPS8</i>	68 % - 80 %	14% - 64%
<i>DPS9</i>	39 % - 73 %	0% - 45%
<i>DPS10</i>	36 % - 80 %	23 % - 77%
<i>DPS Consortium</i>	57 % - 83 %	22% - 79%
<i>Abiotic control</i>	26 % - 38 %	0%

3.2.2 Bezafibrate removal by isolated bacteria and assembled consortia

For Bzf, the removal efficiency displayed by the 10 bacterial strains isolated from estuarine sediments are presented in Table 7. Results show that 4 strains displayed removal efficiency ranging between 67% – 78% after 4 weeks. Moreover, 6 bacterial strains displayed removal efficiencies ranging between

52 % – 64 %. Regarding the bacterial consortium, removal efficiencies between 63 - 67 % were observed for both consortia fed with sodium acetate and yeast extract. No Bzf removal was observed in the abiotic controls in the presence of both carbon sources, indicating that biodegradation occurred in the medium where the bacterial strains were present.

Table 7. Removal efficiency of Bzf for each bacterial strain, for the bezafibrate degrading consortium (DBS) and for the abiotic control, over an experimental period of four weeks.

Bacterial strain	Removal efficiency
<i>DBS1</i>	25 % - 59 %
<i>DBS2</i>	37 % - 53 %
<i>DBS3</i>	29 % - 70 %
<i>DBS4</i>	29 % - 69 %
<i>DBS5</i>	39 % - 67 %
<i>DBS6</i>	37 % - 52 %
<i>DBS7</i>	34 % - 61 %
<i>DBS8</i>	35 % - 57 %
<i>DBS9</i>	40 % - 64 %
<i>DBS10</i>	54 % - 78 %
<i>DBS Consortium A</i>	29 % - 67 %
<i>DBS Consortium Y</i>	39 % - 63 %
<i>Abiotic control A</i>	0 %
<i>Abiotic control Y</i>	0 %

3.2.3 Ketoprofen and Cd removal by enriched microbial consortia

In the enriched culture obtained by exposing *Juncus maritimus* rhizosediments to Cd and/or Kpf, the removal efficiency of Kpf and Cd was assessed during 9 cycles, showing >80% removal of Kpf (in the presence and absence of Cd). However, sediment absorption (between 5-30% and abiotic processes (very variable, between 0 and 20%) can also contribute for the pharmaceutical removal (Figure 8). In the enriched cultures between 80% and 100% of Cd was removed from solution, when only the metal was present, and around between 90 to 100 % when Kpf was also present, due to metal adsorption or internalization in cells and with part of the removal process occurring through sediment adsorption and/or abiotic processes (up to 30%) (Figure 9). The isolation of the bacterial strains from the enriched culture and their characterization is still ongoing.

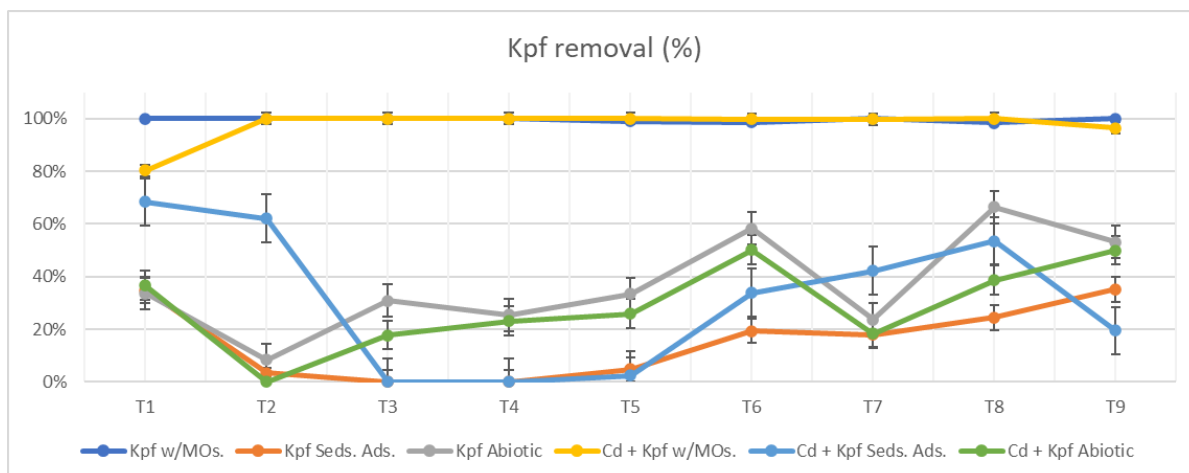


Figure 8. Percentage of Kpf removal by the enriched consortium during nine cycles (T1-T9) of the enrichment experiment using Cd and Kpf. Values are represented for enriched cultures (w/MOs), sediments adsorption controls (Seds. Ads.) and abiotic controls (Abiotic) for microcosms with Kpf or with Cd and Kpf. Error bars correspond to the standard deviation calculated from 3 replicates.

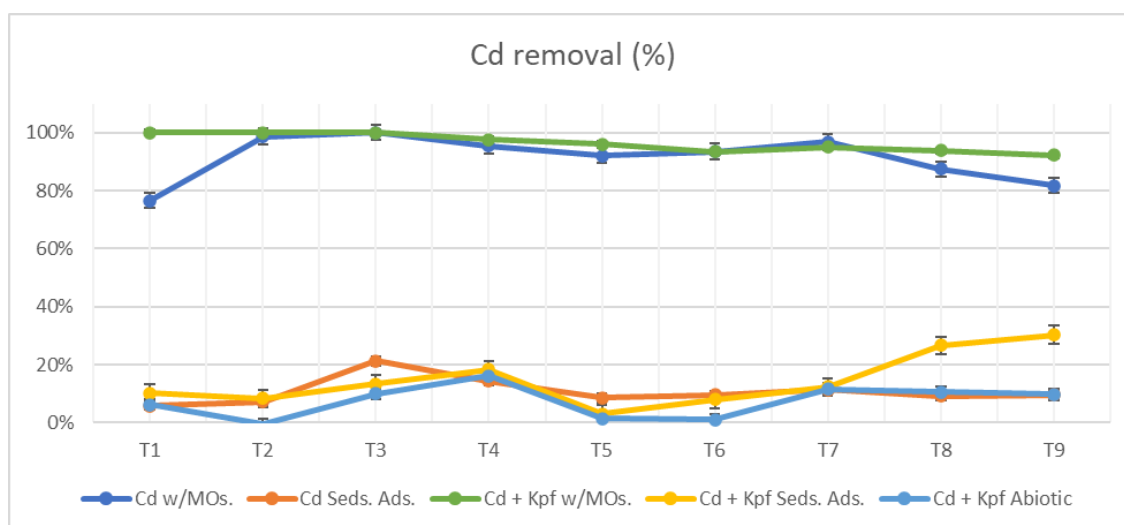


Figure 9. Percentage of Cd removal by the enriched consortium during nine cycles (T1-T9) of the enrichment experiment using Cd and Kpf. Values are represented for enriched cultures (w/MOs), sediments adsorption controls (Seds. Ads.) and abiotic controls (Abiotic) for microcosms with Cd or with Cd and Kpf. Error bars correspond to the standard deviation calculated from 3 replicates.

3.2.4 Conclusions and planned activities

- Results with 20 bacterial strains, isolated from estuarine sediments, highlighted their potential (as isolated bacteria or assembled in consortia) to biodegrade Prx and Bzf in laboratory experiments, using an artificial media.
 - Based on these results, further experiments were design to assess the effects of bioaugmentation, using the selected bacterial consortium, on the dynamics of estuarine

natural community and evaluate the potential of those microorganisms to degrade Prx and Bzf in natural media (estuarine water and sediment). This is very relevant to identify the constraints that can limit the application of bioremediation strategies in natural sediment ecosystems, with important implications for the implementation of mesocosm experimental systems that simulate estuarine environments in WP4 (T4.3).

- Adsorption controls, with sterilized biomass (necromass) would be conducted to evaluate Prx and Bzf capacity to adsorb to bacterial cells during the biodegradation experiments, evaluating its contribution for abiotic removal. Enriched microbial consortia obtained by exposing *Juncus maritimus* rhizosediments to a mixture of inorganic and organic contaminants (Cd and/or Kpf), showed capacity to degrade the pharmaceutical (Kpf) and remove the metal (Cd) from solution.
 - The isolation of the bacterial strains from the enriched culture is still ongoing. These isolates will be tested for their capacity to degrade pharmaceuticals and will be subjected to metal tolerance/resistance assays to different metals, undergo minimum inhibitory concentration assays for metals and pharmaceuticals and will be tested for Plant growth-promoting characteristics.
 - The best performing isolates will be combined in bacterial consortia that will be tested in combination with the plant *J. maritimus*, in microcosm lab experiments that simulate on a small scale the estuarine natural conditions. The selection of the best plant-bacteria systems will be based on the ability of the biosystems to remove from the sediments the target contaminants, which involves: i) the phytoremediation efficiency of the plants and ii) the biodegradation efficiency of the microbial consortia. The most promising plant-bacteria systems will then be tested in a mesocosm experimental systems that simulate estuarine environment in WP4 (T4.3).
 - Adsorption controls will be assembled in biodegradation experiments with the isolated strains to evaluate the adsorption capacity of Kpf to adsorb to sterilized bacterial cells (necromass), evaluating a possible contribution for abiotic removal.

3.3 Results on the microbes and microbial consortia from the Lima Estuary (Site 5)

Estuarine environments, characterized by their unique mix of freshwater and seawater, face increasing challenges due to human activities, including the input of pharmaceutical compounds. Lima River estuary (site 5) is an example of urbanized-industrialized estuarine environment affected by different anthropogenic activities. This environment presents different potential pollution sources in particular metals but also pharmaceuticals like paroxetine and bezafibrate (Cunha et al., 2024; Fraga-Santiago et al., 2022). Enriched microbial consortia, and bacterial strains isolated from enriched cultures, exposed to select contaminants (metals and/or pharmaceuticals), were tested in laboratory assays to evaluate their potential to degrade pharmaceuticals, as well as their capacity to resist to metals, and remove metals from solution. The identification of autochthonous bacteria and consortium able to degrade pharmaceuticals and resist/remove metals may facilitate the bioremediation of polluted estuarine environments.

3.3.1 Paroxetine removal by enriched microbial consortium

Removal efficiency of Prx displayed by the enriched culture exposed to this pharmaceutical are presented in Table 8. The enriched culture displayed an unstable removal after the cycle 8, however, high removal efficiencies were observed after the cycle 16.

Table 8. Prx Removal efficiency performed by Prx enriched culture, after 21 cycles of 15 days.

Removal efficiency											
	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T21
Prx Enriched culture	>95%	>95%	>95%	75 % - 95 %	56 % - 84 %	27 % - 81 %	48 % - 68 %	78 % - 86 %	85 % - 90 %	87 % - 95 %	87 % - 95 %

Regarding the prokaryotic community dynamics, it was only possible to evaluate the community until the 10th cycle. Data regarding the microbial community structure from the 10th until the 21st cycle are being analysed. For Prx enriched cultures, there was no clear pattern throughout time. The genus *Arcobacter* was consistently represented in most replicates across all cycles, the genera *Lentimicrobium* and *Desulfovibrio* were present in most of the cultures over time. Culture dependent methods allowed the isolation of 22 bacterial strains, in which the genera *Pseudomonas* and *Alcaligenes* were the most abundant.

3.3.2 Bezafibrate removal by enriched microbial consortium

Removal efficiency of Bzf o displayed by the enriched culture exposed to this pharmaceutical are presented in Table 9. The enriched culture displayed an unstable removal after the cycle 6, however, high removal efficiencies were observed after the cycle 18.

Table 9. Bzf removal efficiency performed by Bzf enriched culture, after 21 cycles of 15 days.

Removal efficiency											
	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T21
Bzf Enriched culture	>95 %	>95 %	>95 %	0 % -64 %	0 % - 20 %	7 % - 14 %	0 % - 21 %	59 % - 63 %	55 % - 84 %	75 % - 95 %	79 % - 95 %

Regarding the prokaryotic community dynamics, it was only possible to evaluate the community until the 10th cycle. Data regarding the community structure from the 10th until the 21st cycle are being analysed. For Bzf enriched cultures, the genus *Arcobacter*, while initially underrepresented, was one of the most dominant genera by the end of the 10th cycle. Moreover, both *Xanthobacter*, *Sphaerochaeta* and *Desulfovibrio* were consistently present in most replicates over time. Culture dependent methods allowed the isolation of 24 bacterial strains, in which the genus *Pseudomonas* was the most abundant.

3.3.3 Venlafaxine and Cu removal by enriched microbial consortium

The removal efficiency of Vfx and Cu by an enriched culture that used Douro river estuarine sediment was assessed during 12 exposure-cycles, the Vfx efficiency removal being under analysis. For Cu, preliminary results (Figure 10) indicate that removal from the medium was >80% in the absence and > 60% in the presence of Vfx due to metal adsorption or internalization in cells and with part of the removal occurring through sediment adsorption (up to 20%). From these cultures, it was possible to isolate 57 bacterial strains, which are currently being characterized.

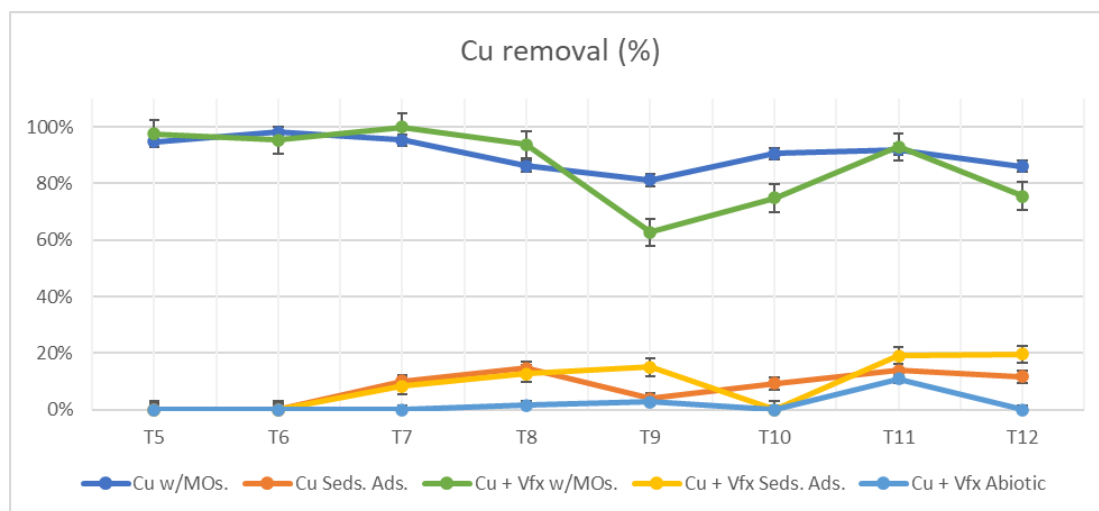


Figure 10. Percentage of Cu removal by the enriched consortium during eight cycles (T5-T12) of the enrichment experiment using Cu and Vfx. Values are represented for enriched cultures (w/MOs), sediments adsorption controls (Seds. Ads.) and abiotic controls (Abiotic) for microcosms with Cu or with Cu and Vfx. Error bars correspond to the standard deviation calculated from 3 replicates.

3.3.4 Cu tolerance of isolated bacteria

Regarding Cu and other metals tolerance, until now, only 3 strains from the 57 isolates obtained from the enrichment cultures (using Cu and Vfx) were tested. The minimum inhibitory concentration for all was at 128 mg/L. All three strains showed tolerance to Cu and Zn and presented some growth inhibition for Cr and Cd between 5 and 10 nM.

3.3.5 PGP traits of isolated bacteria

From the 57 bacterial strains isolated from the enriched cultures (using Cu and Vfx), 10 bacterial strains were assessed so far, in which three tested positives for P-solubilization. The first two bacterial strains tested positive after 72 hours, while the third bacterial strain exhibited a positive result only after one week. Among the remaining isolates, four bacterial strains were characterized as 'negative with growth'. Subsequent evaluations for these isolates will be conducted in PVK-Y media, where glucose has been substituted with yeast extract, and in PVK-T media, where glucose has been replaced with tryptone, aiming to assess the carbon source used.

3.3.6 Conclusion and planned activities

Enriched microbial consortia obtained by exposing *Phragmites australis* rhizosediments to organic and/or inorganic contaminants (Prx, Bzf, Vfx, Cu), showed capacity to degrade the pharmaceutical (Prx, Bzf, Vfx,) and remove the metal (Cu) from solution. A total of 92 bacterial isolates were obtained from these enrichment cultures, which are being tested for their capacity to degrade pharmaceuticals and are being subjected to metal tolerance/resistance assays to different metals, undergoing minimum inhibitory concentration assays for metals and pharmaceuticals and are being tested for Plant growth-promoting characteristics.

- The best performing isolates will be combined in bacterial consortia that will be tested in combination with the plant *P. australis*, in microcosm lab experiments that simulate on a small scale the estuarine natural conditions. The selection of the best plant-bacteria systems will be based on the ability of the biosystems to remove from the sediments the target contaminants, which involves: i) the phytoremediation efficiency of the plants and ii) the biodegradation efficiency of the microbial consortia. The most promising plant-bacteria systems will then be tested in a mesocosm experimental systems that simulate estuarine environment in WP4 (T4.3). Adsorption controls will be assembled in biodegradation experiments with the isolated bacterial strains to evaluate the adsorption capacity of selected pharmaceuticals to adsorb to sterilized bacterial cells (necromass), evaluating a possible contribution for abiotic removal.

4 Optimization of microbes and microbial consortia from wastewater

The objective of this activity is to improve the plant-bacteria interaction to improve metal removal in water/wastewater. To achieve this task, water from Site 6-Major non-ferrous smelter in Belgium (D1.1) was used (groundwater). This site exhibits a pH ranging from 3.1 to 3.7 and an electrical conductivity of 4,960-5,320 mS/cm. Additionally, due to metallurgical activities and the leakage of electrolytes and acids, it is highly contaminated with heavy metals such as As (0.073 mg/L), Cu (207.167 mg/L), Cd (2.533 mg/L), Ni (142 mg/L), and Zn (80.33 mg/L). Following results of D1.2 the DNA content was very low due to the high concentration.

Results from previous projects and activities

Within GREENER project (GA: 826312) UBU has been working with this polluted site to optimize the phytoremediation technology for metal removal in water. From this research project the following knowledge has been generated:

- Ten plant species were exposure to metal(loid)s polluted groundwater from Site 6. Results are published: <https://doi.org/10.1016/j.nbt.2023.12.003>
- Tolerance experiment of ten plant species was conducted. Results indicated that three macrophytes (*P. australis*, *S. holoschoenus*, and *T. angustifolia*) were capable of performing stabilization of metals. *Paper under revision*

- Among this three plants *P. australis* showed significantly superior response compared to other plants when it was exposure to polluted groundwater from Site 6.

Considering these results UBU decided to work in BIOSYSMO with the specie *P. australis*.

Pilot experiment for the optimization of plant-microorganism systems

As it is described in D1.1, Site 6 is an aquifer contaminated with different type of metal(loid)s. Considering that there is not vegetation to study the plant-microorganisms interaction, within WP3-Task 3.1, UBU set-up a plot experiment in which *P. australis* were exposed to this contaminated water for two months. For experiment the plant seedlings in growing pellets of *P. australis* were purchased from Viveros La Dehesa, Valdeobispo, Spain. The exposure to polluted groundwater was performed in 2 different setups, targeting at different exposure routes (Figure 11):

- Abrupt and acute exposure:** For this experimentation, uniform-sized plants were used, and were directly exposed to the polluted groundwater. The experiment was performed in a batch, using one 4-liter bucket, with 2 plant pellets per treatment. Plants were exposed to these conditions for 2 months.
- Chronic exposure:** For this experimentation, *P. australis* plants were chronically exposed to the contaminated water, meaning water was gradually added over those months.
- Control water:** *P. australis* were growing in 4-L bucket using clean water as control experiment.

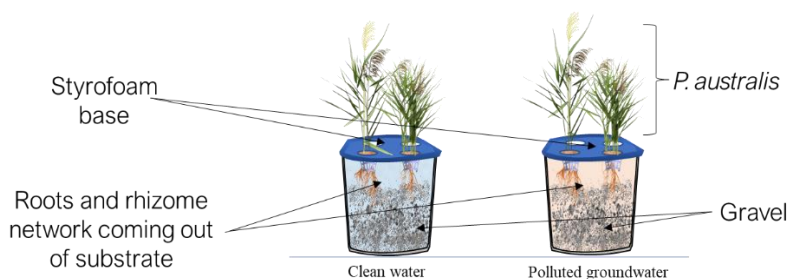


Figure 11. Representation of bucket used for plot experiment with *P. australis*

4.1 Methodology

4.1.1 Isolation of epiphytic and endophytic bacteria from pilot experiment

After two months of *P. australis* plot phytoremediation experiments conducting as mentioned above using the groundwater (Site 6), the isolation of epiphytic and endophytic microorganisms was performed for both the epiphytic and endophytic communities was carried out as described by CIIMAR in D1.2, under section 2.1 detailing the methodology. This protocol outlines specific steps for isolating microorganisms from each community.

For the epiphytic community isolation, a portion of the root was separated without cleaning the sediments. The root was then washed with 0.85% sterile saline solution. Subsequently, the root was thoroughly washed with a pipette in another Falcon tube containing 0.85% sterile saline solution. After completing the previous step, the sample was diluted in several tenfold dilutions using 0.85% saline solution. Finally, 50 µL of each dilution was plated on Tryptic Soy Agar (TSA) plates (15.0 g of Tryptone,

5.0 g of Peptic Digest of Soybean 5.0 g of Sodium Chloride and 15.0 of Difco Agar at a pH of 7.3 ± 0.2) and incubated at room temperature.

On the other hand, for endophytic community isolation, additional root pieces with minimal rhizosediment were cut. The root was washed twice with 70% ethanol for 5 minutes in two consecutive 50 mL Falcon tubes. Then, the root was placed in a 50 mL Falcon tube containing a 0.9% sodium hypochlorite solution, followed by washing the root with sterile distilled water five times. Afterward, the root was macerated with a mortar and pestle using 0.85% sterile saline solution. The macerated sample was diluted in several tenfold dilutions using 0.85% saline solution, and 50 μ L of each dilution was plated on TSA plates and incubated at room temperature.

After 48 hours of incubation, potential bacterial colonies with different morphologies were identified on the plates. Bacterial colonies were randomly picked and spread three times on the same medium to obtain pure bacterial cultures. The pure bacterial strains were cryopreserved by dissolving several loops of each isolate in 20% glycerol and preserved at -80°C .

4.1.2 Characterization of PGP traits for groundwater bacteria

4.1.2.1 Siderophore production

Various tests were conducted to measure siderophores, both total siderophores using the CAS method (Louden et al., 2011) and specific pyoverdine (which is the primary siderophore within the *Pseudomonas* genus). For total siderophores, the bacterial strains were pre-cultured in 5 mL of TSB medium for 24 hours at room temperature with agitation (180 rpm). Subsequently, 2 μ L of pre-culture was added to CAS agar plates, and the plates were checked daily for a week. A positive result was indicated by a colour change from the blue CAS medium to orange and red. On the other hand, for pyoverdine assays, streaks of different bacterial strains were made on SGA plates (sucrose glutamic acid media). Similarly, the plates were checked daily, and a positive result was indicated by a change in colour to phosphorescent yellow.

4.1.2.2 Phosphate solubilization

The measurement of the phosphate solubilization capacity of each of the isolated bacteria and/or microorganisms was conducted using the NBRIP method (National Botanical Research Institute Phosphate growth media) described by Nautiyal (1999). Each strain was pre-cultured for 2 days on TSA plates (Tryptic Soy Agar media), and a colony from each strain was punctured onto NBRIP plates and allowed to grow for a week at room temperature. Plates were daily monitoring to check for a positive reaction, indicated by the appearance of a solubilization halo around the colony. The size of the halo was measured, determining the phosphate solubilization index using the method described by Sitepu et al. (2014).

4.1.3 Minimum Inhibitory Concentration (MIC) Assays

To evaluate the tolerance of the isolates to water contaminated with heavy metals, minimum inhibitory concentration (MIC) assays were conducted using 96-well plates. The MIC assays utilized polluted water collected from site 6, which had been previously filtered at 0.22 μ m and supplemented with Tryptic Soy Broth (TSB) medium at a 1:10 ratio. Serial dilutions were carried out using this polluted water. In each row of the plate, 100 μ L of TSB (17.0 g of Tryptone, 3.0 g of Peptic Digest of Soybean, 2.5 g of

Glucose, 5.0 g of Sodium Chloride, and 2.5 g of Dipotassium Phosphate at a pH of 7.3 ± 0.2) Along the columns, 8 serial dilutions were performed starting from the contaminated water, resulting in a final well volume of 100 μL . Additionally, 5 μL of a microbial culture suspension, prepared to a McFarland turbidity standard of 0.5, were added to half of the columns for visual comparison to assess if contaminants could interfere with result measurements. Each plate included a positive control with TSB medium and bacterial suspension without polluted water, and a negative control with only TSB medium. The plates were incubated at room temperature for 2-3 days to allow bacterial growth. The minimum inhibitory concentration was determined by plating 100 μL of dilutions from the different wells onto Tryptic Soy Agar (TSA) plates (15.0 g of Tryptone, 5.0 g of Peptic Digest of Soybean 5.0 g of Sodium Chloride and 15.0 of Difco Agar at a pH of 7.3 ± 0.2) and comparing them with the positive control, as water contamination interfered with the microplate reader analysis.

4.1.4 Isolates identification

The identification of the isolates was initiated in collaboration with CIIMAR, making use of their facilities and the E.Z.N.A. for Bacterial DNA kit (Omega BIO-TEK). After DNA extraction, quantification was carried out using a DeNovix DS-11 series spectrophotometer/fluorometer. The samples were then subjected to PCR amplification targeting the variable region of the 16S rDNA gene, using the primers 27F 5'-AGAGTTTGATCCTGGCTCAG3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'. The PCR amplification took place in a Veriti™ 96-well thermocycler from Applied Biosystems™, involving 30 cycles of denaturation (95°C for 2 min), annealing (95°C for 30s; 55°C for 30s; 72°C for 1 min), and extension (72°C for 7 min). Subsequently, 2-3 μL of the PCR products were sent for Sanger sequencing, providing genus-level identification for the different isolates. In order to identify the genus and species level of the isolates full length amplicon (16S) sequencing Pacbio Sequel II/III was conducted. This is crucial for subsequent use in plant inoculation and bibliography research. The DNA extraction for this sequencing was also performed using the previously mentioned extraction kit.

4.1.5 Plant systems and optimization of plan-bacteria system

4.1.5.1 Seed disinfection protocol tuning

The seeds of *P. australis* were acquired through Viveros La Dehesa. For this reason, to ensure that the seeds do not carry any other microorganisms beyond those intended for inoculation, the seeds underwent sterilization using two different protocols for in vitro growth. In this way, we can determine which one produced better results.

The first protocol followed was that described by Devries et al. (2020). The seeds were surface-sterilized with 95% ethanol (V/V) for 5 minutes (to inhibit dormancy), followed by another phase with 1% sodium hypochlorite for 10 minutes. Subsequently, they were washed three times with sterile ddH₂O. After this, the seeds were left in ddH₂O overnight. The next day, they were surface-sterilized again with 70% ethanol (V/V) for 2 minutes, followed by incubation in a solution with 1% sodium hypochlorite for 10 minutes. The three washes with sterile ddH₂O were repeated in the same manner.

The second protocol followed was that used by Podlipná et al. (2013) with some modifications. Initially, surface sterilization was conducted using 70% ethanol (V/V) for 1 minute. Afterward, they were exposed to a solution of 1% sodium hypochlorite and 2 μL /10mL Tween 20 for 10 minutes, followed by several washes with sterile ddH₂O until the foam disappears.

4.1.5.2 Selection of optimal *in vitro* plant growth method

Due to inherent challenges in the *in vitro* germination and growth of the plant, three different media were tested, and seeds were plated on them. The first medium contained Murashige and Skoog (MS) medium including vitamins (Duchefa) at 4.4 g/L, the second with 4.4 g/L of MS medium including vitamins and 20 g L⁻¹ of sucrose, and the last one with 4.4 g L⁻¹ of MS medium including vitamins and 40 g L⁻¹ of sucrose. In all cases, ddH₂O was used, and Bacto-agar was added at 1.5%, followed by adjusting the pH to 5.6-5.9 using KOH.

The plates were incubated in a growth chamber (Test Chamber MLR-352H, Panasonic) under controlled conditions for optimal growth (30/18 °C day/night temperature and 16/8h day/night photoperiod). Subsequently, differences in germination and development were observed among the three media.

4.1.5.3 Bacterial inoculation assays in *P. australis*

For the inoculation of bacteria in the plant, three different protocols were tested, and the plant substrate used was peat moss with continuous irrigation. For all three protocols, F113 strain belonging to *Pseudomonas fluorescens*, described as a PGPR, was pre-cultured in 5 mL of MH (Mueller-Hinton) medium for 24 hours with agitation (180 rpm). Afterward, 20 ml of fresh MH medium was inoculated to an OD₆₀₀ of 0.04 and allowed to grow with agitation at 30 °C until reaching at least an OD of 0.7. which is equivalent to 107 CFUs/mL.

The first protocol starts with previously sterilized seeds following the protocol by Podlipná et al. (2013) (see section 4.1.5.1). The pre-cultured bacterial strains were centrifuged at 4,000 rpm for 15 minutes, resuspended in 15 mL of 10 mM MgSO₄ and incubated in this solution for 1 hour at 30 °C and 120 rpm (Pagnani et al., 2018). Control seeds were incubated with the 10 mM MgSO₄ solution in the same conditions. Finally, they were placed on the substrate surface and transferred to the growth chamber.

The other two protocols initiate from plants with 30 days of growth under controlled conditions in the growth chamber. Similarly, the pre-cultured bacterial strains were centrifuged at 4000 rpm for 15 minutes and resuspended in 10 mM MgSO₄. For one of the protocols, inoculation was performed in the soil after adding 1 mL of the bacteria solution per 100 g of soil, while for the other, plant roots were immersed in 15 mL of the solution with bacteria for 1 minute (Gobelak et al., 2015). Subsequently, the plants were transferred to the substrate for growth in the chamber. The assays were conducted under optimal plant growth conditions (see section 4.1.5.2).

4.2 Results on the isolation of microbes from *P. australis*-contaminated groundwater experiment (Site 6)

4.2.1 Isolation of epiphytic and endophytic bacteria from pilot experiment

A total of 34 bacteria and 5 fungal isolates were obtained (Table 10). Concerning the epiphytic community, UBU identified 9 bacteria on control plants, 3 on acutely exposed plants, and 5 on chronically exposed plants. On the other hand, regarding the endophytic community, we observed 8 different bacterial species in plants exposed to the control water, 2 in those acutely exposed to contaminated water, and 6 in those chronically exposed to the same water.

Table 10. Bacteria isolated from the epiphytic, endophytic, and total communities of *P. australis* roots exposed to three different treatments.

Plant Treatment	Bacteria		Fungi		Total
	Epiphytic Community	Endophytic Community	Epiphytic Community	Endophytic Community	
Control Exposure	9	8	2	1	20
Acute Exposure	3	2	1	0	6
Chronic Exposure	5	7	1	0	13
Total	17	15	4	1	39

4.2.2 PGP traits of isolated microorganisms

The different PGP traits assays have been performed only with bacterial isolates and not with the isolated fungi. After conducting various assays to determine whether the bacteria exhibited PGP traits, as described in section 4.1.2, it was observed that most isolated bacteria, both from the epiphytic and endophytic communities, showed PGP characteristics. At least they tested positive in some of the experiments, as shown in Table 11 and Table 12.

Among all the isolates exhibiting PGP traits, those that achieved the best results, either by testing positive in 2 or more experiments or by having higher values in the phosphate solubilization index, were identified in Table 11 and Table 12 as follows: TEP-4, TEP-5, TEP-6, TEP-7, AEP-2, and CEP-4 from the epiphytic community; and CEN-1, CEN-2, CEN-3, CEN-4, CEN-5, CEN-6, and CEN-7 from the endophytic community. Subsequently, these isolates will be considered for inoculation assays after their identification at the species level, along with a literature review. The preliminary identification results are described in section 4.2.4.

Table 11. PGP traits of bacteria isolated from the epiphytic community of *P. australis* roots under control, acute or chronic exposure. TEP, Tap EPiphytic; AEP, Acute EPiphytic; CEP, Chronic EPiphytic.

Treatment	Isolate	Phosphate Solubilization	Pyoverdine Production	Total Siderophore Production
Control	TEP-1	✓	X	X
	TEP-2	X	X	X
	TEP-3	X	X	X
	TEP-4	✓	✓	✓
	TEP-5	✓	X	X
	TEP-6	✓	X	✓
	TEP-7	✓	✓	✓
	TEP-8	X	X	X
	TEP-9	✓	X	X
Acute Exposure	AEP-1	X	X	X
	AEP-2	✓	✓	✓

	AEP-3	X	X	X
Chronic Exposure	CEP-1	X	X	✓
	CEP-2	X	X	X
	CEP-3	X	X	X
	CEP-4	✓	X	X
	CEP-5	X	X	X

Table 12. PGPR traits of bacteria isolated from the endophytic community of *P. australis* roots under control, acute or chronic exposure. TEN, Tap ENDophytic; AEN, Acute ENDophytic; CEN, Chronic ENDophytic.

Treatment	Isolate	Phosphate Solubilization	Pyoverdine Production	Total Siderophore Production
Control	TEN-1	X	X	✓
	TEN-2	X	X	X
	TEN-3	✓	X	X
	TEN-4	✓	X	X
	TEN-5	X	X	✓
	TEN-6	X	X	X
	TEN-7	X	X	X
	TEN-8	X	X	X
Acute Exposure	AEN-1	X	X	X
	AEN-2	X	X	X
Chronic Exposure	CEN-1	✓	X	✓
	CEN-2	✓	✓	✓
	CEN-3	✓	✓	✓
	CEN-4	✓	X	✓
	CEN-5	✓	X	✓
	CEN-6	✓	✓	✓
	CEN-7	✓	X	✓

4.2.3 MICs assays using contaminated groundwater

With respect to the MIC assays, in our case, we exposed the isolated bacteria to polluted water from Site 6, both directly and to serial dilutions of the same. It can be observed that despite being isolated from an experiment in which plants are exposed to this water, neither acutely exposed bacteria nor

chronically exposed bacteria can directly tolerate the contaminated water. However, they do show better results than bacteria isolated in control water, as shown in Table 13. This could be clarified by considering that these bacteria might require a relationship with the plant to tolerate, growth, and survive when exposed to contaminated water. In the case of bacteria exposed chronically, the better performance could be attributed to the gradual exposure to contaminated water.

Table 13. MIC assays of bacteria isolated from the epiphytic and endophytic communities of *P. australis* roots exposed to three different treatments.

Treatment	Epiphytic		Endophytic	
	Isolate	Polluted Water Dilution	Isolate	Polluted Water Dilution
Control Exposure	TEP-1	1/32	TEN-1	1/16
	TEP-2	1/8	TEN-2	1/128
	TEP-3	>1/128	TEN-3	1/128
	TEP-4	1/8	TEN-4	Unconclusive results
	TEP-5	1/8	TEN-5	1/16
	TEP-6	1/8	TEN-6	1/16
	TEP-7	>1/128	TEN-7	1/16
	TEP-8	1/8	TEN-8	<1/128
	TEP-9	>1/128		
Acute Exposure	AEP-1	Unconclusive results	AEN-1	<1/128
	AEP-2	>1/128	AEN-2	Unconclusive results
	AEP-3	Unconclusive results		
Chronic Exposure	CEP-1	1/32	CEN-1	1/8
	CEP-2	1/8	CEN-2	1/8
	CEP-3	Unconclusive results	CEN-3	1/128
	CEP-4	1/8	CEN-4	1/8
	CEP-5	1/8	CEN-5	1/8
			CEN-6	1/8
			CEN-7	1/8

4.2.4 Identified isolates

UBU has collaborated with CIIMAR in the identification of isolates. The identification of sequences obtained from the PCR product was performed using Geneious Prime software. Subsequently, these sequences were cross-referenced and verified against different databases such as NCBI or EzBioCloud to compare the results obtained and achieve a more reliable outcome. , UBU found various bacterial species, with the majority belonging to the genus *Pseudomonas* sp. However, we also found

hypothetical species belonging to the genus *Rahnella* sp. and *Priestia* sp. (*Bacillus* sp.). Due to the inability to reach species-level identification, we decided to perform full-length amplicon (16S) sequencing using the Pacbio Sequel II/Ile, as described in section 4.2.

4.2.5 Plant system and optimization of plant-bacteria system

4.2.5.1 Seed disinfection protocol and *in vitro* plant growth medium tuning

Regarding the seed disinfection protocols described in section 4.1.5.1, it was observed that the first protocol (SP1), despite delaying the start of the experiment by requiring two days, exhibits a lower seed germination rate, as shown in Table 9. Additionally, a significant presence of microbial contaminations was noted when evaluating the protocol mentioned above, indicating that it is not the optimal disinfection method.

Focusing on the second disinfection protocol (SP2), it was observed that the highest seed germination percentages were associated with this protocol. Considering the shorter disinfection time and fewer contaminations compared to the first protocol, it was decided to proceed with the second protocol for subsequent substrate and root inoculation assays.

If we consider the various growth media described in section 4.1.5.2., which were used for subsequent selection as the optimal *in vitro* growth method, we can observe that the medium containing MS including vitamins plus sucrose at a concentration of 20g/L (MS2) achieves the highest germination rates, as seen in Table 14.

Additionally, we can observe that germination does not vary significantly between pre-stratifying the seeds or not; in some cases, non-stratification even yields better results. Therefore, for the upcoming substrate and root inoculation experiments, we will proceed using the medium, namely, MS2, without stratification, as it saves time to initiate the various experiments.

Table 14. Germination percentages of *P. australis* seeds concerning their disinfection protocol (SP), growth medium and whether they underwent stratification or not.

Growth Medium	Disinfection Protocol	Total	
		Stratified (Germination % ± S.D)	No Stratified (Germination % ± S.D)
MS1 (MS including vitamins)	SP1	61.94 ± 17.85	67.52 ± 8.47
	SP2	79.36 ± 10.99	80.13 ± 6.69
MS2 (MS including vitamins + sucrose 20g/L)	SP1	56.64 ± 23.17	58.33 ± 15.28
	SP2	87.6 ± 3.71	89.52 ± 5.97
	SP1	58.94 ± 19.98	64.08 ± 16.22

MS3 (MS including vitamins + sucrose 40g/L)	SP2	88.95 ± 3.01	87.54 ± 3.65
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4.2.5.2 Bacterial inoculation assays in *P. australis*

To initiate the inoculation experiments, it was decided to select the seed disinfection protocol by Podlipná et al. (2013), which yielded the best results, to inoculate only our microorganisms of interest into the plant and observe whether they promote its growth or tolerance to polluted water. Similarly, establishing an *in vitro* growth protocol for *P. australis* was necessary for both subsequent experiments with the plant and the following inoculation assays, with the medium containing MS including vitamins plus sucrose at a concentration of 20 g L⁻¹ (MS2) providing the best results.

Regarding the inoculation protocol, three different methods described in section 4.1.5.3 were tested. In the first method, bacterial inoculation was directly performed on the seeds, in the second method, it was done in the substrate where 30-day-old seedlings were later planted, and in the third method, the roots of 30-day-old seedlings were submerged in a solution containing bacteria. The bacteria used was *Pseudomonas fluorescens* F113, described as a PGPR and non-human pathogenic, and the growth medium for the seedlings was the previous mentioned growth medium, MS (Murashige and Skoog) including vitamins plus sucrose at a concentration of 20g/L (MS2). The seeds were disinfected using the protocol by Podlipná et al. (2013).

Various phenotypic measurements were taken after 70 days in the first method and 45 days in the other two: number of leaves per plant, plant height, wet and dry biomass, comparing each inoculation method with a control without inoculated bacteria.

Table 15 illustrates that, for the seed inoculation protocol, the different measurements show better results for the control than for the inoculated seeds. Similarly, seedlings inoculated with the bacterial solution at the root exhibited lower or similar values compared to the control. Interestingly, the bacterial inoculation method in the soil where 30-day-old seedlings would later be planted showed better results compared to the control. At this point, these are only preliminary results since a replication is pending to begin statistical analysis of the data. However, we are currently considering continuing with the soil inoculation method after identifying our bacterial isolates that have exhibited PGP traits at the species level.

Table 15. Phenotypic measurements of *P. australis* inoculated with the PGPR bacteria *P. fluorescens* F113, according to the inoculation method employed: seed inoculation, soil inoculation or root inoculation.

Inoculation Method	Treatment	N°Leaves /Plant	Fresh Biomass(g)			Dry Biomass(g)		Height(cm)
			Roots	Aerial	Total	Roots	Aerial	
Protocol 1. Seeds	Control	6.977 ± 1.164	0.462 ± 0.153	0.526 ± 0.234	1.014 ± 0.153	0.290 ± 0.025	0.176 ± 0.005	4.409 ± 0.437
	Inoculated	6.264 ± 1.647	0.236 ± 0.167	0.240 ± 0.141	0.488 ± 0.167	0.139 ± 0.037	0.091 ± 0.036	3.731 ± 1.394

Protocol 2. Soil	Control	11.952 ± 1.503	1.673 ± 0.702	0.728 ± 0.178	2.579 ± 0.485	0.389 ± 0.088	0.290 ± 0.074	7.251 ± 0.427
	Inoculated	13.514 ± 1.206	1.756 ± 0.747	0.761 ± 0.178	2.858 ± 0.929	0.474 ± 0.081	0.318 ± 0.049	7.321 ± 1.371
Protocol 3. Roots	Control	11.497 ± 1.717	1.527 ± 0.056	0.566 ± 0.091	2.316 ± 0.081	0.354 ± 0.001	0.230 ± 0.047	7.078 ± 1.133
	Inoculated	12.940 ± 3.181	1.531 ± 0.226	0.680 ± 0.078	2.346 ± 0.276	0.356 ± 0.034	0.277 ± 0.016	6.564 ± 1.230

4.2.6 Conclusions and planned activities

- Several isolates were obtained from the epiphytic and endophytic communities of *P. australis* plants exposed to control water or contaminated water from Site 6 acutely or chronically exposed. Subsequent tests were conducted to observe if the isolates exhibited PGP traits, with the majority yielding favourable results for one or more experiments. Typically, isolates from the endophytic community of plants chronically exposed to contaminated water exhibited more pronounced PGP traits, possibly due to the progressive exposure to contaminated water in the experiment and their close interaction with the plant.
- MIC assays were carried out to determine if the isolated bacteria could grow when exposed directly to contaminated water and its dilutions. However, none were able to grow under direct exposure. This may be attributed to the bacteria requiring plant interaction or progressive exposure to contaminated water. Supporting this, the best results were obtained from isolates in the chronic exposure experiment.
- The identification of some bacterial isolates exhibiting prominent PGP traits has commenced for subsequent species-level identification and literature review. This evaluation will determine their suitability for advancing the experiments and tasks within this project.
- Within the bacterial inoculation experiments on the plant, three different protocols were implemented: on the seeds, in the soil, or at the root. The best results in various phenotypic measurements were obtained with soil inoculation, indicating that this is the most optimal inoculation method. However, these are preliminary results, and a third replication is needed for further confirmation.
 - In the future, we will continue conducting further assays with the isolated bacteria to complete their characterization as PGPRs. We will plan to conduct assays for measuring ACC deaminase activity.
 - Similarly, an assay will be performed to verify whether any of the isolated bacteria, in addition to possessing PGP traits, can utilize diesel as a carbon source. In this way, these isolates can be employed as a more holistic tool applicable in various contaminated environments.
 - Regarding the remaining unidentified bacteria, sequencing and subsequent identification will be continued. Meanwhile, those already identified at the genus level will be

attempted to be identified at the species level. Following this, upon the completion of the third replication of the conducted inoculation assays, further inoculation assays will be carried out using some of our target bacteria in phytoremediation assays.

- UBU in collaboration with JSI will develop strategies for consortia aggregation and immobilization using the microorganisms isolated from *P. australis* and the microorganisms isolated in Site 6 (D1.2). Different strategies for microbial inoculation will be tested such as rhizosphere inoculation by electrostatic attachment and determination of biofilm growth on root surface.
- In relation with task 4.1, where phytoremediation experiments are framed, to elucidate the mechanism of metal removal from the water (absorption, translocation, or sequestration), plant tissues will be digested to analyze metal distribution within the plant (metal localization). The water will be analyzed to determine the total bioavailable metal(loid)s. The remaining substrate will undergo sequential metal extraction to identify the metal species present. Comparisons will be made between the clean control, the abiotic control (no plants or bacteria), and the treatment groups (including plant and bacterial treatments) to assess the changes in the following fractions: Exchangeable, Carbonates, Fe and Mn oxides, Organic matter, and Residual metal fractions.

5 Synthesis and conclusions

The work developed in task T1.1 allowed the successful identification of 155 microbial isolates with potential capacities to degrade or tolerate pollutants, obtained from samples of rhizospheric soils, landfill sediments, rhizosediments, estuarine sediments, estuarine water or groundwater, as detailed in D1.2. In the present task T3.1, some of these strains were further characterized for their resistance to the metals found on the sites, their ability to promote plant growth (production of growth regulators, increased nutrient collection, etc.) and to degrade organic pollutants.

- Tolerance to metals was achieved by some microorganisms (bacteria and fungi) from contaminated soils (sites 1, 2, and 10). The earlier screening procedures allowed for selecting the most relevant microbes that showed Hg tolerance at site 2, Cd, Cu, Pb and Zn tolerance at sites 1, 5, 10. In addition, Cd and Cu removal capacities of microbial consortia from site 4 and site 5, respectively, was achieved. Tolerance to metals was also observed on microbes isolated from site 6, with better tolerance when microbes were exposed chronically. Metal tolerance mechanisms will be further assessed in WP2 on a subset of microbes, both fungi and bacteria, combining genome sequencing and modelling approaches.
- High removal capacities of some pharmaceuticals, up to 95%, was clearly achieved by microbial consortia isolated from sites 4 (Bzf, Prx and Kpf) and 5 (Bzf, Prx and Vfx).
- Plant growth promotion traits measured by biochemical assays were achieved by some of the isolated microbes from all investigated sites, while these capacities varied greatly between microbes from the same site. The best PGP isolates were selected for further experiments, e.g. confrontation with plants.

- Inoculation of model plants (Salix, Phragmites) with selected microbes allow for selecting the best performing microbes in terms of plant growth enhancement. The soil inoculation method for site 6 will be retained for further experiments *with P. australis*, while the perlite inoculation procedure will be applied to microbial consortia (fungi + bacteria) from site 1, 2 and 10 with the model plant developed in WP3 (poplar 717 1 B4), in cooperation with UPM. For sites 4 and 5, the best performing isolates will be combined in bacterial consortia that will be tested in combination with the plants *J. maritimus* and *P. australis*, in microcosm lab experiments that simulate on a small scale the estuarine natural conditions, to optimize the most promising plant-bacteria systems to be tested in a mesocosm experimental systems that simulate estuarine environment in WP4 (T4.3).

6 References

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