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Definition of key indicators and criteria for assessment of technology/bioremediation performance

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Publishable Executive Summary

The GREENER project aims at developing green, sustainable, efficient, and low-cost solutions for soil/sediment and water bioremediation that will effectively accelerate the remediation time of a range of organic and inorganic pollutants of high concern. WP3 aims at characterizing physically, chemically and microbially the selected contaminated sites and to identify the best available bioremediation techniques for these sites. This deliverable is the first version of *D3.8 Definition of key indicators and criteria for assessment of technology / bioremediation performance*.

This document provides an overview of the general and specific Key Performance Indicators (KPIs) considered for 12 different GREENER technologies. A semi-quantitative approach for evaluating multiple KPIs in an integrative manner is described. This approach can be either used to compare different treatments of the same technology or different technologies. In addition, the approach can be extended to perform a cost-benefit analysis. For each technology a brief summary is provided regarding the state-of-the-art as well as the experimental setting under which the technology will be tested. For those technologies with the best perspective to advance into a higher Technology Readiness Level (TRL) the experimental set-up and tentative KPIs at the higher TRL are described as well.

Performance evaluation is critical for any remedial technology and, particularly, for novel technologies at an early developmental stage. Performance can be measured in different ways. For remediation of contaminated soil and groundwater general technical criteria include: i) the reduction in contaminant concentrations for the matrix of interest (soil, water, soil vapor), ii) the removal of a certain amount of contaminant mass over a defined concentration as well as iii) the time needed to achieve the remediation goal(s). General technical performance indicators are helpful because they provide an evaluation framework that allows comparison of different remedial technologies and also the basis to assess whether or not the remediation goals or legislative contaminant thresholds are met.

Nonetheless, remedial goals cannot be achieved at any cost. With increasing prices for natural fuels and the climatic and environmental crisis that we are currently facing sustainability-related aspects and by extent KPIs such as energy and/or materials consumption, waste generation and emissions are additional criteria to be considered for developing and selecting a particular remedial technology.

Within GREENER multiple technologies for biological treatment of contaminated soil and groundwater are proposed. While all technologies rely on the activity of microorganisms and plants to reduce contamination and toxicity of affected soil and water the treatment principles behind many of the technologies differ significantly from each other. For instance, biological elimination of inorganic contaminants is based on the precipitation and/or removal from solution of metallic and metalloid species, whereas the elimination of organic compounds is based on the mineralization of the target substance to CO_2 and water, its breakdown into intermediate products or its transformation to innocuous end-products.

Such reactions depend on the presence of specific organisms, the expression of specific genes and the activity of specific proteins. Is the transformation observed due to the growth and activity of the organisms stimulated or augmented in the system or due to other factors (abiotic reactions, dilution or concentration effects or unexpected leaking of the target compounds, etc.)?

Technologies using bioelectrochemical systems can, for instance, generate added-value products such as H_2-gas or electricity form the breakdown of organic contaminants. In such cases system performance is also measured by the generation of such products in addition to the elimination/reduction of the target contaminants.

In consequence the evaluation of technology performance solely using general performance indicators provides a partial picture of what can be achieved by a particular technology as well as an incomplete evaluation framework for improving and developing novel technologies such as those proposed in Greener. The definition of specific KPIs along the general technical and sustainability KPIs presented here provides the foundation for the scaling-up of the most promising technologies (Task 6.1), the evaluation of technology development advancement (Tasks 6.3 and 6.4) and ultimately the basis for selecting (Task 6.5), dimensioning and evaluating the technologies to be implemented at field scale in pilot demonstrations (Task 6.6).

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Abbreviations and acronyms

Abbreviation	Definition
BES	Bioelectrochemical systems
BTEX	Benzene, Toluene, Ethylene, Xylene
KPI	Key Performance Indicator
MEC	Microbial Electrochemical Cell
METs	Microbial Electrochemical Technologies
MFC	Microbial Fuel Cell
PAH	Polycyclic Aromatic Hydrocarbons
SMFCs	Soil Microbial Fuell Cells
TPH	Total Petroleum Hydrocarbons
TOC	Total Organic Carbon
TRL	Technology Readiness Level
cVOCs	Chlorinated Volatile Organic Compounds

Abbreviation	Partner
UBU	University of Burgos
AXIA	Axia Innovation
MIB	Microgen Biotech Limited
SIE	Sustainable Innovations Europe
TAUW	Tauw GmbH
JSI	Institute Josef Stefan
ITC	Institute of Technology Carlow
MANO	Materia Nova
MEN	Mendel University in Brno
LEI	Leitat
UAM	Universidad Autonoma de Madrid
BATH	University of Bath
UNICA	Universita Degli Studi di Cagliari
USUR	University of Surrey
ACC	Acciona Construcción
SDAS	Ecology Institute of Shandong Academy of Sciences
JIAU	Jiangsu University
NTU	Nanjing Tech University
QUST	Qingdao University of Science and Technology

1. Introduction

The GREENER project aims at developing green, sustainable, efficient, and low-cost solutions for soil/sediment and water bioremediation that will effectively accelerate the remediation time of a range of organic and inorganic pollutants of high concern. WP3 aims at characterizing physically, chemically and microbiologically the selected contaminated sites and identifying the best available bioremediation techniques for them. The specific aims include:

- To select different contaminated soils/sediments and waters (surface and ground waters) sites at different locations in Europe and China with different environmental conditions and contamination.
- To identify and characterize the mixture of contaminants in the soils/sediments and waters of the impacted sites and determine the concentration of each compound.
- To evaluate the relevant physico-chemical factors of the contaminated sites that might negatively or positively influence on bioremediation processes.
- To characterize the autochthonous microbial communities of contaminated sites by advanced molecular and bioinformatics computational techniques and, thus, evaluate potential changes in the microbial community dynamics and functionality.
- To identify potentially pathogenic microorganisms within the enrichment consortia and define techniques for their elimination without affecting the non-pathogenic microbial community.
- To define Key Performance Indicators (KPIs) for evaluation of treatment efficacy as well as specific bioremediation treatability protocols depending on the technology assessed.

Deliverable 3.8 is the sole deliverable for Task 3.7 “Key performance indicators for the selection of best bioremediation techniques”. The main aim of this task is to define key indicators for each specific bioremediation technology upon which an assessment protocol can be developed allowing for evaluation of overall technology/bioremediation treatment performance. This task will be also an important input for Tasks 6.3 and 6.4 (Pilot scale experiments for soil and water technologies, respectively), for Task 6.5 (Decision-making tool) and finally for Tasks 6.6 and 6.7 (Field testing of the developed technologies in contaminated water and soil).

The document is structured in the three main parts:

- Introduction to KPIs
- KPI rating
- Description of GREENER technologies and their specific KPIs

In the first part, the concept of KPIs, general and specific KPIs is introduced. The notion why KPIs are important to evaluate treatment performance is further discussed.
A semi-quantitative method for evaluation of general and/or specific KPIs is further presented that allows comparison between different treatments/variations of the same technology as well as between different technologies.
Finally, specific KPIs for each GREENER technology are defined that support i) progress/fine-tuning of the respective technology and ii) specific evaluation of technology performance.

2. Introduction to KPIs

2.1 General and specific KPIs

What are KPIs?

A quantifiable or semi-quantifiable measure used to evaluate the success of the remediation technology and the achievement of the remedial objectives previously set.

Why use KPIs?

- Metric to evaluate technology performance and progress
- Structured evaluation and objective determination for advancing the TRL
- Comparison between different treatments of the same technology and also between different technologies

General and specific KPIs

General KPIs are technology-independent and can be used to compare technology/treatment performance with other technologies/approaches

Specific KPIs are technology-dependent, in other words, characteristic of a specific technology or remediation approach. Specific KPIs can be particularly helpful for technology scaling-up, selecting between two treatments/variations of the same technology as well as for improving remediation/biodegradation monitoring and evaluation at field scale.

General KPIs

General KPIs should be relevant, with potentially some minor differences, to all partners and technologies. The following table provides an overview of potential general KPIs. These can be divided into two main categories: technically-oriented and sustainability-oriented.

Table 1: *Summary of general KPIs*

KPI	Description
Technically-oriented	
Contaminant concentration	Change in contaminant concentration in the target matrix (soil, water, etc.) during the remediation treatment. *Usually aimed at achieving a target remediation level in accordance with a legislative threshold*
Contaminant mass reduction [%]	Amount of contaminant mass removed from the system during treatment. *It should be noted that a 90%-95% contaminant mass removal does not necessarily mean that the regulatory limits to claim the remediation as completed are reached.*
Decontamination cost [EUR] (Efficiency) [EUR/ton or EUR/m^3]	Cost to treat 1 ton of soil or 1L or 1 m^3 of contaminated water. *The aim is to be competitive against conventional treatment technology*
Decontamination time [weeks]	Time required to achieve the desired remediation goal *Long decontamination times may prevent the use of the technology*
Sustainability-oriented	
Residue generation [ton or m^3]	Generation of non-reusable or disposable product following treatment
Materials (kg) / Energy consumption (kwh)	Use of materials/substrate/electricity to implement the technology (e.g. addition of N or P to stimulate microbial growth, electrodes for BES systems, electricity to inject or recirculate reagents)
Emissions	Generation of noise, odor or gaseous emissions

It should be noted that for implementation of GREENER technologies requirements for health & safety (e.g. safety measures for applying microorganisms and/or chemicals) as well as ethical aspects (e.g. biosafety level of microorganisms, use of GMOs) must be taken into consideration. In case of testing at higher TRL, for instance, in a pilot study acceptance by the pertinent site owners and authorities is necessary. In some cases public and stakeholder opinion may be relevant for technology implementation and need to be addressed.

3. KPI rating

3.1 KPI rating

To make technology/treatment comparison easier with the aid of KPIs a weighting rating system is proposed. The approach described follows the working scheme of the MALBO techno-economical assessment for remediation technologies used in the Federal State of Nordrhein-Westphalia in Germany (**Table 2**).

Table 2: *Summary of general KPIs*

range	weighting factor	KPI	Treatment 1 Points	Treatment 1 Weight. Points	Treatment 2 Points	Treatment 2 Weight. Points
		General Technical KPIs				
30-50	30	Conc. reduction	4	1,2	3	0,9
20-40	20	Mass removal	4	0,8	3	0,6
10-30	5	Time	2	0,1	3	0,15
				2,1		**1,15**
		General Sustainability KPIs				
10-20	10	waste generation	3	0,3	4	0,4
10-20	10	emission	3	0,3	4	0,4
10-20	10	energy consumption	4	0,4	5	0,5
				1,0		**1,3**
		Technology Specific KPIs				
5-10	5	specific KPI1	4	0,2	4	0,2
5-10	5	specific KPI2	4	0,2	5	0,25
5-10	5	specific KPI3	4	0,2	4	0,2
				0,6		**0,65**
		Weighed evaluation				
	55	**Technical KPI**	2,1	**1,16**	1,15	**0,63**
	30	**Sustainability KPI**	1,0	**0,30**	1,3	**0,39**
	15	**Specific KPI**	0,6	**0,09**	0,65	**0,10**
Treatment 1 shows better overall performance				**1,55**		**1,12**
		Total cost in EUR		85.000		70.000
		Cost-Benefit		85.000/1,55*10^4		70.000/1,55*10^4
Treatment 1 shows better cost-benefit ratio				**5,5**		**6,25**

MALBO Bd. 11 (MUNLV NRW - Materialien zur Altlastensanierung und zum Boden-schutz, Bd. 11)

The basic aspects of the rating include:

- KPIs are divided into three main categories: technical, sustainability and specific. Each category is assigned a specific weighting factor between 0-100% (or between 0 – 1) depending on its relevance to the evaluation.

- Each category is composed, in turn, of various KPIs. In a similar manner the KPIs are assigned a specific weighting factor between 0 – 100 % (or 0 – 1) depending on its relevance within the specific category.

- A performance score is assigned to each KPI depending on the treatment/technology performance for that particular indicator: 1 (very low); 2 (low); 3 (medium); 4 (high) and 5 (very high)

- For each KPI the weighting factor (0-1) is multiplied by the score (1-5)

- For each category the weighting factor*score results are added up

- The same operation is performed at the category level, that is, the category weighting factor is multiplied by the sum of the individual KPIs scores. A category score is generated.

- The category scores (e.g. scores for technical, sustainability and specific) are added. The treatment/technology with the highest sum result is the best performing technology.

Since the best-performing technology may not necessarily be the most-economically meaningful option a cost-benefit analysis can be performed that combines both the total costs for the technology and performance results based on the KPI assessment.

For the cost-technical assessment the following steps are taken:

- the total costs for the technology are calculated
- the KPI score is multiplied by an appropriate 10^{th} factor, generally between 10^4 - 10^6
- the total cost is divided by the corrected (multiplied) KPI score

The technology with the lowest cost:KPI quotient represents the best cost-benefit ratio and, thus, is the most feasible option for implementation at a site.

4. Phytoremediation in constructed wetlands (UBU)

4.1 Technology Overview and Aims

Phytoremediation is a cost-effective technique to remove, detoxify, or stabilize inorganic and organic pollutants in both soil and water matrixes. Constructed wetlands are semi-natural systems for treatment of urban or industrial contaminated water streams based on contaminant removal from the water matrix by various means including [1]:

- rhizodeposition/rhizoprecipitation (inorganic compounds)
- absorption via root-uptake (inorganic and organic compounds)
- degradation (organic compounds)

For inorganic compounds the accumulation capacity of trace metals and metalloids by plant species is based on their ability to absorb and transport these compounds from the soil to the roots and, in turn, from the roots to their aerial parts where they may further accumulate. In case of significant accumulation in areal parts, the biomass can be harvested and the metals/metalloids are removed from the system. It should be noted that the effectiveness of metal/metalloid uptake depends on multiple factors including: accumulating capacity of the plant, plant biomass, type of metal/metalloid species, concentration of target species in the wastewater stream, presence of other competing metal/metalloid species, etc. [2].

The selection of plant species for constructed wetlands is critical for successful metal uptake/precipitation and bioaccumulation. The capacity for the accumulation of trace metals such as Cd, Pb, and Zn in aerial tissue has been shown to differ 47, 60, and 121 fold, respectively, amongst 19 aquatic plant species [3].

Bioaccumulation in aerial parts is influenced by metal bioavailability, rate of absorption by the plant roots, and translocation from the roots to the aerial tissue [4]. Most plant species have direct/indirect root bioactivation mechanisms that enhance root absorption such as: (i) acidification by exudation of organic acids, (ii) adsorption by formation of chelates, (iii) release of exoenzymes that increase available ion concentrations, and/or (vi) promotion of microbial activity [5].

Table 3: *Technology Overview*

GREENER PARTNER	University of Burgos (UBU)
Type of Technology	Phytoremediation
Process	*Phytoextraction of metals from contaminated water in constructed wetlands*
Target compounds	Trace (heavy) Metals and Metalloids
Test Matrix (contaminants)	Water (surface, groundwater, wastewater)
Current TRL / Goal TRL	4 /5

Within GREENER Simple Small-Scale Wetland Systems (SSWS) are developed to evaluate the ability of a constructed wetland system to treat wastewaters or polluted groundwater as a consequence of industrial activities [6].

The specific aims of the phytoremediation approach developed by GREENER are:

1) to establish a robust and efficient phytoremediation system for water (surface/groundwater/wastewater) polluted with trace metals and metalloids. For this purpose, the following actions will be conducted: (i) screening of ten aquatic plants adapted to temperate environmental conditions with potential metal (hyper)accumulation capacity and (ii) designing of a small scale wetland, operating with horizontal water flux and a floating anchor for aquatic plants.

2) to study the effect of nutrients and chelating agents in the efficiency of phytoextraction of trace metals and metalloids, alone or in combination with plant growth promoting rhizobacteria.

3) to build Microcosm Floating System (MFS) (TRL 4) to test the phytoremediation capacity of four aquatic plant species in three different treatments: control, polluted water, and microbial consortium addition. The best performing combination will be selected for the Small Scale Constructed Wetlands (SSCW) (TRL 5).

4.2 Activities at lower TRL and KPIs

Target compounds

For testing of phytoremediation technology at lower TRL4 the following target compounds will be tested:

- Cu, Ni, Zn, Cd, Pb, Cr, Hg, As

The concentration range includes:

- high range: 240, 200 and 76 for Cu, Ni, Zn, respectively
- low range: 13, 6 and 4 for Zn, As and Cr, respectively

Materials

The Microcosm Floating System (MFS) for testing the suitability of different species of aquatic plants consists of a 4 L buckets that holds a floating system of extruded polystyrene which, in turn, holds 4 plastic baskets in which plantlets are introduced (**Figure 1**).

The aquatic species to be tested include: *Carex riparian, Cyperus rotundus, Cyperus longus, Iris pseudacorus, Juncus effusus, Lythrum salicaria, Mentha acuatica, Phragmites australis, Scirpus holoschoenus, Typha angustifolia*.

Groundwater used for testing the MFS system consists of synthetic water mimicking the water composition from the GREENER sites 8 and 9:

- GW1: Melter 1. Sample No. 8101. Brine sample with heavy metals.
- GW2: Melter-2. Mixture 1:1:1 (in volume) of samples of wells No. 7, 31 and 45. Groundwater samples of moderate salinity.

Figure 1: Microcosm Floating System (MFS)

Experimental design and monitoring

Experiment 1

The experimental design will consist of the following treatments:

- Control (C), 200 mL of a concentrated Hoagland nutrient solution (x20) diluted to a final volume of 4 L.
- Groundwater (GW2), 200 mL of a concentrated Hoagland nutrient solution (x20) and 200 mL polluted water Melter1 (x20 pollution concentrations) diluted to 4 L.
- Evaluation of PGPR

For each aquatic plant species there will be one MFS per treatment including four plantlets (four replicates).

Monitoring of mesocosms will include the parameters and instrumental equipment as shown in **Table 4**.

Experiment 2

Tolerance Experiment: Exposure of 10 plant species to real contaminated water. Selection of 3 species for scaling-up experiments

Experiment 3

Definition of Hydraulic Retention Time (HRT) for scaling-up wetlands.

Table 4: *Monitoring of testing at low TRL*

Parameter	Instrumental equipment
General GW-parameters	pH, EC, TDS with portable multiparametric probe
Nutrients: ammonia, nitrate and o-phosphate	Segmented flow analyzer SKALAR San+
Total C, Total N, Inorganic C	Elemental Analyzer TOC/TN (Shimadzu)
Macroelements: Na, K, Ca, Mg and Trace Metals in plant tissues	Microwave assisted acid digestion with H2O2+HNO3 (c) ICP-OES Arcos (SPECTRO), ICP-MS/MS Triple Quadrupole (8900 Agilent)
Total N in plant tissues	Elemental Dry combustion analyzer TruSpec CN (LECO)

Samples (40 mL aliquots) for monitoring the parameters described in Table 2 will be performed weekly until 90 days of incubation. At the end of the incubation period plants will be harvested and examined for:

- above- and belowground biomass
- length of stems and roots
- element composition both below- and aboveground

General and specific KPIs

For the phytoremediation approach the following general and specific KPIs are defined (**Table 5**).

Table 5: *General and specific KPIs for the phytoremediation approach in constructed wetland*

General KPI	Definition
Contaminant concentration:	pH, EC, TDS with portable multiparametric probe
Contaminant mass reduction:	Segmented flow analyzer SKALAR San+
Decontamination time:	Elemental Analyzer TOC/TN (Shimadzu)
Materials consumed:	Costs for pH correction, nutrients supplement, PGPOs or other biological agents supplied to improve plant growth
Specific KPI	**Definition**
Plant biomass	above and below ground plant biomass production. Comparison to available data from literature
Bioconcentration Factor (BCF)	ratio of trace metals concentration in plant tissue to (ground)water concentration
Transference Factor (TF)	ratio of trace metals concentration in above ground plant tissue to plant roots

4.3 Activities at lower TRL and KPIs

Target compounds

For testing of phytoremediation technology at higher TRL5 the following target compounds will be tested:

- Cu, Ni, Zn, Cd, Pb, Cr, Hg, As

The concentration range includes:

- high range: 240, 200 and 76 for Cu, Ni, Zn, respectively
- low range: 13, 6 and 4 for Zn, As and Cr, respectively

Materials

The testing system for phytoremediation will be performed in Small Scale Constructed Wetlands (SSCW) that allow horizontal flow and treatment of the polluted (ground)water as the water circulates through the wetland system. First the SSCW will installed in a control room to evaluate at optimal condition the operational and removal capacity of the wetland. In a second phase of the scaling-up, the SSCW will be placed outdoors but equipped with a protective cover and tested for different aquatic plant species (Figure 2). In detail the SSCW system consists of various components as described in **Table 6**.

Table 6: *Components of the SSCW system*

Component	Description
Plastic container	40x80x30 cm (width, length, height) in black PVC material, 90 L capacity. Twelve units.
Floating layer	Perforated extruded polystyrene layer with twelve (12) holes in which plastic buckets were introduced for plantlets introduction. Additional hole is made for pumping case.
Sediment mixture	sand and gravel (coarse grain) and vermiculite (Termite, coarse grain). Ratio 1:1 (v/v), 10 cm bottom layer.
Water pump and pipes	Recirculating circuit with a 12 v aquatic pump, flow rate between 1-5 L day^{-1}, inserted in a filter case PVC Ø 90 mm, protected with a PE filter of 0.5 mm mesh. Twelve units and 16 mm black PE connecting pipes.
Control unit	with electric distribution panel, timer and connections.
Greenhouse	3x4.5x2 m (width, length, height) with transparent polyethylene (PE) cover and removable aluminum structure

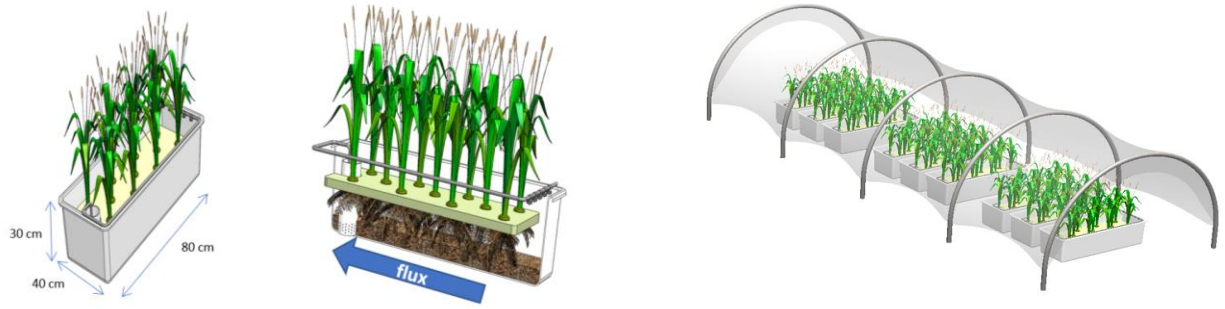

Figure 2: Small Scale Constructed Wetlands (SSCW)

Experimental design and monitoring

As there are twelve (12) SSCW units only the best three performing plants from the lower TRL will be considered for the higher TRL test. A control without plants will be considered as well to account for removal by adsorption to the sediment matrix. Real water from Melter 1 will be used for the higher TRL test. In addition, the system will be coupled with BES to evaluate the performance of the hybrid system.

Three aquatic plant species will be selected by the tolerance experiment will be used. Monitoring of mesocosms will include the parameters and instrumental equipment as shown in **Table 7**.

Table 7: *Monitoring of testing at high TRL*

Parameter	Instrumental equipment
General GW-parameters	pH, EC, TDS with portable multiparametric probe
Nutrients: ammonia, nitrate and o-phosphate	Segmented flow analyzer SKALAR San+
Total C, Total N, Inorganic C	Elemental Analyzer TOC/TN (Shimadzu)
Macroelements: Na, K, Ca, Mg and Trace Metals in plant tissues	Microwave assisted acid digestion with H2O2+HNO3 (c) ICP-OES Arcos (SPECTRO), ICP-MS/MS Triple Quadrupole (8900 Agilent)
Total N in plant tissues	Elemental Dry combustion analyzer TruSpec CN (LECO)
Microbial community characterization	Community Level Physiological Profiling (CLPP) and enzymatic profiling in microplate assays. Microplate Readers (Tecan, BioTek)
Metagenomics	Characterization of metal resistant/tolerant bacterial strains in ITC consortium

At the end of the experiment plants will be harvested and examined for:

- above- and belowground biomass
- length of stems and roots
- element composition both below- and aboveground
- biochemical characteristics

General and specific KPIs

For the phytoremediation approach the following general and specific KPIs are defined (**Table 8**).

Table 8: *General and specific KPIs for the phytoremediation approach in constructed wetland*

General KPI	Definition
Contaminant concentration:	Change in Trace Element concentration in GW
Contaminant mass reduction:	Percentage of change in the concentration of trace elements in GW
Decontamination time:	Time needed to achieve the contaminant levels according EU legislation
Efficiency or total cost	Decontamination cost in EUR or EUR/m3. Costs for materials purchased, electricity costs and recirculation system installation and maintenance need to be taken into account.
Energy consumed	Total Kwh required to operate the recirculation system during treatment
Materials consumed	Nutrients, ITC microbial consortium, chelating agents
Health and Safety	No biosafety level 2 or > microorganisms are found wetlands after culture addition.
Ethics and acceptance	The ITC microbial consortium does not contain any GMOs (Genetically Modified Organisms).
Specific KPI	**Definition**
Plant biomass	above and below ground plant biomass production, phenotypical response and biochemical characteristics. Comparison to available data from literature.
Bioconcentration Factor (BCF)	ratio of trace metals concentration in plant tissue to (ground)water concentration
Transference Factor (TF)	ratio of trace metals concentration in above ground plant tissue to plant roots

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5. Biostimulation/Bioaugmentation for Enhanced Treatment of Soil in the Construction Sector (ACCIONA)

5.1 Technology Overview and Aims

Combined contaminations of hydrocarbons and metals/metalloids in soil are common and account for significant disposal costs in the construction sector. Bioremediation strategies in soil are generally targeted to degrade organic compounds, as inorganic contaminants cannot be degraded. Bioremediation approaches for soil treatment are based on biostimulation and bioaugmentation strategies that are generally conducted in soil (bio)piles and supported via active (mechanical) mixing.

Biostimulation refers to the addition of specific nutrients to a contaminated soil to stimulate microorganisms capable of biodegrading pollutants. It involves identifying and adjusting certain physical and chemical factors (such as soil temperature, pH, moisture content, nutrient content, soil gradation, aeration, etc.) that may enhance biodegradation rate of the contaminants by the indigenous microorganisms present in the soil.

Bioaugmentation is the application of external wild type microorganisms to the contaminated soil that are capable of degrading the target compounds. Bioaugmentation is generally used in soils with low or non-detectable number of autochtonous degraders, or soils with compound-mixes that require the action of multiple microorganisms.

The bioremediation approach considered here is aimed at tackling soils contaminated with both hydrocarbons and heavy metals. Since hydrocarbons can be degraded, but metals not, soils with low to moderate metal levels will be considered. At the same time, some metals are taken up as micronutrients by the microorganisms and, thus, may have a stimulatory effect.
Various aspects will be considered to improve current biopile technology including optimized nutrient supply and the use of microbial carriers that improve the success of bioaugmentation. The final aim is to improve biodegradation rates in biopiles and biodegradation of more recalcitrant hydrocarbons. Particular emphasis will be put also on metal concentrations and leachability following microbial treatment. Microbial activity may increase metal availability by lowering the soil pH or decrease it by uptake into microbial biomass.

Table 9: *Technology Overview*

GREENER PARTNER	Acciona (ACC)
Type of Technology	Bioremediation (Biostimulation/Bioaugmentation)
Process	*Biodegradation of hydrocarbons in soils co-contaminated with metals/metalloids*
Target compounds	Aliphatic hydrocarbons, Metals and Metalloids
Test Matrix (contaminants)	Soil
Current TRL / Goal TRL	3 /4

5.2 Activities and KPIs at lower TRL

Target compounds

For testing of biostimulation/bioaugmentation strategies for hydrocarbon treatment in soil the following target compounds have been tested:

- Long-chained hydrocarbons >C21-C35 up to 3.000 mg/kg and heavy metals (10 – 1.000 mg/kg)

Materials

Batches of laboratory tests at micro and mesocosm scale were carried out in order to check the efficiency of the treatments. These tests are useful for the determination of the degrading efficiency and the conditions before the on-site scaling up.

Several experiments were carried out to evaluate the application of the optimized bioremediation strategies (bioestimulation and bioaugmentation). For bioaugmentation strategies the consortia enriched by GREENER partners (e.g diesel consortium by UAM or other hydrocarbon-degrading consortia by ITC) were used. For nutrient supply (biostimulation) both inorganic (N:P:K fertilizer) and organic fertilizers (e.g. vermicompost, biochar) have been evaluated.

The testing systems consisted of batch reactors made of polyethylene (vol) and soil (mass or volume) with or without amendments and humidified to the optimum water holding capacity. The batch reactors were incubated in the dark in an incubation chamber at approx. 20°C temperature. (see also **Table 10**).

Table 10: *Materials for the batch experiments*

Component	Description
Soil	Soil contaminated with both hydrocarbons and heavy metals
Batch containers (size)	Polyethylene containers (material chosen for its low permeability and minimum hydrocarbon absorption)
Incubation chamber	For controlling incubation temperature
Microbial carriers + consortia	Microbial carriers design by JSI
Vermicompost	Commercial vermicompost of organic wastes, classified as type A according Spanish law of Fertilizers (RD 506/2013)
Biochar amendment	produced by pyrolisis at 450ºC of pine sawdust pellets at laboratory scale
Rhamnolipids	Commercial

Experimental design and monitoring

Contaminated soil samples and the selected treatments were added to closed containers and periodic sampling were carried out in order to evaluate the degradation of hydrocarbons under different conditions.

The incubation were performed in a chamber under controlled conditions of temperature and humidity (22 ºC, 40% field soil capacity). Additionally, the soil were aerated and the moisture content controlled two times a week to accelerate the degradation of hydrocarbons. The following treatments have been studied.

Table 11: *Materials for the batch experiments*

Treatment	Description
Natural attenuation (CT)	moisture is adjusted in the soil to values close to its field capacity.
Biostimulation (BS):	several nutrients (nitrogen, phosphorus, potassium, etc.) will be added to the soil to enhance microbial growth, especially those microorganisms with degradation capacity of hydrocarbons that were adapted to the conditions of the place of application.
Bioaugmentation (BA)	application of indigenous microorganisms isolated from the polluted soils in order to accelerate the removal of undesired compounds. Application of microbial carriers and biochar will be tested. Application of synthetic community developed in the framework of WP3

At the end of incubation period, a sample is dried at 30ºC for chemical parameters measurement and the rest, frozen at -20ªC for microbial analysis.

Samples were periodically to an accredited laboratory for the evaluation of the hydrocarbons degradation under the tested conditions. Monitoring of batch reactors included the parameters and instrumental equipment as shown in Table 12.

Table 12: *Monitoring parameters at lowTRL*

Parameter	Instrumental equipment
pH, EC and redox potential	portable multiparametric probe
TOC	TOC Analyzer
Nutrients: N, P,	Segmented flow analyzer SKALAR San+
available, Ca, Mg, Na and K	extraction with ammonium acetate and determination via ICP-OES
Microelements (Fe, Mn, Cu and Zn)	Extraction with DTPA and determination via ICP-OES
Total Cr, Pb and Hg	ICP-OES

Parameter	Instrumental equipment
Total As, Cu, Fe, Mo, Se and Zn	ICP-OES and ICP-MS (As, Mo and Se)
Petroleum Hydrocarbons (TPHs)	GC-FID

General and specific KPIs

For the biostimulation/bioaugmentation approach the following general and specific KPIs are defined (**Table 13**).

Table 13: *General and specific KPIs for the biostimulation/bioaugmentation*

General KPI	Definition
Contaminant concentration :	Change in the compounds under study: TPHs in presence of potentially toxic metals and metalloids. Total concentrations for TPHs, metals and metalloids in soil + leachable metals and metalloids
Contaminant mass reduction:	Percentage of change in the concentration of hydrocarbons in soil
Decontamination time:	Time needed to achieve the goal concentration or mass reduction
Efficiency or total cost	Decontamination cost in EUR or EUR/m^3. Costs for materials purchased, electricity costs and recirculation system installation and maintenance need to be considered.
Materials consumed	Nutrients, vermicompost and microbial inoculants to stimulate biotransformation processes
Specific KPI	**Definition**
Degrader counts	total HC-degrading bacteria counts or cells/kg of soil e.g. via MPN-technique
Functional genes	Quantification of genes involved in hydrocarbon degradation via qPCR

5.3 Activities and KPIs at higher TRL

Depending on the success of the remedial approaches tested at the lower TRL for biodegradation of most recalcitrant hydrocarbon fractions (>C21-C35) experiments will be conducted at higher TRL using biopile technology in a similar context to that for the construction industry. The most appropriate remedial approaches will be upscaled at two different scale: pilot scale (1 ton) and field real scale (biopile).

For testing of biostimulation/bioaugmentation approach at higher TRL5 the following target compounds will be tested:

- Long-chained hydrocarbons >C21-C35 up to 3.000 mg/kg and heavy metals (10 – 1.000 mg/kg)

Materials

1Tn mesocosm experiments and biopile technology trials using biostimulation/bioaugmentation approaches will be performed at the ACCIONA facilities, considering the results obtained at laboratory scale. The activities will be carried out at two different scale: pilot scale (1 ton off site treatment) and field real scale at ACCIONA Machinery Park in Noblejas (Toledo).

Table 14: *Materials for the outdoor biopile experiments*

Component	Description
Soil biopiles	Soil biopiles contaminated with total petroleum hydrocarbons and heavy metals.
Bacterial consortium	Best-performing Hydrocarbon-degrading consortia from lower TRL
BES	Bioelectrochemical systems (snorkel with graphit rodes)
Bacterial Carriers	Different bacterial carriers will tested and the selected ones will be applied in the biopile.

Experimental design and monitoring

Biostimulation/Bioaugmentation of the biopile system: a Biopile treating petroleum impacted soil will be set up. Different scales will be inoculated with the different microbial consortia developed in the lower TRLs and biostimulation approaches will be also incorporated. The pilot scale will consist of 1 m^3 of contaminated soil stored in an IBC tank, and the field real scale biopile will be dimensioned (L x W x H).

Table 15*: Treatments for biostimulation and bioaugmentation tests*

Treatment	Description
Pilot scale (1Tn Experiment)	Different bioremediation strategies will be tested:

Treatment	Description
	• Control • Organic amendment and bioaumentation: Vermicompsot+Bioaugmentation using microbial consortia isolated from Site_001 • Organic amendment + Bioaugmentation + BES
Field real scale (Biopile)	Depending on the results obtained at 1 Tn experiment. • Organic amendment and bioaumentation: Vermicompsot+Bioaugmentation the synthetic comunity developed in WP3 • Bacterial carriers → developed by JSI and tested at microcosm scale by UBU • BES (developed by LEITAT)

Various physical-chemical and biological properties will be monitored regularly in the biopiles by taking samples approximately every 1 month over the course of 6 months. For the biological properties, samples will be analyzed through omics techniques. Sequential description of the action at pilot scale and field real scale, including recommendations for execution, start-up, control and the necessary means:

- Excavation and disposal of contaminated land
- Preparation of the action area
- Homogenization, spreading and application of the bioremediation treatment
- Maintenance of treated soil
- Monitoring

Table 16: *Monitoring parameters at high TRL*

Parameter	Instrumental equipment
pH, EC and redox potential	portable multiparametric probe
TOC	TOC Analyzer
Nutrients: N, P,	Segmented flow analyzer SKALAR San+
available, Ca, Mg, Na and K	extraction with ammonium acetate and determination via ICP-OES
Microelements (Fe, Mn, Cu and Zn)	Extraction with DTPA and determination via ICP-OES
Total Cr, Pb and Hg	ICP-OES
Total As, Cu, Fe, Mo, Se and Zn	ICP-OES and ICP-MS (As, Mo and Se)
Petroleum Hydrocarbons (TPHs)	GC-FID
Biochemical analysis (enzimatic activity, PLFAs)	Tecan spectrophotometer
Microbiome	Sequencing, omics, qPCR

General and specific KPIs

For the biopile field trials following general and specific KPIs are defined:

Table 17: *General and specific KPIs for biostimulation/bioaugmentation in biopiles*

General KPI	Definition
Contaminant concentration:	Change in the compounds under study: TPHs in presence of potentially toxic metals and metalloids. Total concentrations for TPHs, metals and metalloids in soil + leachable metals and metalloids
Contaminant mass reduction:	Percentage of change in the concentration of hydrocarbons in soil
Decontamination time:	Time needed to achieve the goal concentration or mass reduction
Efficiency or total cost	Decontamination cost in EUR or EUR/m^3. Costs for materials purchased, electricity costs and recirculation system installation and maintenance need to be taken into account.
Materials consumed	Nutrients, vermicompost and microbial inoculants to stimulate biotransformation processes
Specific KPI	**Definition**
Degrader counts	total HC-degrading bacteria counts or cells/kg of soil e.g. via MPN-technique
Functional genes	Quantification of genes involved in hydrocarbon degradation via qPCR

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6. Ecopiling (ITC / MicroGen)

6.1 Technology Overview and Aims

Combining bioremediation strategies such as bioaugmentation and biostimulation with phytoremediation appears to be an effective way to remove recalcitrant hydrocarbons from large volumes of contaminated soil. Ecopiling is a modification of traditional passive biopiling in that, instead of enclosing the biopile with black plastic, the pile is planted with suitable phytoremediation plants in order to promote rhizoremediation.

The Ecopile process involves bio-stimulation of indigenous hydrocarbon degraders, bio-augmentation through inoculation with known PAH-degrading consortia and phytoremediation, through the effect of root growth and penetration throughout the soil and the resulting stimulation of microbial activity in the rhizosphere.

Table 18: *Technology Overview*

GREENER PARTNER	Institute of Technology Carlow (ITC)
Type of Technology	Ecopiling
Process	*Aerobic biodegradation and phytoremediation of Total Petroleum Hydrocarbons*
Target compounds	Aliphatic hydrocarbons
Test Matrix (contaminants)	Soil
Current TRL / Goal TRL	6/7

Ecopiles are typically constructed using contaminated soil excavated, treated with nutrients (nitrogen and phosphorus fertilizer) and seeded with hydrocarbon degrading microbial inoculants. The base layer of soil (~0.5 m deep) is placed over a heavy-duty polythene liner and 50 mm perforated drainage pipe placed at approximately 1 m centers, laterally across the pile to allow for passive ventilation (Figure 1). The Ecopile is then raised in consecutive 0.5 m layers, comprising contaminated soil and drainage piping to a height of 2-3 m. The Ecopiles are constructed trapezoidal in shape with a 2:1 slope from base to top. Finally, each Ecopile is capped with uncontaminated topsoil (~5 cm deep) and seeded with suitable plant species such as clover and ryegrass.

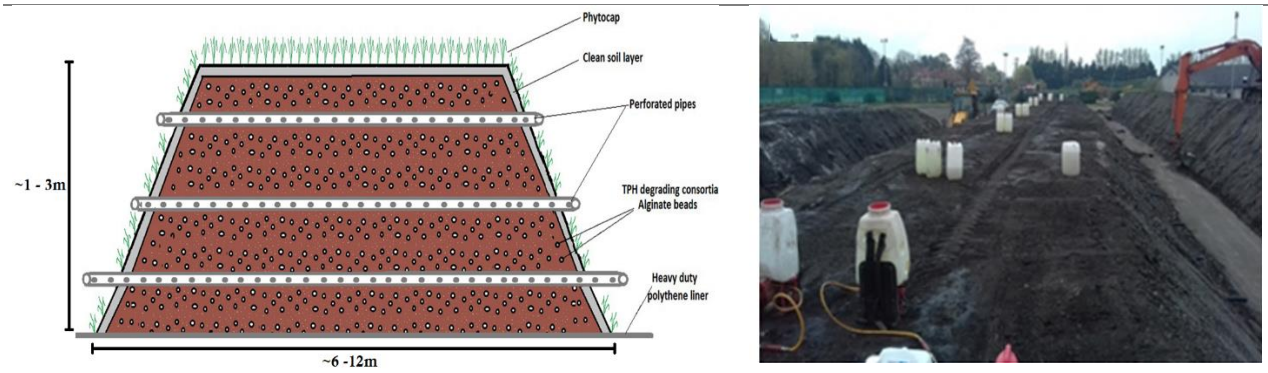

Figure 3: Structure of an ecopile

6.2 Activities and KPIs at lower TRL

Target compounds

For testing of improved ecopile technology the following target compounds will be tested:

- Petroleum hydrocarbons

The concentration range includes:

- high range: up to 1000 ppm of TPH

Materials

Phytoremediation trials using plants or a combination of plants and microbial consortia will be performed in a greenhouse setting (**Table 19**).

Table 19: *Materials for the greenhouse experiments*

Component	Description
Soil	Soil contaminated with total petroleum hydrocarbons from sugar factory (Carlow, Ireland)
Plant pots (size)	Plant ports for with capacity to support 1 kg of soil
Greenhouse	For controlling incubation temperature
Plants	Sinapsis alba, Lolium perenne (var. Abergain) + Festuca rubra (50/50) Lolium perenne (var. Abergain) + Lolium perenne (var. Abergreen) Sugar factory mix: Lolium perenne (var. Kerry) 40.0%, Lolium perenne (var. Solas) 30.0%, Lolium perenne (var.Aspect) 25.0%, Trifolium repens (var. Coolfin) 2.5% Trifolium repens (var. Buddy wc) 1.5% Trifolium repens (var. Rivendale) 1.0%
Bacterial consortia	Hydrocarbon-degrading consortia from ITC/MIB and UAM

Experimental design and monitoring

Experiment series 1: Phytoremediation preliminary trials: a greenhouse experiment will be carried out to test a number different grass mixtures for their ability to grow on contaminated soil and stimulation degradation of the contaminants. The testing system will consist of plant pots including 1 kg of soil (5 randomized replicates per treatment) incubated in the greenhouse under artificial light and controlled temperature (**Table 19**).

Experiment series 2: Combination of plants and microbial consortia for effective remediation of the soil: the best performing grass mixture will be tested in combination with a number of different bacterial consortia (from ITC/MIB and UAM). Soils will be soaked with overnight cultures of the consortia and seeds of the selected plants will be sown. The pots (5 replicates per treatment) will be randomized and cultivated for 12 weeks.

Table 20: *Treatments for phytoremediation tests using solely plants and a combination of plants and microorganisms*

Treatment	Description
Synapsis alba	moisture is adjusted in the soil to values close to its field capacity.
Lolium perenne	several nutrients (nitrogen, phosphorus, potassium, etc.) will be added to the soil to enhance microbial growth, especially those microorganisms with degradation capacity of hydrocarbons that were adapted to the conditions of the place of application.
Sugar factory mix	application of indigenous microorganisms isolated from the polluted soils in order to accelerate the removal of undesired compounds.
Plant + ITC consortium	best performing plant from phytoremediation test + TPH-degrading consortium from ITC/MIB
Plant + UAM consortium	best performing plant from phytoremediation test + TPH-degrading consortium from ITC/MIB

The following parameters will be monitored during the greenhouse trials (**Table 21**).

Table 21: *Monitoring parameters at low TRL*

Parameter	Instrumental equipment
TPH – Total Petroleum Hydrocarbons	Gravimetric and via GC-FID (Gas-Chromatography coupled to Flame Ionization Detection) previous Soxhlet extraction (1;1 acetone :hexane)
Plant biomass	Gravimetric (dry weight)

General and specific KPIs

For the phytoremediation trials in the greenhouse the following general and specific KPIs are defined (**Table 22**).

Table 22: *General and specific KPIs for phytoremediation in the greenhouse*

General KPI	Definition
Contaminant concentration:	Change in parent compounds: TPH and PAHs
Contaminant mass reduction:	Percentage of change in the concentration of hydrocarbons in soil
Decontamination time:	Time needed to achieve the corresponding percentage of parent compound transformation
Materials consumed	Amount of electron donor(s) added and other chemicals (e.g. for pH adjustment) to enable biotransformation
Specific KPI	**Definition**
1st order decay rate	For both TPH (aromatics and aliphatics) and PAHs
End-products	Toxicity of the soil before and after treatment (cress, lettuce, nematode, earthworm, Daphnia toxicty assessment assays)
Plant biomass	Amount of root and total biomass produced

6.3 Activities and KPIs at higher TRL

Target compounds

For testing of improved ecopile technology the following target compounds will be tested:

- Petroleum hydrocarbons

The concentration range includes:

- high range: up to 1000 ppm of TPH with high percentage of recalcitrant hydrocarbons.

Materials

Ecopile trials using a combination of plants and microbial consortia with or without passive electrodes (hybrid system) will be performed at the former Irish Sugar site in Carlow (**Table 23**).

Table 23: *Materials for the outdoor ecopile experiments*

Component	Description
Soil piles	Soil piles contaminated with total petroleum hydrocarbons from sugar factory (Carlow, Ireland) in outdoor facility
Plant	Best-performing plant from lower TRL
Bacterial consortium	Best-performing Hydrocarbon-degrading consortia from lower TRL in combination with plant
Electrodes/Snorkels	Passive electrodes for testing of hybrid technology

Experimental design and monitoring

Bioaugmentation of the Ecopile system: a number of Ecopiles treating petroleum impacted soil will be set up These will typically be 10m x 5m x 1.5m (L x W x H) in dimension. Different Ecopiles or different parts of the same Ecopile with be inoculated with the different microbial consortia developed in the lower TRLs. The microbes will be cultured in 25L sanitized containers in low nutrient levels media (1/10 diluted Nutrient Broth) to an OD value of 1.0. These will be diluted 1/10 (while ensuring a minimum of 10^8 CFU/ml) and will be applied by mechanical sprayers at a rate of 1L/m^3

Development of hybrid Ecopiling and BES systems: Passive bioelectrical stimulation systems will be inserted into the ecopiles to stimulate microbial activity and biodegradation within the contaminated soil.

Table 24: *Treatments for phytoremediation tests using solely plants and a combination of plants and microorganisms*

Treatment	Description
Ecopile with Plant + consortium 1	moisture is adjusted in the soil to values close to its field capacity.
Ecopile with Plant + consortium 2	several nutrients (nitrogen, phosphorus, potassium, etc.) will be added to the soil to enhance microbial growth, especially those microorganisms with degradation capacity of hydrocarbons that were adapted to the conditions of the place of application.
Ecopile with Plant + consortium 1 + passive electrodes	application of indigenous microorganisms isolated from the polluted soils in order to accelerate the removal of undesired compounds.
Ecopile with Plant + consortium 2 + passive electrodes	best performing plant from phytoremediation test + TPH-degrading consortium from ITC/MIB

Various physical-chemical and biological properties will be monitored regularly in the Ecopiles by taking samples approximately every 3 months over the course of 2 years (**Table 25**). For the biological properties samples will be analyzed through NGS of amplicon libraries of the bacterial 16S biomarker genes and the Fungal and nematode 18S biomarker genes, in addition to samples taken for metagenomic and proteomic analysis.

Table 25: *Monitoring parameters at high TRL*

Parameter	Instrumental equipment
General soil parameters	pH, electrical conductivity, redox potential, moisture and organic matter content
TPH – Total Petroleum Hydrocarbons and PAHs	Gravimetric and via GC-FID (Gas-Chromatography coupled to Flame Ionization Detection) previous soxhlet extraction (1;1 acetone :hexane)
Soluble nutrients (Nitrate and phosphate)	via ion-chromatography
16S /18S biomarkers	DNA extraction, Amplicon library preparation and DNA sequencing. Bioinformatic analysis of sequence data using DADA2 and QIIME2 pipelines
Total bacterial and TPH degrader counts	stanardard plate count methods and MPN method for TPH degraders

General and specific KPIs

For the ecopile trials outdoors following general and specific KPIs are defined (**Table 26**).

Table 26: *General and specific KPIs for phytoremediation in ecopiles*

General KPI	Definition
Contaminant concentration:	Change in parent compounds: TPHs (aromatics and aliphatics) and PAHs (mg/kg)
Contaminant mass reduction:	Percentage of parent compound transformed between start and termination of the experiment (taking toxic intermediates and non-toxic end-products into account)
Decontamination time:	Time needed to achieve the corresponding percentage of parent compound transformation (months/years)
Efficiency or total cost:	Decontamination cost in EUR or EUR/m3.
Energy consumed	Total Kwh required to construct the system during treatment
Materials consumed	Amount of fertilizer, inoculum, and aeration piping used
Specific KPI	**Definition**
1st order decay rate	For both TPH (aromatics and aliphatics) and PAHs
End-products	Toxicity of the soil before and after treatment (cress, lettuce, nematode, earthworm, Daphnia toxicty assessment assays)
Plant biomass	High plant biomass growing on the ecopiles. Good root biomass and penetration into the ecopiles.
Soil Biology	Bacterial, fungal and nematode biodiversity and abundance will be determined in treated and untreated soil from the NGS data.
Hybrid systems (Ecopiles-BES)	Time taken to achieved desired level of contaminants in the soil.

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7. Soil Microbial Fuel Cells – SMFCs (BATH)

7.1 Technology Overview and Aims

Microbial fuel cells (MFCs) are bioelectrochemical systems that generate electricity thanks to the action of so-called electroactive bacteria. An organic substrate is oxidized at the anode, generating protons, electrons and CO_2. The protons move towards the cathode while the electrons flow across an external circuit (generating current) to combine with an oxidant (generally oxygen) and the protons and form water. The technology has been widely investigated as a means to treat wastewater while generating energy [21].

A very attractive type of MFCs is the soil microbial fuel cells (SMFCs). Here the soil, acts as the source of both electroactive microorganisms and nutrients and as the separator between the two electrodes. The technology is characterized by an extreme design and operation simplicity with respect to traditional MFCs. Still, SMFCs have not been widely investigated yet, and only in recent years there appear to be some literature on their potential for soil remediation. In SMFC remediation, the existence of the anode accelerates the capability of the electroactive microorganisms to provide more electrons to promote the metabolic reaction rates of anaerobic bacteria that degrade the contaminants.

In some cases, SMFCs have been tested as a means to remove heavy metals in soil. In this case, however, the process simply consists on the movement of metal ions from the anode to the cathode. So the process is better described as the metal's migration from the anode to the cathode. As a consequence, after remediation, the heavy metal concentration increases in the cathode regions and decreases in the anode regions [22]. An alternative is to use a microbial electrolysis cell (MEC), where the application of an input potential would promote the electrodeposition of the heavy metals at the cathode.

More promising is the removal of organic pollutants via the electrochemical oxidation at the anode. The organic substrates are degraded under microbial catalysis with electron production, which leads to the generation of useful electricity [23].

Table 27: *Technology Overview*

GREENER PARTNER	Univ. of Bath (Bath)
Type of Technology	Soil Microbial Fuel Cell; Bioelectrochemical systems for soil remediation
Process	Electrochemical oxidation
Target compounds	pesticides: Lindane, Atrazine, diazinon, methiocarb, dieldrin, Hexochlorobenzene, Isodrin, Dichlorvos, Glyphosate, Cypermethrin) and TPHs (ITC and Acciona soil)
Test Matrix (contaminants)	soil
Current TRL / Goal TRL	3/7

Goals

The main aims of the SMFC technology are:

- To develop an effective and easy-to-scale-up SMFC technology for soil remediation
- To design an effective and low cost SMFC with the use of materials that are compatible with scale-up and mass production. This include avoiding the use of expensive oxygen reduction reaction catalysts at the cathode (i.e. Pt) and expensive proton exchange membranes.
- To assist the design with a mathematical model that, by combining transport phenomena, bio-electrochemical and electrochemical reactions, predicts current and potential distribution, and inform on the best geometrical design and the route to scale-up.

The SMFC consists of an air cathode and an anode buried inside the soil. Three designs are tested: ceramic tubular SMFC, flat-plate geometry and vertical anode geometry. Figure 4 shows the three geometries. In the first one, a ceramic tube provides the structure and separates the two electrodes. In the other two geometries, the SMFC simply consists of two carbon-based electrodes (Figure 4).

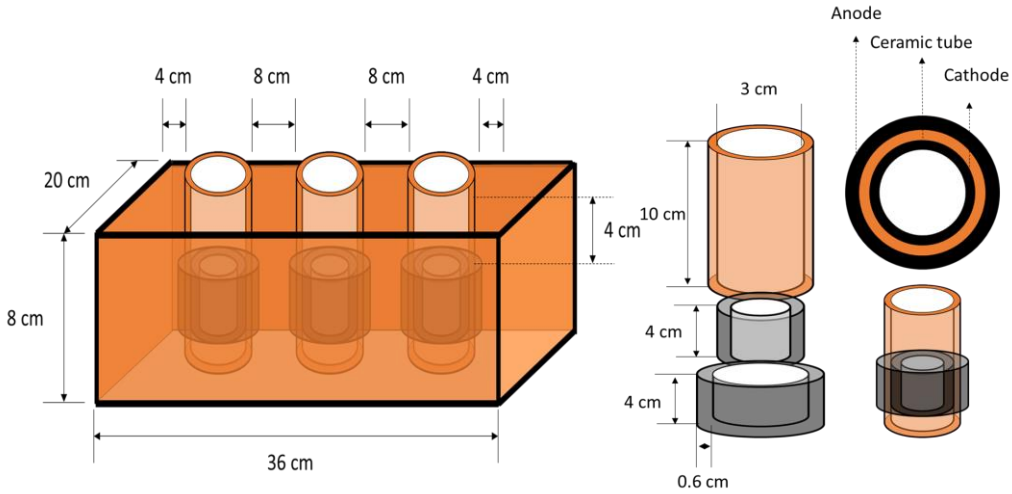
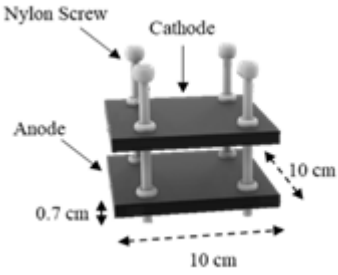
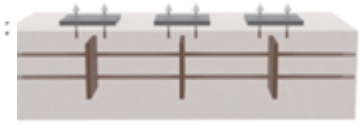


Figure 4: Structure of the SMFC





7.2 Activities and KPIs at lower TRL

Target compounds

For testing the new develop SMFC technology the following target compounds will be tested:

- Pesticides tested: imidacloprid, prochloraz, clothianidin, lindane, oxadiazon, flufenacet and atrazine. Each at a concentration of 5 mg/kg
- PAH in soil provided by ACCIONA and ITC (concentration range to be confirmed by the respective partners)

Materials

Bioelectrochemical remediation of pesticides will be performed under controlled lab conditions in incubation chambers filled with soil (Figure 4). The experimental setting consists of the following components (**Table 28**).

Table 28: *Materials for the SMFC experiments*

Component	Description
Soil	Soil collected from the university of Bath (pH 6.5, Nitrogen Low, Phosphorous None, Potassium Low, Moisture Content 52.77 %, Organic Matter Content 17.44 ± 1.5 50%, C/N 18/1 g, CTC 80 M.MOL C/Kg)
SMFC	Flat-plate geometry Graphite felt as electrodes Ti wires as current collectors
Data acquisition system	The voltage generated by the system is monitored with a data acquisition system

Experimental design and monitoring

Prior to performing remediation experiments preliminary tests will be performed with the following materials to improve performance of the SMFC:

- testing of metal-based catalyst at the cathode (provided by Leitat)
- Testing commercial Pt-functionalised carbon cathode
- Assess the incidence of electrode orientation and design on the electrochemical performance of the SMFCs

To test the efficiency of the bioelectroremediation approach three different treatments will be set up (**Table 29**).

The SMFCs were operated in a glass house, under controlled conditions of temperature and each in individual PVC boxes. The soil moisture was kept constant by providing daily tap water. The anode and cathode were connected to an external resistance of 510 Ω and to a voltmeter to monitor the voltage over time.

Table 29: *Treatments for SMFC bioelectroremediation*

Treatment	Description
Treatment 1	soil spiked with a target contaminant (control 1)
Treatment 2	SMFC in open circuit operation (no current generation) in soil spiked with a target pollutant (control 2)
Treatment 3	SMFC in closed circuit in soil spiked with a target pollutant

For each treatment from **Table 29** the soil will be incubated with the target contaminant for a defined period of time prior to start of the test to allow the adaptation of the microbial consortia to the new environment. The following treatment combinations will be performed for each case:

- Case 1: individual pollutant
- Case 2: co-existence of two or more pollutants
- Case 3: combination with biostimulation and bioaugmentation by integrating in the soil the methodology from Task 5.1 (*for higher TRL*)
- Case 4: combination with phytoremediation by integrating the best condition found in Task 5.2 (*for higher TRL*)

The following parameters will be monitored during the SMFCs trials (**Table 30**).

Table 30: *Monitoring parameters at low TRL*

Parameter	Instrumental equipment
voltage	Data logger
pH and EC	pH and EC sensors
K, P and F	Colorimetric assays using commercial kits
Pollutant and relative metabolites	UPLC-MS/MS

General and specific KPIs

For the electrobioremediation trials the following general and specific KPIs are defined (**Table 31**).

Table 31: *General and specific KPIs for the SMFCs tests*

General KPI	Definition
Contaminant concentration:	Change in parent compounds: TPH and pesticides
Contaminant mass reduction:	Percentage of parent compound transformed to end-products between start and termination of incubation (taking toxic intermediates and non-toxic end-products into account)
Decontamination time:	Time needed to achieve the corresponding percentage of parent compound transformation
Materials consumed	For the case of combination with biostimulation/bioaugumentation: Amount of electron donor(s) added and other chemicals (e.g. for pH adjustment) to enable biotransformation
Specific KPI	**Definition**
1st order decay rate	these data are not available in the literature for SMFCs. Comparison will be made with other methodologies in the literature and in the case of TPHs also with the outcomes from the other tasks in WP5
metabolites	Biodegradation progress by formation and further transformation of intermediate metabolite compounds over time

General KPI	Definition
End-products	bioenergy and metabolites of interest (to be investigated)
Structural genes	Assesment of 16S-RNA genes at DNA level for total bacteria and specific groups (e.g. Dechlorinators - Dehalococcoides, Geobacter, Dehalobacter) or other key organisms (e.g. Sulfate reducers) in the anodic biofilm that develops in SMFCs operating either in open circuit mode or closed circuit mode and in the soil. Each gene may be consider a different KPI.

7.3 Activities and KPIs at higher TRL

The scale-up tests will build on the experiments and design from the lower TRL to demonstrate the possibility to treat larger amount of soil and more complex soils (real samples). Stacks of SMFCs will be generated to boost the power output (and therefore the electrochemical processes to speed up the treatment) and enlarge the area to be treated.
The mathematical model, validated by the tests in the lab at lower TRL will predict the radius of action of each SMFC to plan the most effective way of designing the SMFCs stack and provide specifications on the basis of the area to be treated.
Large scale tests will be performed in the lab (controlled environment) with contaminated soil under the best conditions identified at low TRL with the combination of other methodologies from Tasks 5.1 and 5.2. Possibly experiments in combination with Ecopiles may be considered.

Information for the lower TRL regarding the target compounds, materials, the experimental design, monitoring and KPIs is applicable to the higher TRL in case of successful results from the lower TRL.

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[22] doi: 10.3390/microorganisms7120697

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8. Aggregate and Microbial Carriers (JSI)

8.1 Technology Overview and Aims

Co-cultures are hard to optimize due to different growth dynamics of the strains involved and the large distances between cells, particularly when complex substrates need to be broken down by one strain and the products released are to be used by other strains. The oxygen distribution within a co-culture is not easy to monitor. Generally, co-culture processes are often separated into two steps: aerobic and microaerophylic/anaerobic. The direct combination of autotrophic processes (CO_2-consuming) with heterotrophic processes (CO_2-producing) is currently only possible in suspensions or capsules of genetically modified autotrophic organisms which are capable of transmembrane exporting of carbohydrates with limited success.

In contrast, aggregation enables close contact between cells and more intensive interactions. The interactions facilitate not only the exchange of growth substrates, secondary metabolites as well as quorum sensing molecules, but also local scavenging of oxygen resulting in the formation of anaerobic niches within the aggregates (e.g. natural cosms in the biologically water treatment processes) [24].

The most important factors in cell aggregation are the size of the aggregates, the distribution of cells within the heterocellular aggregates (aggregate structure) and the number of the aggregates. The structure of the aggregates determines how cells are distributed within the aggregate. This is extremely important since the distribution of different types of cells determines the cascades of the biotechnological processes within the aggregate. Cells closer to the surface are involved in the earlier steps (e.g. degrading lignocellulose) than cells deeper within the aggregates which are involved in the later steps (e.g. fermentation and production of biofuels or acetate, lactates). Moreover, oxygen scavenging occurs in the most outer layers of the aggregates, enabling anaerobic processes to occur within the aggregates (e.g. nitrification and denitrification coupling of different microbial cells).

The method of electrostatic activation of bacterial cells is based on the deposition of polyelectrolytes on cells. If cells are covered with positively charged polyelectrolytes, they tend to attach to other surfaces, including other cells [25]. These depositions also can change some physiological properties by inducing higher production and release of exo-proteins [26].

Alginate is a very useful matrix for development of carrier technology, since it is relatively cheap, can be degraded and is not toxic. Currently the alginate matrix is used in combination with Ca ions to make a gelatinous structure. Since organic contaminants such as Polycyclic Aromatic Hydrocarbons (PHAs) are hydrophobic and the alginate structure omit cells to get in contact with the pollutants, it is necessary to create alginate with a certain degree of hydrophobicity [27]. One major goal is to develop an alginate-based matrix that can incorporate single cells as well as aggregates. Currently, the interaction of such alginates with bacterial cells and pollutants such as PAHs is not known. At the same time if the alginate is too hydrophobic it may limit nutrient transfer between cells; hence, the combination of hydrophobic and hydrophilic alginate requires optimization. Finally, the development of alginate matrix with Zn ions as alternative to Ca ions remains to be explored.

Table 32: *Technology Overview*

GREENER PARTNER	Josef Stefan Institute (JSI)
Type of Technology	Electrostatic aggregation of microbial cells and binding aggregates in matrix
Process	Intercellular coupling of degradation metabolic compunds of complex polutants and partitioning of hydrophobic pollutants within the matrix.
Target compounds	PHA, lignin, chlorinated ethenes, ethanes and methanes (chlorinated Volatile Organic Compounds -cVOCs)...
Test Matrix (contaminants)	soil
Current TRL / Goal TRL	2/4

Goals

The main goal of the aggregate technology is to establish a method of electrostatic preparation of aggregates that are formed from microbial cells of different species.

The specific (step-wise) goals include:

(i) the preparation of an initial electrostatic method of cell modification to the level of coupling of bacterial cells together (TRL2)
(ii) sorting out and determining the robustness of consortia that are capable of degradation of PAH
(iii) development of a method for isolation of the most robust and efficient consortia
(iv) improve the efficiencies of the biodegrading activities of consortia due to the faster exchange of nutrients.

The final goal is to combine functionally operative aggregates with the carrier technology to reach TRL4.

8.2 Activities and KPIs at lower TRL for aggregate technology

The aggregate technology consists in the electrostatic aggregation of microbial cells for resilient intercellular coupling of metabolic degradation potential of contaminants such as PHAs.

Target compounds

The target compounds that will be tested for the aggregate technology include: PAHs, lignin as well as chlorinated ethenes, ethanes and methanes (chlorinated Volatile Organic Compounds -cVOCs)

Materials

Aggregate formation will be performed using microfluidic devices. The experimental setting consists of the following components (**Table 33:** Materials for the aggregation experiments).

Table 33: *Materials for the aggregation experiments*

Component	Description
polyelectrolites	The polyelectrolytes must be selected to be highly charged, biodegradable and not toxic for cells; thus, they will be modified to assure no or very low toxicity for aggregated cells.
cultures	single species combined with cells degrading cellulose (proof of interaction and control of processes), microbial consortia degrading PAH, microorganisms (cells) from contaminated environments for determining degradation potential of consortia.
monitoring of microbial activity	multiplate reader with cultivation capability, shakers, incubators
growth	shakers, incubators
aggregation equipment	syringe pumps, mixer or disintegrator, droplet generator, microflow chip, micro cnc for chip construction

Experimental design and monitoring

Different types of aggregation will be tested including:

1. separated non-aggregated cells
2. doublets
3. triplets up to 20 cells
4. larger aggregates

Two different types of aggregation approaches will be considered:

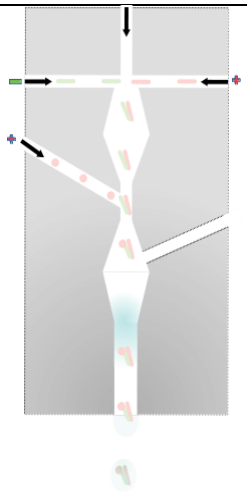
1. bottom up: using microfluidic to combine cells together (Fig.5a)
2. top down: using appropriate mixing conditions, dossing and concentrations of cells for appropriate aggregation - determined number of cells (Fig.5b)

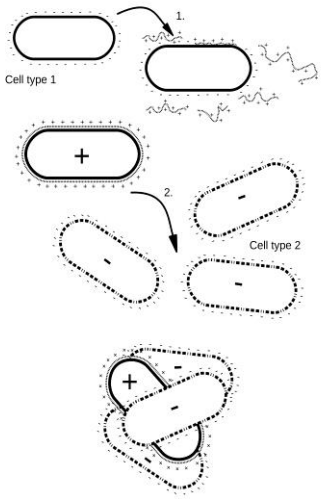
The dosage and mixing should be coupled in a simple automated production line (see example in a Fig. 5c).

Microbial growth will be monitored via:

1. size of aggregates (flow cytometry using FD and SS parameters)

2. number of active cells (flow cytometry with syto labeled cells)
3. activity (measurement of CO_2 production, 2,6 DCPIP, tetrazolium salts)





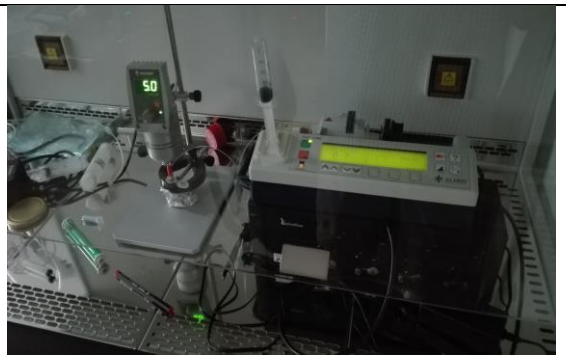


Figure 5: a) Bottom-up approach | b) Top-down approach | c) prototype of automatization of top-down approach

During the aggregation tests degradation and organization of cells in aggregates will be monitored in microplates (200µL) daily for 10 days followed by single monitoring events on day 20 and 30 (DCPIP, tetrazolium). Similarly, microbial activity will be monitored in larger Erlenmayer flasks (100 - 200mL of liquid volume) daily for 10 days followed by single monitoring events on day 20 and 30. The sample volumes and monitoring parameters are shown in **Table 34**.

Table 34: *Monitoring parameters at low TRL*

Parameter	Instrumental equipment
Aggregate structure	100 µL for flow cytometry [24] and 10 µL for microscopy [25]
General medium parameters	5 mL of aqueous phase for pH, electrical conductivity and redox conditions using specific sensors
Community structure and activity	10 mL for genetic material (microbial community structure and activity)
Volatile organic compounds	Fermentative products via GC-MS [28]

General and specific KPIs

For the aggregation trials the following general and specific KPIs are defined (**Table 35**).

Table 35: *General and specific KPIs for the aggregation tests.*

General KPI	Definition
Contaminant concentration:	Change in parent compounds: TPH and pesticides
Contaminant mass reduction:	Percentage of parent compound transformed to end-products between start and termination of incubation (taking toxic intermediates and non-toxic end-products into account)
Decontamination time:	Time needed to achieve the corresponding percentage of parent compound transformation
Specific KPI	**Definition**
aggregate size and stability	Size of aggregates and consortia added to enable biotransformation, robustness of the system
1st order decay rate	Degradation of PAH and comparison to available decay rates from literature
metabolites	Volatile compounds wide range - nontarget analysis
End-products	Pushing system of communities toward complete degradation to CO_2
Determining coupling of intercellular metabolic pathways:	RNA sequencing of aggregated cells. Bioinformatic screening of transcribed genes and their relative quantification.
Structural genes	Assessment of 16S-RNA genes at DNA level for total bacteria and specific groups. Detecting relative division rate by the dissolution of DNA bound dyes of different microbial cells used in the aggregation building (via fluorescent and confocal microscopy). Each aggregate might be considered a different KPI. The number of the members of consortia will represent a different activity of aggregation.

General KPI	Definition
Functional genes	Functional genes involved in the degradation of PAHs within the randomly formed aggregates will be determined by RNA sequencing. In experiments of known isolates the transcription will be quantified on a basis of genes involved in the degradation process.

8.3 Activities and KPIs at lower TRL for microbial carrier technology

The carrier technology consists in the incorporation of microbial aggregates within a hydrophobic alginate matrix that allows both the incorporation of hydrophobic contaminants such as PAHs and the intercommunication and molecule exchange between cells.

Target compounds

The target compounds that will be tested for the aggregate technology include: PAHs, lignin as well as chlorinated ethenes, ethanes and methanes (chlorinated Volatile Organic Compounds -cVOCs)

Materials

For production of alginate-based microbial carriers the following components will be used (**Table 36**).

Table 36: *Materials and instrumentation for production of microbial carriers.*

Component	Description
alginate matrix	alginate with Ca and Zn ions that turn alginate into hydrophobic mesh pH 7, T=22oC, $ZrOCl_2 \cdot 8H_2O$ (up to 3%) and $CaCl_2$ (up to 5%), sodium alginate (0.5% to 5%)
solid support	natural porous stones, expanded clay - perlite, calcite
cultures	single species combined with cells degrading cellulose and aggregates from aggregation technology
impregnation	vacuum chambers
bead preparation	encapsulator
culture growth	shakers and incubators
growth monitoring	multiplate reader with cultivation capability, shakers, incubators, GC-MS, HPLC-MS

Experimental design and monitoring

Two types of carriers will be prepared: (i) simple, a composite of different alginates is put together and (ii) solid, mineral or organic solid porous support is impregnated together with the alginate matrix, aggregates and separated cells, respectively (**Figure 6**).

1. Simple bead
   a) hydrophilic core + pollutant degrading cells + hydrophobic alginate
   b) hydrophobic alginate + hydrophilic alginate + pollutant degrading cells
2. Impregnated solid matrix
   a) organic degradable matrix (sterile straw, hay, cellulose)
   b) inorganic matrix (perlite, calcite, natural pebles, zeolites)

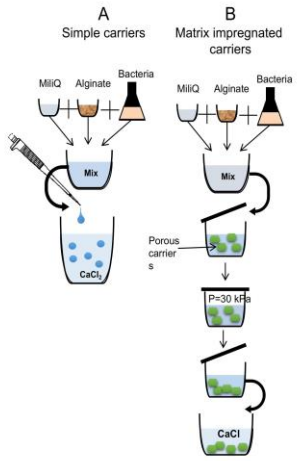
Matrix preparation:
Different concentrations of alginate and ions will be tested to determine physical compatibility with
1. Separated non-aggregated cells
2. Doublets
3. Triplets up to 20 cells
4. Larger aggregates

Matrix viscosity and efficiency of impregnation will be tested when impregnating porous mineral supports via vacuuming and re-pressurization at variable pressure levels. The optimal number of cycles of vacuuming and re-pressurizing will be determined.

Design of upscaling:
The amount of carriers produced per time will be determined in small sets. The upscaling effects of stability of carriers will be made by the measurement of efficiencies of impregnation per mass of carrier.

Growth and activity monitoring:
1. qPCR and 16S rRNA gene sequencing
2. Degradation of PAHs
3. Activity (measurement of CO_2 production, 2,6 DCPIP, tetrazolium salts)

Figure 6: Simplified design of experiments and preparation of carriers: (A) simple bead like carriers and (B) impregnated porous mineral or organic carriers with hydrophobic alginate containing cells.



Microbial activity within the carriers will be monitored daily for 10 days followed by single monitoring events on day 20 and 30. The sample volumes and monitoring parameters are shown in **Table 37**.

Table 37: *Monitoring parameters at low TRL*

Parameter	Instrumental equipment
Aggregate structure	100 µL for flow cytometry [25] and 10 µL for microscopy [28]
General medium parameters	5 mL of aqueous phase for pH, electrical conductivity and redox conditions using specific sensors
Community structure and activity	10 mL for genetic material (microbial community structure and activity)
Volatile organic compounds	Fermentative products via GC-MS [29]

General and specific KPIs

For the microbial carrier technology the following general and specific KPIs are defined (**Table 38**).

Table 38: *General and specific KPIs for the aggregation tests.*

General KPI	Definition
Contaminant concentration:	Change in parent compounds
Contaminant mass reduction:	Percentage of parent compound transformed to end-products between start and termination of incubation (taking toxic intermediates and non-toxic end-products into account)
Decontamination time:	Time needed to achieve the corresponding percentage of parent compound transformation
Materials consumed:	Number of cells, amount of matrix and supporting material that enable biotransformation, increase of the efficiency of the system when compared to dispersed cells

General KPI Specific KPI	Definition Definition
1st order decay rate	Degradation of PAH and comparison to available decay rates from literature
metabolites	Volatile compounds wide range - nontarget analysis
End-products	Pushing system of communities toward complete degradation to CO_2
Determining coupling of intercellular metabolic pathways:	RNA sequencing of aggregated cells. Bioinformatic screening of transcribed genes and their relative quantification.
Structural genes	Assessment of 16S-RNA genes at DNA level for total bacteria and specific groups. Detecting relative division rate by the dissolution of DNA bound dyes of different microbial cells used in the aggregation building (via fluorescent and confocal microscopy). Each aggregate might be considered different KPI. The number of the members of consortia will represent a different activity of aggregation.
Functional genes	Functional genes involved in the degradation of PAHs within the randomly formed aggregates will be determined by RNA sequencing. In experiments of known isolates the transcription will be quantified on a basis of genes involved in the degradation process.

8.4 Activities and KPIs at higher TRL for aggregate-microbial carrier technology

Activities at higher TRL will be performed in combination with other partners (e.g. UBU for bioaugmentation). A scaling-up for growing culture and producing aggregate-carriers in amounts large enough for field application will be performed based on the optimal approached developed at lower TRL.

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9. Decolorization of Wastewater by Microalgae – Phycoremediation (MENDELU)

9.1 Technology Overview and Aims

More than 10,000,000 types of synthetic dyes (azodyes) are generated worldwide with an annual production of around 7×10^5 metric tones. These dyes are widely used in the textile, paper, food, cosmetics and pharmaceutical industries. Up to 50% of unused dyes are discharged into wastewater sewers without treatment [30][31].

More than 30 azo compounds have been shown so far to be biodegraded and decolorized by different species of microalgae including *Chlorella Pyrenoidora*, *Chlorella vulgaris* and *Oscillatoria tenvi*s. The resulting biodegradation products of azo dyes are simple aromatic amines. The azoreductase enzyme of algae is responsible for breaking the azo linkage.

Table 39: *Technology Overview*

GREENER PARTNER	Mendel University (MENDELU)
Type of Technology	Phycoremediation
Process	*bioremediation of water by microalgae selected and designed on their capabilities for sorption biotransformation of azodyes*
Target compounds	Azodyes
Test Matrix (contaminants)	Synthetic water with known concentrations of dyes and industrial wastewater from site 4
Current TRL / Goal TRL	3/ 4-6

Goals

The main goal of the present approach is to develop a solid basis for microalgal biodegradation technology.

The specific goals include:

1. biobank screening, metabolomic analysis and expression analysis for selection of microalgae with the ability to degrade azo dyes
2. Comparative metabolomic and transcriptomic analysis of microalgae cultures to better understand their physiology in degradation experiments

9.2 Activities and KPIs at lower TRL

Target compounds

For testing of phycoremediation technology the following target compounds will be tested:

- Azodyes (Congo Red, Eriochrome Blue, and/or Malachite Green, etc.) within the concentration range 0.1 – 200 mg/L
- Industrial wastewater from site 4 at different dilutions (1x; 2x; 10x; 20x; 50x; 100x; 500x; 1000x)

Materials

For phycoremediation trials using microalgae at lower TRL the following materials will be used (**Table 40**).

Table 40: *Materials and instrumentation for phycoremediation experiments*

Component	Description
Microalgae and natural consortia	*Scenedesmus quadricauda*; *Chlorella sorokiniana*; *Chlorela vulgaris*; *Chlamydomonas reinhardtii* Enriched consortium sampled from nature (contaminated site)
Bioreactors	Tubular (8 tubes in one reactors) bioreactors - controlled pH, temperature, CO_2, quantity and quality of the light (Figure below). ATS system (Figure below) surface area 60x40x7.5 cm, volume up to 50 L for one ATS; maintaining a constant temperature and relative humidity; light intensity 500 µmol m²/s with photoperiod 16/8h.
Culture media	Minimum mineral medium with CO_2, azodyes or wastewater from site 4 (coloring industry)

Experimental design and monitoring

The experimental design is shown graphically in the following figure.

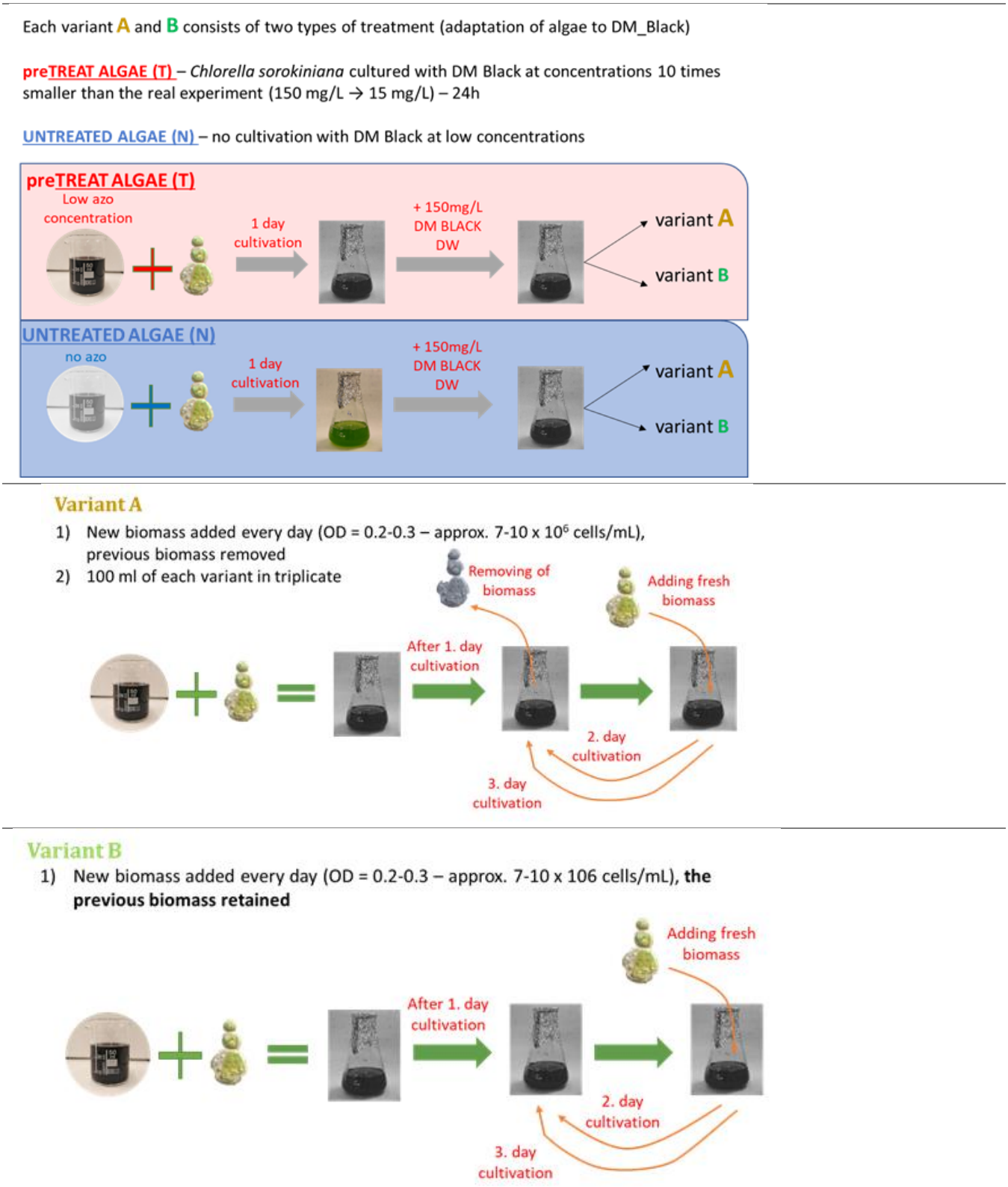

Figure 7: Schematic view for phycoremediation experiments at low TRL

The following parameters will be monitored during the phycoremediation biodegradation tests (**Table 41**).

Table 41: *Monitoring parameters at low TRL*

Parameter	Instrumental equipment
algae growth	temperature, pH, light, CO2, medium nutrients - Petri dish and Erlenmeyer flask
azodyes	HPLC/MS-MS and spectrophotometry
Targeted metabolites relevant for glycolysis, TCA cycle and carotenoids	UHPLC-MS/MS
Non-targeted metabolites	Advanced ionization and mass spectrometric methods for metabolomic analysis DESI-MS/DART-MS Desorption Electrospray Ionization (DESI)/Direct Analysis in Real Time
Gene expression related to stress ROS	Real-time PCR Microsopy, spectrophotometry, flow-cytometry

General and specific KPIs

For the phycoremediation treatment the following general and specific KPIs are defined (**Table 42**).

Table 42: *General and specific KPIs for phytoremediation in the greenhouse*

General KPI	Definition
Contaminant concentration:	Change in parent compounds azodyes and secondary metabolites
Contaminant mass reduction:	Percentage of change in the concentration of hydrocarbons in soil
Decontamination time:	Time needed to achieve the correspoding percentage of parent compound transformation
Materials consumed	Light (intensity and quality), temperature and nutrients supply for optimal microalgae growth.
Specific KPI	**Definition**
1st order decay rate	For azodyes and microalagae species. Comparison to available decay rates from literature
metabolites	Biodegradation progress by the formation and further transformation of intermediate metabolite compounds over time: secondary metabolites (polyphenols, carotenoids, chlorophylls, flavonoids) [32]
End-products	Accumulation of non-toxic end-products (still unknow and must be identified)
Functional genes	Identification of genes involved in biodegradation of azo dyes; first focusing on azoreductases [33]

9.3 Activities and KPIs at higher TRL

Phycoremediation experiments at TRL 5-6 will consist of 23 bioreactors (2L) supplied with CO_2 and operated in parallel under controlled conditions (temperature, pH, light intensity) as well as ATS systems 3 bioreactors (100 L) with natural microalgae consortia under artificial/natural light conditions.

Target compounds

For testing of phycoremediation technology the same azodyes and industrial wastewater as for the lower TRL will be tested:

- Azodyes (Congo Red, Eriochrome Blue, and/or Malachite Green within the range 0.1 – 200 mg/L
- Industrial wastewater from site 4 at different dilutions (1x; 2x; 10x; 20x; 50x; 100x; 500x; 1000x)

Materials

For phycoremediation tests using microalgae at higher TRL the following materials will be used (**Table 40**).

Table 43: *Materials and instrumentation for phycoremediation experiments at higher TRL*

Component	Description
microalgae	Likely the most successful *Scenedesmus quadricauda* and *Chlorella sorokiniana* species from the lower TRL and natural consortia for ATS system.
reactors	Tubular (8 tubes in one reactors) bioreactors - able control, pH, temperature, CO_2, quantity and quality of the light (Figure below). And ATS system (Figure below) surface area 60x40x7.5 cm, volume up to 50 L for one ATS; maintaining a constant temperature and relative humidity; light intensity 500 µmol m²/s with photoperiod 16/8h.  
Culture media	BBM; BG-11 and TAP medium
Light	500 µmol, 12/12h or 16/8h
Temperature	23 – 30°C

Experimental design and monitoring

For experiments at higher TRL the following aspects will be considered:

Upscaling

A stepwise approach will be used to:

- scale up the optimum treatments/strains obtained in 5 mL plates and Erlenmeyer flasks to 2 L bioreactors.
- optimize incubation conditions to shorten treatment times and increase remediation efficiency

Monitoring and sample processing – extraction

- harvest biomass after 24h, 48h, 72h or 10 days (approx. 30 mg sample/1 mL of 80% ethanol)
- TLC scanning analysis of carotenoids
- HPLC analysis of potential degradation products (using standards) and identification of novel metabolites via HPLC MS/MS non-targeted analysis

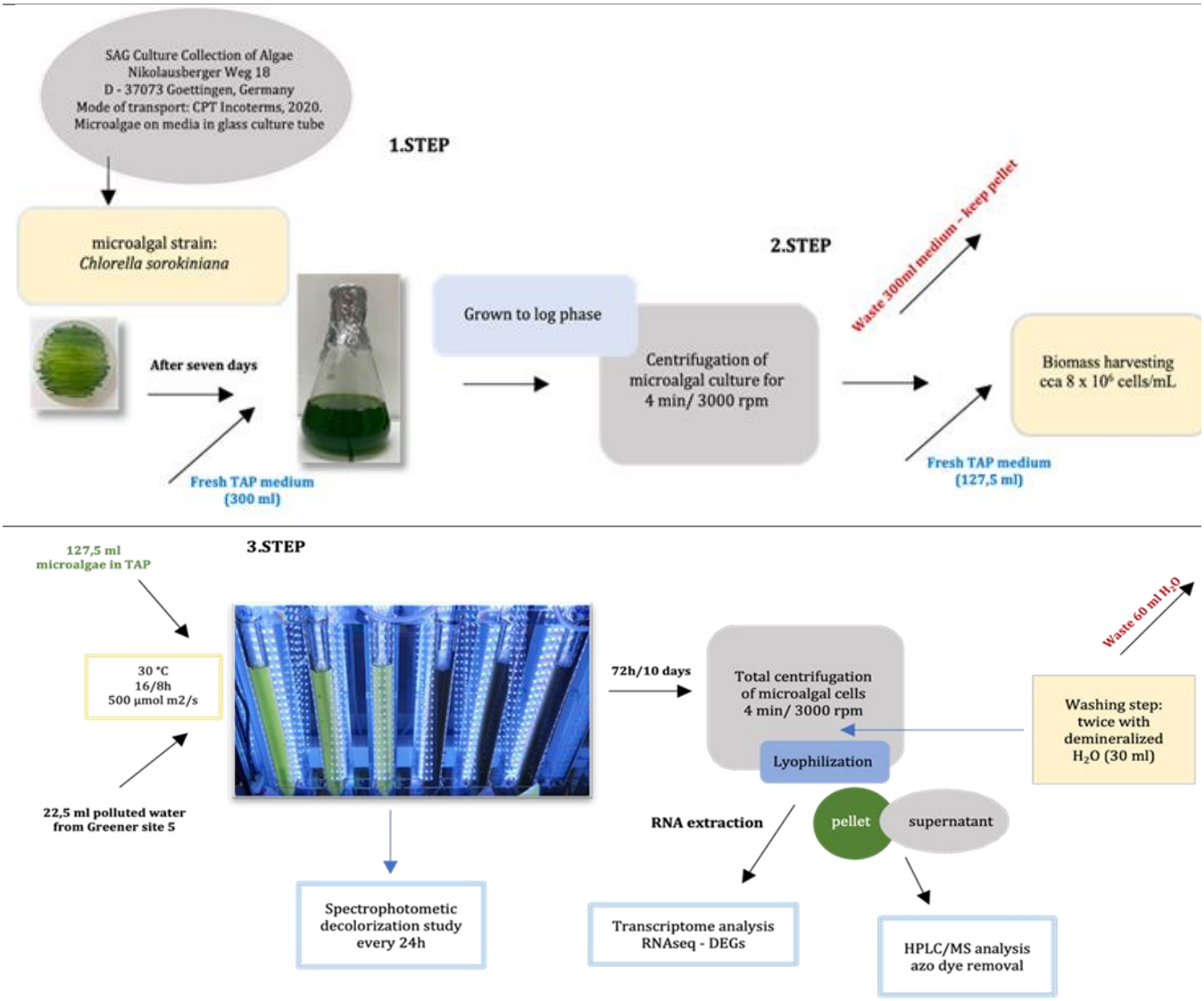

Figure 8: Schematic view for phycoremediation experiments with single strains at higher TRL

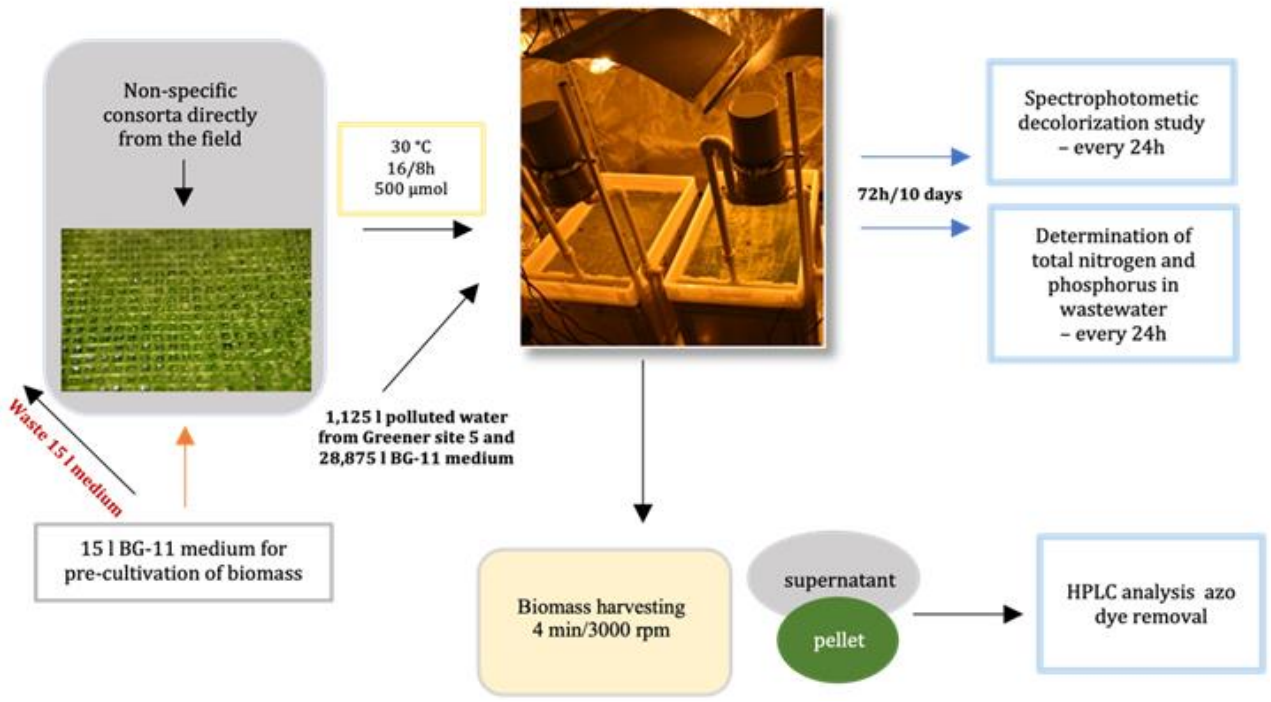

Figure 9: Schematic view for phycoremediation experiments with microbial consortia at higher TRL

Table 44: *Treatments for phycoremediation at higher TRL*

Treatment	Description
Industrial wastewater from site 4 (azodye DM Black)	DM Black diluted with TAP medium or water or natural non-specific consortia in BG-11 medium

The following parameters will be monitored during the phycoremediation biodegradation tests (**Table 41**).

Table 45: *Monitoring parameters at higher TRL*

Parameter	Instrumental equipment
algae growth	temperature, pH, light, CO2, medium nutrients - Petri dish and Erlenmeyer flask
azodyes	HPLC/MS-MS and spectrophotometry [32]
Targeted metabolites relevant for glycolysis and TCA cycle	UHPLC-MS/MS
Non-targeted metabolites	Advanced ionization and mass spectrometric methods for metabolomic analysis DESI-MS/DART-MS Desorption Electrospray Ionization (DESI)/Direct Analysis in Real Time
Gene expression related to stress	Real-time PCR [33]

General and specific KPIs

For the phycoremediation tests at higher TRL the following general and specific KPIs are defined (**Table 42**).

Table 46: *General and specific KPIs for phycoremediation at higher TRL*

General KPI	Definition
Contaminant concentration:	Change in parent compounds azodyes and secondary metabolites
Contaminant mass reduction:	Percentage of parent compound transformed between start and termination of incubation (taking toxic intermediates and non-toxic end-products into account)
Decontamination time:	Time needed to achieve the corresponding percentage of parent compound transformation
Materials consumed	Light and nutrients supply for optimal microalgae growth; energy for maintaining stable temperature
Specific KPI	**Definition**
1st order decay rate	Comparison to available decay rates from lower TRL
metabolites	Biodegradation progress by the formation and further transformation of intermediate metabolite compounds over time: secondary metabolites (polyphenols, carotenoids, chlorophylls, flavonoids)

General KPI	Definition
End-products	Accumulation of non-toxic end-products (still unknow and must be identified)
Functional genes	Identification of genes involved in biodegradation of azo dyes; first focusing on azoreductases, Identification of genes involved in biodegradation of azo dyes; first focusing on azoreductases, maping of whole travbsriptome by RNAseq - connected to the metabolite analysis and biodegraded end products through the specification of genes and consequent enzymes.
Heavy metals	Determination of heavy metals in biomass after remediation or biodegradation

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10. Bioremoval of Metals via Nanoparticle Formation under Aerobic and Anaerobic Conditions (MANO)

10.1 Technology Overview and Aims

Due to their properties, nanoparticles have gained significant interest for a wide variety of applications, ranging from biosensing and catalysts to memory schemes, electrometers, etc [34]. Different organisms have been studied for their ability to produce metallic nanoparticles as a substitute to conventional physical and chemical methods: plants, fungi and bacteria can produce intra- or extra- cellular nanoparticles of different size, shape and composition. Moreover, the optimization of process conditions can modulate the nanoparticle morphology and production rate.

In particular, bacteria can be considered as ideal candidates for nanoparticles synthesis because of their resistance to harsh environmental conditions (resistance to multiple contaminants, even at high concentration) [35][36][37][38].

Formation of nanoparticles has been shown so far for both aerobic and anaerobic bacteria. For aerobic bacteria nanoparticle formation has been observed for the following metals and strains: Au (Cupriavidus metallidurans) and Cu (Morganella psychrotolerans, Pseudomonas stutzeri). For anaerobic bacteria: Fe (Magnetospirillum gryphiswaldense, Geobacter sulfurreducens,), Cu (Morganella psychrotolerans, Shewanella oneidensis), palladium (Shewanella oneidensis, E. coli) and Ag (Shewanella oneidensis).

Despite a significant potential for metal removal there are only few reports on nanoparticle formation for bioremediation of contaminated water. Though, this promising approach enables the combination of bioremoval of metals with the synthesis of nanoparticles for high-value applications. In line with circular economy concepts, the process optimization to real waste streams could be highly valuable in the future.

Table 47: *Technology Overview*

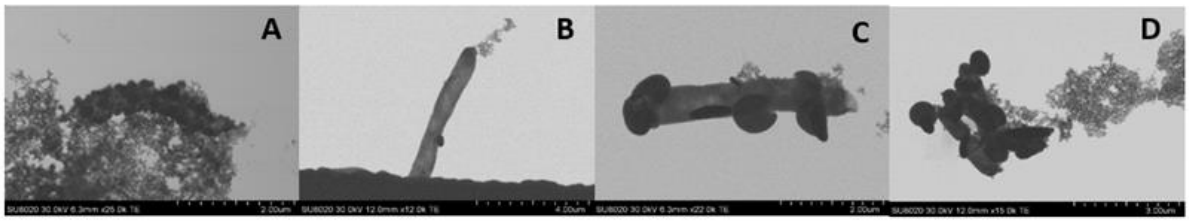
GREENER PARTNER	Materia Nova Institute of Materials (MANO)
Type of Technology	Metal bioremoval via nanoparticle formation
Process	*Aerobic and anaerobic production of nanoparticles by bacteria*
Target compounds	metals
Test Matrix (contaminants)	water
Current TRL / Goal TRL	3/4

Goals

The main goal is to develop robust and efficient anaerobic and aerobic bioremediation processes that allow the removal of metals in solution and the recovery of nanoparticles.

The specific aims are:

- Stimulate nanoparticle formation in batch laboratory studies with solutions mimicking environmental water streams
- Reproduce nanoparticle formation for metal removal both under aerobic and anaerobic conditions using a complex water matrix (water from a contaminated GREENER site)
- Perform nanoparticle and metal removal upscaling with the most promising processes (aerobic and/or anaerobic) and the best-performing bacterial strains



MEB pictures of the individual bacteria incubated for 72 hours in 8003. (A) *Magnetospirillum gryphiswaldense*, (B) *Shewanella oneidensis*, (C) *Morganella psychrotolerans*, (D) *Pseudomonas stutzeri*

Figure 10: Nanoparticles

10.2 Activities and KPIs at lower TRL

Target compounds

For metal removal via nanoparticle formation the following metal species and concentrations ranges will be considered:

- Cu, Fe, Ni, Zn and As

The concentration range includes:

- high range: 1500, 950 and 1000 mg/L for Cu, Fe and Ni, respectively
- low range: 140 and 20 for Zn and As respectively

Materials

The testing system for bioremediation will be performed in small glass bottles with rubber caps that allow agitation, aerobic or anaerobic conditions and treatment of the polluted water. First the bacterial inoculum will be done in aerobic condition to obtain an optimal optical density (2,0 or more). Bottles will be installed in an incubator with agitation to evaluate at optimal condition the metal removal capacity of the bacteria. In a second phase of the scaling-up, the bacterial inoculum will be placed in 2L bioreactor (Figure 8). Incubations with different metal species and bacterial strains in batch settings will be performed using the following materials (**Table 48**).

Table 48: *Materials for nanoparticle formation at low TRL*

Component	Description
bacterial strains (aerobic)	Pseudomonas sp.
bacterial strains (anaerobic)	Shewanella sp and Morganella psychrotolerans. Bacillus megaterium
Batch reactors	100 mL glass bottles sealed with rubber caps, agitation 100rpm and incubation between 20-30 °C
metallic salts	Polluted water: iron sulfate, copper sulfate

Figure 8: Bioreactor 2L

Experimental design and monitoring

As there are a lot of experiments for metal removal, only the best result from the lower TRL will be considered for the higher TRL test. The most promising bacteria species will be selected and consortia of two different bacteria following by one bacteria treatment will be used. The experimental design will consist of the following strains and metal combinations (**Table 46**).

Table 46: *Treatments for bioremoval of metals using nanoparticle formation*

Treatment	Description
Step 1: Consortia of bacteria	Addition of two bacteria inoculum with an OD of 2,0 in polluted water in bioreactor
Incubation time	Incubation in anaerobic condition for 48 hours at 30°C
Recuperation of treated water	Removal of the bacteria by centrifugation
Step 2: one bacteria	Addition of Bacillus megatherium to polluted treated water
Incubation time	Incubation in anaerobic condition for 48 hours at 30°C
Recuperation of treated water	Centrifugation to recover bacteria and water treated with the two bacteria steps

The following parameters will be monitored during the batch incubation studies (**Table 47**).

Table 49: *Monitoring parameters at low TRL*

Parameter	Instrumental equipment
general culture parameters	pH and Oxygen level (specific sensors), OD (spectrophotometry)
metal concentrations in solution	spectrophotometer, ICP, colorimetric tests
Metallic nanoparticles	ICP, XRD, SEM, TEM, magnetism (by spectrophotometry)
Functional and structural genes	PCR, Real time PCR, metagenomics

Sampling of the batch reactors will performed after 0, 2h , 6h and then every 24h. Specifically, the following sampling volumes and sampling frequency are expected:

- For metals, 1ml of culture for OD measurements at specific wavelenghts (all sampling times).
- For all, 5ml of culture for metallic concentration and nanoparticle characteristics (every 24h)
- For all, 1ml of culture for determination of functional and structural genes (0 and end of culture)

General and specific KPIs

For the batch incubations for nanoparticle formation the following general and specific KPIs are defined (**Table 50**).

Table 50: *General and specific KPIs for metal removal via nanoparticle formation*

General KPI	Definition
Contaminant concentration:	Change in parent compounds: metals
Contaminant mass reduction:	Percentage of change in the concentration of metals in water
Decontamination time:	Time needed to achieve the correspoding percentage of parent compound transformation
Materials consumed	Culture materials added at the start of incubation and added during the incubation
Specific KPI	**Definition**

General KPI	Definition
Viability of organisms	Important bacterial optical density at the inoculation step
Production of nanoparticles	Measurement of magnetism or number of nanoparticles/cells or number of nanoparticles in supernatant
Genetic stability	Assessment of the presence and abundance of Functional genes (specific for each strain) involved in the production of nanoparticles
Size and homogeneity of nanoparticles	Size measurement of intracellular nanoparticles by SEM/EDX and of extracellular nanoparticles by nanosizer, XRD
Elementary composition of nanoparticles	Quality and purity of the nanoparticles obtained SEM/EDX, XRD
Recovery yield	Determination of extracted/ recovered metals compared to the residual metals in the biomass

10.3 Activities and KPIs at higher TRL

Depending on results from TRL 3, activities at higher TRL will be conducted using the best-performing species in real contaminated water samples from a GREENER site in combination or not with other GREENER technologies (e.g. in a sequential process with BES or in a BES system).

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11. Bioprecipitation of Metals in Microbial Electrochemical Cells (USUR)

11.1 Technology Overview and Aims

In Microbial Electrochemical Technologies (METs) the chemical energy stored in biodegradable compounds is converted into electricity, and/or electrical energy is used as the energy source for microbial reactions.

Groundwaters and industrial waters contaminated with heavy metals present a significant environmental risk due to the toxicity of the heavy metals; the toxicity and bioavailability of heavy metals varies depending on their oxidation state [39].

Certain micro-organisms can utilize metal ions as terminal electron acceptors, reducing the metals either to elemental form or to a less-toxic oxidation state. In METs, the cathode electrode can serve as the electron donor for metal-reducing micro-organisms either directly or via a mediator [40]. In addition, metals can be removed via precipitation due to the formation of biologically generated reaction products (e.g. sulfide) or a change in solution conditions (e.g. pH).

METs with a cathodic microbial catalyst have been used to remove Cr(VI) [41], U(VI) [42], V(V) [43], Cd(II) [44], Co(II) [45] and Zn(II) [46].

Table 51: *Technology Overview*

GREENER PARTNER	Univ. of Surrey (USUR)
Type of Technology	Bioremediation with bioelectrochemical systems
Process	*Metal removal via microbial reduction or precipitation in double chamber microbial electrochemical cells*
Target compounds	Metals (Cr6+, Co3+, Cu2+, Ni2+, Zn2+)
Test Matrix (contaminants)	Water (industrial wastewater streams containing metals and contaminated groundwater)
Current TRL / Goal TRL	3/4

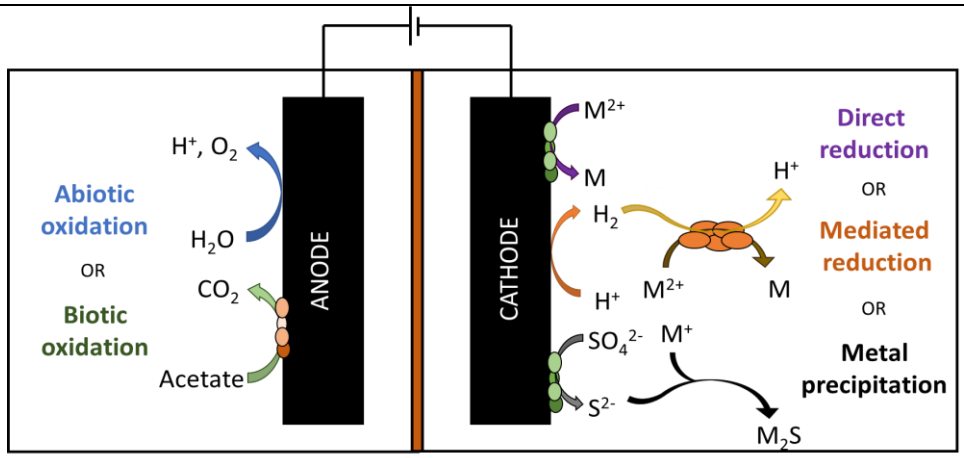


Figure 11: Overview of metal transformations in a microbial electrochemical cell: i) reduction on cathode, ii) direct or mediated reduction and iii) metal

Goals

The main goal is to enrich robust and efficient bioelectroactive microbial consortia and develop optimum operational conditions for microbial electrochemical cells for effective metal removal via precipitation from contaminated waters (wastewater and groundwater).

The specific aims are:

- To enrich efficient microbial cultures for microbial electrochemical reduction of metals.
- To reduce the toxicity of contaminated groundwaters resulting from the presence of heavy metals in batch laboratory studies (TRL 3).
- To optimize the operational conditions (pH, applied voltage, metal load)

11.2 Activities and KPIs at lower TRL

Target compounds

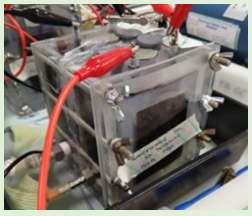
For metal removal via precipitation using MECs the following metal species and concentration ranges will be considered:

- for aerobic conditions: Cr^{6+}, Co^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+} (1 – 6.000 mg/L)

Materials

Incubations with different metal species and electroactive microbial consortia in MECs will be performed using the following materials (**Table 52**).

Table 52: *Materials for bioelectrochemical metal precipitation*

Component	Description
microbial consortia	enriched consortia from GREENER sites and other locations: GREENER sites 3 and 9 (cathode side) and enriched consortium from anaerobic digestion sludge (Burgess Hill, UK) (anode side)
reactor	plexiglas chambers, tightening rods, connectors, tubes, electrolyte tanks (up to 1L pyrex bottles), peristaltic pumps 
electrodes	carbon brush and carbon felt
separators	cation and anion exchange membranes

Experimental design and monitoring

Various experiments will be performed under different anodic and cathodic operating conditions (**Table 53**).

Table 53: *Summary of treatment for metal removal using MECs*

Treatment	Description
Treatment A1 (anodic)	abiotic (water oxidation)
Treatment A2 (anodic)	biotic (acetate oxidation).
Treatment C1 (cathodic)	direct bioelectrochemical reduction
Treatment C2 (cathodic)	mediated bioelectrochemical reduction
Treatment C3 (cathodic)	precipitation with reaction products

The following parameters will be monitored during the MECs studies (**Table 54**).

Table 54: *Monitoring parameters at low TRL*

Parameter	Instrumental equipment
general culture parameters	pH, EC and redox potential using specific probes (specific sensors)
Dissolved metal concentrations	spectrophotometric methods, Kits Hach Lange (LCK313, LCK360, LCK337 and LCK329)
Total metals concentration	ICP-MS (Inductively-Coupled Plasma)
Electrochemical control and data recording	Arbin battery tester, PalmSens potentiostat, power sources

Monitoring of the MECs will be performed either in hourly or daily time intervals depending on the initial metal concentrations. Electrochemical monitoring will include voltage, linear sweep voltammetry, cyclic voltammetry and electrochemical impedance spectroscopy measurements.

For water quality analysis the following volumes will be sampled:

- 5 mL of aqueous phase for determination of pH, metal concentrations and ions at all sampling times
- 5 mL of aqueous phase for determination of total metals with ICP-MS

General and specific KPIs

For the metal bioprecipitation experiments using MECs the following general and specific KPIs are defined (**Table 55:** General and specific KPIs for metal bioprecipitation).

Table 55: *General and specific KPIs for metal bioprecipitation*

General KPI	Definition
Contaminant concentration:	Aiming to reach concentrations below the limits set in European legislation
Contaminant mass reduction:	Higher than 95%
Decontamination time:	Hours or days depending on the initial concentration
Materials consumed	Amount of electron donors and other chemicals (e.g. for pH adjustment) added
Specific KPI	**Definition**
External voltage requirement	Amount of external electrical voltage required to run the oxidation and reduction reactions
Current output	Generated electrical current
Coulombic efficiency	Conversion rate between electrical and chemical energy
Size and homogeneity of nanoparticles	Recovery of metals in elemental form, nanoparticles, precipitates (e.g. metal sulphides or metal hydroxides) or remaining as ions in solution
Form of the end product	Quality and purity of the nanoparticles obtained SEM/EDX, XRD
Reduction in toxicity	The reduction in toxicity of the water

11.3 Activities and KPIs at higher TRL

Depending on results from the TRL 3, activities at TRL 4 will be conducted by increasing treatment volumes and reducing treatment times (e.g. operation of multiple MECs in parallel).

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12. Bioprecipitation of Chromium and Decolorization of Wastewater in Microbial Electrochemical Cells (USUR)

12.1 Technology Overview and Aims

Azo dyes are often incompletely degraded by conventional wastewater treatment processes, which causes the risk of release of dye-containing water to the environment. Dye-contaminated water can not only compromise the aesthetic quality of water bodies, but also increase the biochemical and chemical oxygen demand as well as impair photosynthesis and inhibit plant growth. Moreover, dye contamination may be associated to toxicity, mutagenicity and carcinogenicity [47].

In Microbial Electrochemical Cells (MECs) an applied electrical voltage is used as the energy source for microbial reduction of target compounds at the biocathode. Microbial communities can be enriched in MECs containing azo-dye and chromium contaminated wastewater, thereby selecting for species which can utilize the cathode as an electron donor, whilst simultaneously utilizing the target compounds as electron acceptors. MECs with a cathodic bacterial community have been demonstrated to decolorize several different azo dyes [48].

Table 56: *Technology Overview*

GREENER PARTNER	Univ. of Surrey (USUR)
Type of Technology	Bioremediation with bioelectrochemical systems
Process	*Azo -dye decolorisation / degradation in microbial electrochemical cell*
Target compounds	Azo dye 'DM Black', Cr6+ (Propietary Dye from TAUW client)
Test Matrix (contaminants)	Water (industrial wastewater streams containing metals and contaminated groundwater)
Current TRL / Goal TRL	3/4

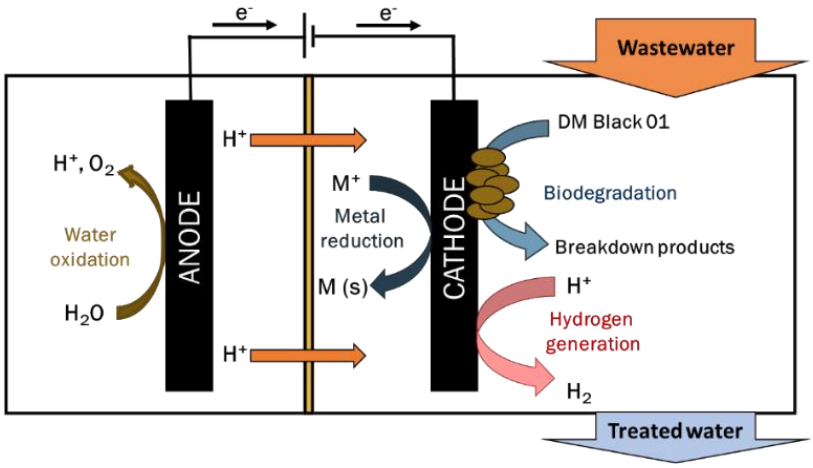


Figure 12: Overview of bioelectrochemical mechanisms for dye decolorization

Goals

The ultimate goal is to develop a bioelectrochemical treatment approach for a mixed industrial wastewater containing elevated concentrations of azodyes and chromium (III).

The specific aims are:

- to enrich microbial cultures for efficient microbial electrochemical reduction of DM Black azo-dye and chromium
- to reduce the toxicity of contaminated wastewater resulting from the presence of DM black and chromium in batch laboratory studies (TRL 3)
- to optimize the operational conditions of the MEC (pH, applied voltage, cycle time)

12.2 Activities and KPIs at lower TRL

Target compounds

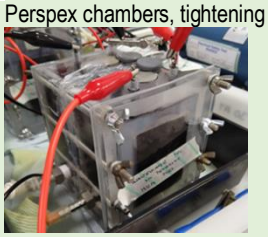
The target compounds include both inorganic and organic species under the following concentrations:

- Cr3+ (1 – 30 mg/L) and AzoDyes as COD-Chemical Oxygen Demand (2.000 mg/L)

Materials

Treatment tests of "DM-Black" wastewater with electroactive microbial consortia in MECs will be performed using the following materials (**Table 57**).

Table 57: *Materials for bioelectrochemical metal precipitation*

Component	Description
microbial consortia	enriched consortia from GREENER sites 3 and 9
reactor	Perspex chambers, tightening rods, connectors, tubes, 
electrodes	carbon brush
separators	cation and anion exchange membranes, nafion membranes

Experimental design and monitoring

Various experiments will be performed under different anodic and cathodic operating conditions (**Table 58**).

Table 58: *Summary of treatment for metal removal using MECs*

Treatment	Description
Treatment A1 (anodic)	abiotic (water oxidation)
Treatment A2 (anodic)	biotic (acetate oxidation)
Treatment C1 (cathodic)	abiotic
Treatment C2 (cathodic)	biotic (sediment inocula)
Treatment C3 (cathodic)	biotic (synthetic community)

The following parameters will be monitored during the MECs studies (**Table 59**).

Table 59: *Monitoring parameters at low TRL*

Parameter	Instrumental equipment
general culture parameters	pH, EC and redox potential using specific probes (specific sensors)
Dissolved metal concentrations	Spectrophotometric methods, Kits Hach Lange (LCK313, LCK360, LCK337 and LCK329)
Total metals concentration	MP-AES (Microwave-Plasma Atomic Emission Spectrometry)
Electrochemical control and data recording	Arbin battery tester, PalmSens potentiostat, power sources

Monitoring of the MECs will be performed initially at 24 hrs, later depending on decolorization rate. Electrochemical monitoring will include voltage, linear sweep voltammetry, cyclic voltammetry and electrochemical impedance spectroscopy measurements.

For water quality analysis the following volumes will be sampled:

- 5 mL of aqueous phase for determination of pH, metal concentrations and ions at all sampling times
- 5 mL of aqueous phase for determination of total metals with ICP-MS

General and specific KPIs

For the phytoremediation trials in the greenhouse the following general and specific KPIs are defined (**Table 60**).

Table 60: *General and specific KPIs for Cr bioprecipitation and dye decolorization in MEC*

General KPI	Definition
Contaminant concentration:	aiming to reach Cr concentrations below the limits set in European legislation, and aiming for 90% decolorization
Contaminant mass reduction:	higher than 90%
Decontamination time:	weeks
Materials consumed	amount of electron donors and other chemicals (e.g. for pH adjustment) added
Specific KPI	**Definition**
External voltage requirement	amount of external electrical voltage required to run the oxidation and reduction reactions
Current output	generated electrical current
Coulombic efficiency	conversion rate between electrical and chemical energy
Form of the end product	quality and purity of the nanoparticles obtained SEM/EDX, XRD
Reduction in toxicity	the reduction in toxicity of the water

12.3 Activities and KPIs at higher TRL

Depending on results from the lower TRL activities at higher TRL (e.g. using larger wastewater volumes and multiple MECs in parallel) will be conducted in combination or not with other GREENER technologies (e.g. in combination with phycoremediation bioreactor system from MENDELU).

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13. Bioelectrochemical Degradation of Total Petroleum Hydrocarbons - TPHs (LEITAT)

13.1 Technology Overview and Aims

Petroleum hydrocarbons are complex pollutants, composed by a mixture of aliphatic and aromatic hydrocarbons. Their bioremediation requires multiple microorganisms, each one degrading specific hydrocarbons (i.e. microbial consortia). These microorganisms can grow on petroleum hydrocarbons as sole carbon and energy source, but they need oxygen [49] . This is usually the limiting factor in contaminated groundwater, characterized by poor oxic/anoxic conditions. For bioremediation purposes, the use of indigenous bacterial consortia (enriched or stimulated from the polluted site) is advantageous compared to bioaugmentation, as the bacteria are already adapted to the physico-chemical characteristics of the water/soil matrix.

Generally O_2 acts as terminal electron acceptor (TEA) for hydrocarbon-degrading bacteria. If the TEA can be replaced by a solid acceptor (i.e. electrode) for bacterial metabolism, this means that the consortium has electroactive properties. The use of a solid electron acceptor (anode in bioelectrochemical systems) can be an alternative to the continuous injection of air/oxygen in groundwater [50]. On the other hand, the electrons collected at the anode must be consumed by a complementary cathodic reduction, for the bioremediation process to continue. At the cathode, the electron acceptor can still be oxygen or other contaminant compounds present in groundwater (e.g. metal ions, usually associated with hydrocarbons contamination [50]. Based on the available electron acceptor, the global process can be energy-positive (electricity can be recovered as side-product) or energy-negative (an external voltage must be applied to the BES system to sustain the process). BES geometry will be different in case of gas-phase TEA (e.g. air-cathode snorkel) or liquid-phase TEA (e.g. metals reduction).

For the purpose of the GREENER project the BES approach is considered for on-site treatment of groundwater, that is, contaminated groundwater is pumped to the surface and directed into a Microbial Fuel Cell reactor for treatment. In-Situ applications by introducing the BES system into the groundwater monitoring wells are not foreseen yet.

Table 61: *Technology Overview*

GREENER PARTNER	LEITAT
Type of Technology	Bioremediation with bioelectrochemical systems
Process	*On-Site treatment of Petroleum-derived hydrocarbons*
Target compounds	Total Petroleum Hydrocarbons (TPHs), BTEX, Polycyclic Aromatic Hydrocarbons (PAHs)
Test Matrix (contaminants)	Contaminated groundwater from GREENER site 7
Current TRL / Goal TRL	3/ 4-5

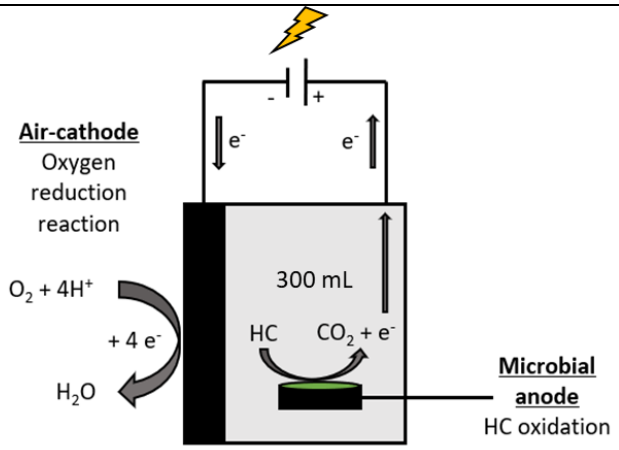


Figure 13: Overview of bioelectrochemical mechanisms for hydrocarbon degradation

Goals

The ultimate goal is to develop a bioelectrochemical treatment approach for groundwater contaminated with various organic contaminants on-site.

The specific aims are:

- determining if proprietary TPH-degrading microbial consortia (from ITC and UAM) has electroactive properties, i.e. can be used for TPH/BTEX/PAH decontamination using BES
- isolating electroactive bacteria from contaminated sites samples, and use them for BES inoculation and laboratory operation
- testing carbon fiber brush as anode, within an air-cathode lab BES setup (membrane and cathode material will be fixed based on experience)
- optimizing BES operational conditions (MFC/MEC mode, voltage, batch treatment time, need of nutrients), in view of technology scaling-up
- developing a scaled-up, air-cathode BES reactor geometry for in-situ application to hydrocarbons-contaminated groundwater.*

*Operation of a pilot reactor at a contaminated site (#7, chemical industry in Tarragona) following positive evaluation of experiments at lower TRL.

13.2 Activities and KPIs at lower TRL

Target compounds

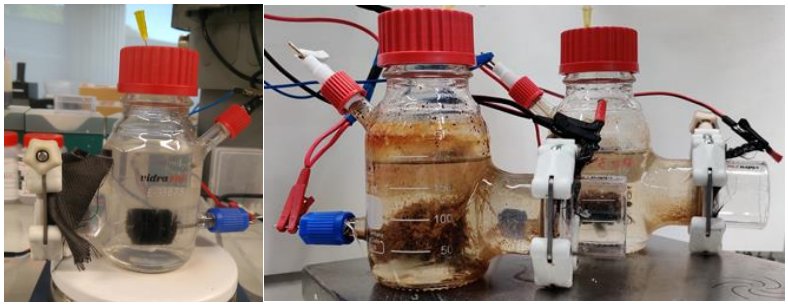
The target compounds include TPHs and BTEX at the following concentrations:

- TPHs up to 11 mg/L; BTEX up to 5 mg/L

Materials

Treatment tests with electroactive microbial consortia in MFCs will be performed using the following materials (**Table 62:** Materials for bioelectrochemical).

Table 62: *Materials for bioelectrochemical hydrocarbon degradation at TRL 3*

Component	Description
microbial consortia	TPH-degrading consortia from partners (ITC, UAM), able to grow on different substrates (crude oil, engine oil, diesel, etc.) enriched electroactive microbial consortia from GREENER site 7
growth medium	minimal medium spiked with 800 ppm kerosene (1 mL/L), as sole carbon and energy source for microorganisms
reactor	single-chamber, glass reactors (300 mL volume), to be used as air-cathode BES 
electrodes	anode of carbon fiber brush (30 cm^2), cathode of unidirectional carbon fibers with MnO_2 catalyst (12.6 cm^2), separator of Tyvek (12.6 cm2)
incubation system	thermostatic bath (28 ºC) + stirrer (140 rpm) to help contaminant diffusion towards the anode (* kerosene is not soluble in water)
Electrochemical monitoring	VMP3 potentiostat to perform electrochemical characterization techniques (anode inoculation, chronoamperometry, cyclic voltammetry)

Experimental design and monitoring

For testing the electroactivity of TPH-degrading consortia the following steps and methodology will be used:

1) routine growth of the consortia in kerosene-spiked minimal medium (see figure), at 28 ºC and 140 rpm, up to OD600 of 0.5-0.6;
2) selection of the 3 consortia achieving higher OD600 values;
3) centrifugation (3000 rpm, 15 min), pellet collection and inoculation of SC-BES reactors (2 BES x consortium, see figure);
4a) SC-BES refilling with minimal medium spiked with kerosene + step-batch feeding with concentrated medium when OD600 reaches max. value (stationary phase)
- OCV cycle (1 month) with regular CV analysis to evaluate electroactivity of consortia (from 0 to 1 V vs Ag/AgCl, 1 mV/s, 3 cycles);
- in case that electroactivity is detected, poising the anode potential at optimal value to sustain EAB growth and TPH degradation;
- in case consortia are not electroactive, reinoculate the anode with lab. MFC effluent and evaluate potential syntrophy between EABs and TPH-degrading consortia;
4b) SC-BES refilling with sodium acetate medium (30 mM) + step-batch feeding (replacing 50% reactor volume) at COD < 500 ppm
- standard anode inoculation protocol developed in LEI for acetate-fed biofilm (3 weeks duration);
- in case that electroactivity is detected, replace acetate with kerosene, poise anode potential at optimal value and see if TPH electroactive degradation takes place;
- in case consortia are not electroactive, reinoculate the anode with lab. MFC effluent and evaluate potential syntrophy between EABs and TPH-degrading consortia;
5) regular monitoring for 1 month, to validate results.

For isolating electroactive bacteria from selected contaminated locations from site 7 (Pz-17, Pz-39 and Pz-19b from chemical site in Tarragona) the following steps and methodology will be used:
- SC-BES refilling with contaminated water + step-batch feeding when TPH < x (x = minimal concentration for EAB activity, to be determined)
- OCV cycle (1 month) with regular CV analysis to evaluate electroactivity of consortia (from 0 to 1 V vs Ag/AgCl, 1 mV/s, 3 cycles);
- in case that electroactivity is detected, poising the anode potential at optimal value to sustain EAB growth and TPH degradation;

The following parameters will be monitored during the MFCs studies (**Table 63**).

Table 63: *Monitoring parameters at TRL 3*

Parameter	Instrumental equipment
general culture parameters	pH, EC and redox potential using specific probes
Hydrocarbons (TPH, BTEX, PAH)	Solid phase extraction + elution + concentration + GC (FID/MS detector) - analytical method under development
Volatile organic acids	GC-FID
Microbial consortia growth	OD600, electrochemical (indirect) techniques, SEM analysis
Functional and structural genes	bacterial DNA extraction and characterization, at the end of experiment

Monitoring of the MFCs will be performed on a weekly basis.

For water quality analysis the following volumes will be sampled:
- 1 mL sample for OD600 determination
- 1 mL for volatile organic acids determination full BES reactor volume for pH, conductivity, TPH/BTEX/PAH determination

General and specific KPIs

For the MFC hydrocarbon degradation tests the following general and specific KPIs are defined (**Table 64**).

Table 64: *General and specific KPIs for bioelectrochemical hydrocarbon removal at TRL 3*

General KPI	Definition
Contaminant concentration:	aiming to reach hydrocarbon concentrations below the limits set in European/national legislation
Contaminant mass reduction:	higher than 90%
Decontamination time:	Week(s)
Materials consumed	amount of electron donors and other chemicals (e.g. for pH adjustment) added
Specific KPI	**Definition**
External voltage requirement	amount of external electrical voltage required to run the oxidation and reduction reactions
Current output	generated electrical current
Coulombic efficiency	conversion rate between electrical and chemical energy
Form of the end product	quality and purity of the nanoparticles obtained SEM/EDX, XRD
Reduction in toxicity	the reduction in toxicity of the water

13.3 Activities and KPIs at higher TRL

Depending on results from the lower TRL, activities at higher TRL (e.g. using larger wastewater volumes and multiple MECs in parallel) may be conducted in at pilot scale at site 7.

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14. Bioelectrochemical Removal of Metals in Microbial Electrochemical Cells (LEITAT)

14.1 Technology Overview and Aims

Microbial Electrochemical Technologies (METs) provide a technological platform that employs microorganisms to transform the chemical energy stored in biodegradable compounds into electricity and/or chemicals. The most common configuration of a MET consists of a two-chamber architecture including an anode and a cathode. In many cases a two-chamber configuration also incorporates a physical separator between the anode and the cathode which permits an optimal transport of the electrons between the two chambers throw an external circuit [52].

The use of such systems for wastewater reclamation processes as well as nutrient recovery has been extensively explored. The main advantage compared to other treatments is the lower energy density compared to conventional techniques. Recently, MET platforms have been reported as a suitable method for the recovery of several metals [53].

All METs, which have been reported for assisted metal recovery to date are associated to metal reduction on the cathodic electrode. Four different mechanisms for the recovery of metals have been reported. The first process involves direct reduction of the metals on an abiotic cathode for all of those metals whose redox potential is higher than the anode one such as Au, V, Cr, Ag, Cu, Fe and Hg among others. In this system, the reduction is thermodynamically favorable and metals are directly used as electron acceptors [54]. The second process is also based on a biotic anode and an abiotic cathode connected to an external power source. In this configuration, metals with lower redox potential than the anodic can be reduced [54]. Examples of these metals are Ni, Pb, Cd and Zn, among others [55]. The third mechanism is associated with microbial reduction of metal oxides on a cathodic electrode [56]. Finally, the fourth mechanism combines methods two and three with a poised potential by the use of a potensiostat and a reference electrode [57].

Table 65: *Technology Overview*

GREENER PARTNER	LEITAT
Type of Technology	Bioremediation with bioelectrochemical systems
Process	*Metal removal by electrodeposition or precipitation in double chamber microbial electrochemical cell*
Target compounds	Cu^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}
Test Matrix (contaminants)	Contaminated groundwater from GREENER site 8
Current TRL / Goal TRL	4/5

Figure 14: Two-chamber bioelectrochemical mechanism for metal removal and recovery

Goals

The ultimate goal is to develop a bioelectrochemical treatment approach for groundwater contaminated with various metal cationic species.

The specific aims are:

- isolating electroactive bacteria from GREENER site contaminated samples that can be used for BES inoculation and laboratory operation
- optimizing BES operational conditions (MFC/MEC mode, voltage, batch treatment time, need of nutrients) in view of technology scaling-up

14.2 Activities and KPIs at lower TRL

Target compounds

The target compounds include Cu^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+} at concentrations between 1 – 6.000 mg/L.

Materials

Treatment tests with electroactive microbial consortia in MECs will be performed using the following materials (**Table 66**).

Table 66: *Materials for bioelectrochemical metal precipitation*

Component	Description
microbial consortia	inoculation with microbial electroactive consortium from an in-house operating MFC or biostimulation of consortia present in GREENER samples
growth medium	reagents for metals solutions production and also for mineral medium based on sodium acetate as carbon source.
reactors	6 bichameral MECs with recirculation and feeding system: plexiglas chambers, tightening rods, connectors, tubes, buffer tanks (up to 1L pyrex bottles), peristaltic pumps (dosing flowrate range), different electrode materials, separators, hydrophobic membrane, ion exchange membranes from more than one supplier.
electrodes	different electrode materials such as stainles steel, unidirectional carbon fibers & carbon felt
Electrochemical monitoring	VMP3 potentiostat (BioLogic) to perform electrochemical characterization techniques (anode inoculation, chronoamperometry, cyclic voltammetry)

Experimental design and monitoring

For testing the electroactivity of metal precipitating microbial consortia the following tests and methodology will be used:

1) Anode inoculation:

- inoculation with biomass from an operating MFC applying an inoculation protocol developed in Leitat

2) Cathode materials:

- 4-5 different materials will be electrochemically studied in separate experiments to evaluate the best-performing material for technology requirements, that is, high oxygen reduction rate and low metals reduction overpotentials.

3) Abiotic electro-reduction study:

- electroreduction will be studied through cyclic voltammetries at different electric conductivity values K (mS/cm) in order to see the influence on the reduction potential as the K range of real groundwater is generally very low. In addition, different metal concentrations and turbulence conditions will be studied.

4) Removal pathway study:

- Different operational conditions will be tested in order to study bioelectrochemical removal pathways. Target metals will be studied individually in synthetic groundwater containing concentrations that mimic real polluted groundwater, that is, initial concentrations from 1 mg/L to some g/L, depending on the target metal.
The best performing treatment/combination will be selected for the TRL 5 field demonstration in combination with a phytoremediation approach.

The following parameters will be monitored during the MECs studies (**Table 67**).

Table 67: *Monitoring parameters at TRL 4*

Parameter	Instrumental equipment
general water parameters	pH, EC and redox potential using specific probes
COD and dissolved metals	Kits Hach lange (LCK360, LCK337, LCK329 & LCK308 for metals and also the traces ones; and LCK 514 and LCK314 for COD)
total metal concentration	ICP-MS (Inductively Coupled Plasma – Mass Spectrometry)
microbial consortia growth	OD600, electrochemical (indirect) techniques, SEM analysis
electrochemical control and data recording	VMP3 potentiostat (BioLogic®)

Monitoring of the MECs will be performed on a hourly basis at 0, 0.17, 0.5, 1, 3, 24h (or similar retention time in case of increasing treated volume).

For water quality analysis the following volumes will be sampled:

- 5 mL of aqueous phase for determination of Metals and COD at all sampling times.
- 5 mL of aqueous phase for determination of total metals with ICPMS (sampling times 0, 3, 24h)
- pH and electrical conductivity will be measured directly in the buffer tanks

General and specific KPIs

For the MEC metal precipitation tests the following general and specific KPIs are defined (**Table 68**).

Table 68: *General and specific KPIs for metal precipitation in MEC*

General KPI	Definition
Contaminant concentration:	aiming to reach concentrations below the limits set in European legislation
Contaminant mass reduction:	approx. 95% metal mass removal
Decontamination time:	hours or few days depending on metal reducing overpotentials and the reaction kinetics
Materials consumed	amount of electron donors and other chemicals, electricity
Specific KPI	**Definition**
External voltage requirement	amount of external electrical voltage required to run the oxidation and reduction reactions
Current output	generated electrical current
Coulombic efficiency	conversion rate between electrical and chemical energy
Form of the end product	quality and purity of the nanoparticles obtained SEM/EDX, XRD
Reduction in toxicity	the reduction in toxicity of the water (via toxicological tests)

14.3 Activities and KPIs at higher TRL

Depending on results from the lower TRL activities at higher TRL will be conducted in combination with phytoremediation technology developed by UBU in an artificial wetland system.

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15. Biological In-Situ Metal Precipitation (TAUW)

15.1 Technology Overview and Aims

Remediation of groundwater impacted with dissolved metals, metalloids, and radionuclides is perhaps one of the biggest challenges in environmental cleanup today [58]. Unlike organic contaminants, metals cannot be destroyed or their recovery easily enhanced. Instead, in-situ approaches for the treatment of metals in groundwater generally involve direct precipitation, coprecipitation, or sorption, with the goal of permanently sequestering and immobilizing the metals in the aquifer soil matrix. The success of precipitation-based in-situ treatment approaches is dependent upon the following: kinetics, equilibrium solubility and durability/permanence.

Biostimulation (electron donor addition) and bioaugmentation (addition of inoculum) have gained significant acceptance as viable approaches for treatment of both organic and inorganic contaminants in the subsurface [59][60]. Some of the major advantages of in-situ treatment include: contaminated material does not need to be transferred elsewhere for treatment, flexible implementation (also if built area), no waste generation, stimulation of natural attenuation and remediation processes.

Long-lasting metal precipitation in aquifers can be achieved by formation of metal sulfides. In natural groundwaters sulfide anions are generally formed by the activity of sulfate-reducing bacteria (SRB). These are anerobic microorganisms that use sulfate as an electron acceptor [61]. In order to stimulate the activity of SRB at contaminated aquifers for in-situ metal treatment the following conditions need to be met: i) strictly anoxic redox conditions, ii) tolerable pH for microorganisms, iii) sulfate must be present in sufficient concentrations and iv) electron donor has to be available in excess.

Despite enormous potential for biological in-situ treatment of groundwater contaminated with metal and metalloids there is lack of field demonstrations, where this approach has been applied successfully at large scale in real environmental settings. Since metals, metalloids and radionuclides are widespread groundwater contaminants resulting in large plumes there is urgent need to development cost-efficient and robust in-situ biological approaches for metal immobilization that represent an alternative to current long-term and costly traditional remediation technologies (e.g. Pump & Treat or soil excavation in the saturated zone).

Table 69: *Technology Overview*

GREENER PARTNER	TAUW
Type of Technology	Biostimulation
Process	*In-Situ Metal Precipitation via sulfate reduction*
Target compounds	As, Ni, Zn
Test Matrix (contaminants)	contaminated groundwater from GREENER site 8
Current TRL / Goal TRL	4/5

$$SO_4^{=} + \text{organic matter} \xrightarrow[\text{bacteria}]{\text{anaerobic}} S^{=} + H_2O + CO_2$$

$$S^{=} + 2H^{+} \longrightarrow H_2S$$

Figure 15: Overview of biochemical and biogeochemical reactions involved in the in-situ metal precipitation via sulfate reduction

The main goal is to establish a robust biostimulation approach for in-situ metal precipitation that can be used for full scale remediation at the GREENER site 8

The specific aims include:

- Perform batch and column biostimulation studies (TRL 4) using aquifer materials (contaminated sediment and groundwater) in combination with various electron donors. The best performing combination will be selected for the field demonstration (TRL 5).
- Perform a field demonstration to show technology success after validation at TRL4. The field demonstration will be conducted at the GREENER site 8 (smelter 1 in Belgium) characterized by low pH (4,5), high sulfate concentrations (500-1.000 mg/L) as well as arsenic, nickel and zinc in the mg/L range.

15.2 Activities and KPIs at lower TRL

Target compounds

The target compounds include As, Ni and Zn at concentrations between 1 – 60 mg/L.

Materials

Treatment tests for in-situ metal precipitation will be performed using the following materials (**Table 66**).

Table 70: *Materials for in-situ metal precipitation at lower TRL*

Component	Description
SRB sulfate-reducing bacteria	indigenous microbial populations that will be stimulated
sediment and groundwater from site 8	site materials (soil and groundwater) will be sampled and used as incubation matrix
batch reactors	250 mL glass bottles for performing batch incubations. 150 ml of slurry with a sediment:water ratio of approx. 1:10. Typically, the remaining headspace is flushed with N_2 gas.
soil column	plexiglass column of 5 cm diameter and 65 cm in length packed filled site sediment (approx. 35 cm segment) and flushed with metal-contaminated groundwater from site 8
electron donor	sodium lactate or molasse (residual fluid product from the potato industry, rich in starch)

Experimental design and monitoring

Batch study: The following 3 treatments will be tested. The best performing biostimulation treatment will be selected for the TRL 5 field demonstration.

- Treatment 1: control without electron donor
- Treatment 2: donor A (rapid fermentation) sodium lactate without inoculum (biostimulation) + pH correction
- Treatment 3: donor B (rapid fermentation) molasse without inoculum (biostimulation) + pH correction

Incubations will be conducted in duplicate batch bottles using 0,25 L vials (50% headspace volume, N_2 gas) at 20°C in darkness in the glovebox. The expected duration is 4-8 weeks.

Column study: The best-performing electron donor from the batch study will be tested in the column study. The study consists, thus, of one test column and three experimental stages:

- Stage 1: recirculation of electron donor and contaminated groundwater with metals to achieve sulfide formation and metal precipitation in sediment
- Stage 2: flushing of column with electron donor
- Stage 3: flushing of column with uncontaminated water (stability of sulfides formed)

The column study is expected to take 2-3 months.

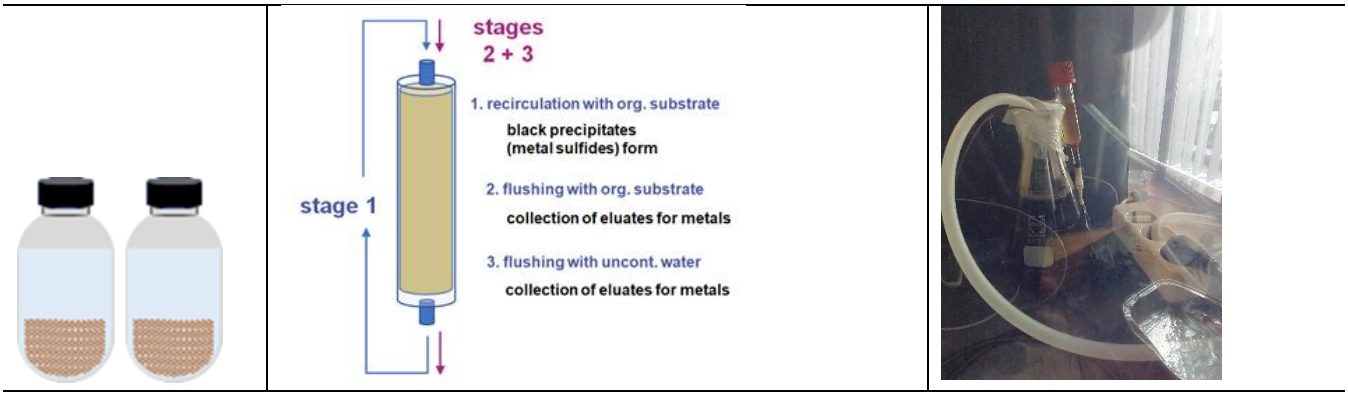

Figure 16: Batch systems with sediment and groundwater; Column system and anaerobic glovebox

The following parameters will be monitored during the batch and column studies (**Table 74**).

Table 71: *Monitoring parameters at TRL 4*

Parameter	Instrumental equipment
general water parameters	pH, EC and redox potential using specific probes
metals in solution	As, Ni and Zn via ICP-OES (Inductively Coupled Plasma - Optical Emmission Spectrometer)
sulfate	via ion chromatography

Monitoring of the batch and column study will be performed according to the following scheme:

- Sampling times for batch study at 0, 2 and 6 weeks. pH as needed.
- Sampling times for column study: general GW parameters weekly, metals and sulfate every 2-4 weeks

The sampling volumes for the parameters in **Table 71:** Monitoring parameters at TRL include:

- approx. 5 mL - 10 mL for metals, sulfate and general GW parameters

General and specific KPIs

For the in-situ metal precipitation tests the following general and specific KPIs are defined (**Table 72**).

Table 72: *General and specific KPIs for in-situ metal precipitation at TRL 4*

General KPI	Definition
contaminant concentration:	change in metal concentrations over time
contaminant mass reduction:	percentage of metal removed (expected 99 %)
decontamination time:	time needed to achieve the corresponding percentage of metal removal (weeks)
materials consumed	amount of electron donor(s) added and other chemicals (e.g. for pH adjustment) to enable biotransformation
Specific KPI	**Definition**
sulfate reduction	change/decrease in sulfate concentrations

15.3 Activities and KPIs at higher TRL

Target compounds

The target compounds include As, Ni and Zn at concentrations between 1 – 60 mg/L.

Materials

Materials for the pilot test include (**Table 73**).

Table 73: *Materials for in-situ metal precipitation at TRL 5*

Component	Description
electron donor	sodium lactate or molasse (residual fluid product from the potato industry, rich in starch)
pH corrector	pH-adjustments will be performed by addition of NaOH according to results from the lower TRL experiments
Injection and monitoring wells	1"-injection wells to inject reagents and 1-2" additional monitoring wells upstream and downstream of well 8062 for gaining groundwater samples

Experimental design and monitoring

Site description: The field demonstration will be conducted at the GREENER site 8 (smelter 1) in the vicinity of Antwerp (Belgium). An area of approx. 20m^2 around the monitoring well 8062 with relatively moderate metal concentrations (in relation to the contamination source) in the range 1 - 60 mg/L and downstream of the source zone is foreseen as optimal location for the pilot test.

The experimental setup will consist of:

- injections: organic substrate and pH corrector is brought into the subsurface via Direct-Push injections at two depths (8-9 and 12-13 m b.g.l.)
- monitoring wells: for monitoring the existing well 8062 filtered at 8-9 m b.g.l. and 12-13 m b.g.l. will be used. Upstream and dowstream of well 8062, approx. 2-5 m apart, additional temporary monitoring wells will be installed with filtered screens at the same depths, that is, 8-9 m and 12-13 m b.g.l.

Monitoring will be performed 2 weeks prior to electron donor injection and pH correction as well as multiple times following donor injection (2 weeks, 1, 2 and 3 months)

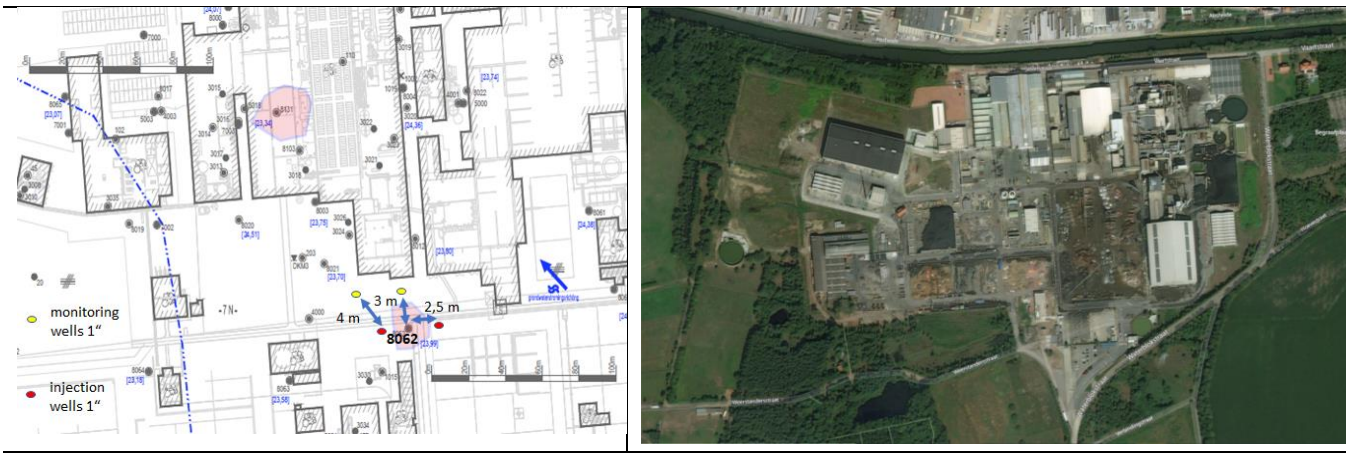

Figure 17: Selected for pilot study around well 8062. Aerial view of GREENER site 8

The following parameters will be monitored during the pilot study (**Table 74**).

Table 74: *Monitoring parameters at TRL 5*

Parameter	Instrumental equipment
general water parameters	pH, EC and redox potential using specific probes
metals in solution	As, Ni and Zn via ICP-OES (Inductively Coupled Plasma - Optical Emmission Spectrometer)
Sulfate and sulfide	via ion chromatography and colorimetric assays
volatile organic acids	via HPLC
functional and structural genes	via real-time PCR

For treatment of each source sampling will be performed every 15 days during the first two months and on a monthly basis for the rest of the pilot demonstration, unless specified otherwise. Sampling will be performed at the corresponding reinjection and extraction wells.

Amount and Parameters: sampling at extraction and defined monitoring wells for each sampling event

- approx. 5-10 L for general GW-parameters (pH, redox, electrical conductivity, temperature and dissolved oxygen) during continuous flow through multiparametric cell
- 1 L of groundwater for determination of volatile organic acids (acetate, formate and butyrate)
- 1 L of groundwater for metals (iron and manganese, metals) and 1 L of groundwater for anions (sulfate and sulfide)
- 1 L of aqueous phase for determination of functional and structural genes

General and specific KPIs

For the in-situ metal precipitation pilot trial the following general and specific KPIs are defined (**Table 75**).

Table 75: *General and specific KPIs for in-situ metal precipitation at TRL 5*

General KPI	Definition
contaminant concentration:	change in metal concentrations over time (mmol/L or mg/L)
contaminant mass reduction:	percentage of metal removed (expected 99 %)
decontamination time:	time needed to achieve the corresponding percentage of metal removal (weeks)
materials consumed	amount of electron donor(s) added and other chemicals (e.g. for pH adjustment) to enable biotransformation
efficiency	decontamination cost in EUR/m^3
Specific KPI	**Definition**
change in electron donor	change in TOC concentrations over time (mmol/L or mg/L)
change in electron acceptor	change/decrease in sulfate concentrations (mmol/L or mg/L)
fermentation of elec. donor	change/build-up of volatile organic acids resulting from fermentation of electron donor (acetate, formate and butyrate)
formation of end-products	accumulation of H_2S over time (mmol/L or mg/L)
structural genes	changes in the microbial community structure following addition and fermentation of electron donor
functional genes	assessment of genes involved in the sulfate-reduction process (drsA and apsA)

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