LIFE sedremed
ENHANCED BIOREMEDIATION OF CONTAMINATED MARINE SEDIMENTS

LIFE20 ENV/IT/000572

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START DATE OF THE PROJECT: **1 October 2021**

DURATION OF THE PROJECT: **42 months**

DELIVERABLE B1.1

Ex situ bioremediation and prototype implementation

DUE DATE OF DELIVERABLE: **March, 2023**

ACTUAL SUBMISSION DATE: **October, 2024**

BENEFICIARY LEADING THIS DELIVERABLE: **IDRABEL**

CONTRIBUTING BENEFICIARY: **EKOGRID, ISODETECT, SZN**

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Justification for late submission

The action B1, and the consequent deliverable were delayed for several reasons:

The technologies were not adapted to seawater, and this adaptation required additional time and study to complete.

1) The first preliminary sediments characterization was completed only in February 2023, and this delayed the beginning of the first trial.
2) The mesocosms were initially foreseen at SZN premises, but because of the fine tuning required, it was decided to perform this at IDRABEL labs, so that IDRABEL could better control the progress. This also caused delays because the sediments required to be shipped and IDRABEL had to recover seawater.
3) The time for the analysis was not taken into account, but it required at least 2 months minimum after the end of each batch to gather results and plan modification accordingly.
4) Also, the timing of the mesocosms run was underestimated, as the planned 4 weeks were simply not enough to observe significative changed and in the last run this was increased to 8 weeks.

Executive summary

This deliverable presents our first achievements in the implementation action B1 carried out within the LIFE SEDREMED project. It provides information on first sampling activities and the whole design sampling campaign to be carried out during the project, and the main results achieved during lab scale experiments through technology set-up till its implementation in mesocosm (ex-situ). In order to develop this deliverable, the partners considered logistical, safety, environmental and administrative aspects. After sediment collection, samples were sent to IDRABEL to run mesocosm experiments, in order to establish a preliminary protocol to be applied for both ex-situ and in-situ tests. The set-up of the best remediation and combination system was carried out by using multiple batches during mesocosm lab scales. The contaminant analysis was performed by ISODETECT and revealed weak degradative activities. This allowed adaptation of the methodology using mesocosm trials. In conclusion, the mesocosm experiments were fundamental to develop a prototype by adapting, integrating and optimizing the 2 technologies (IDRABEL, EKO) designing the most efficient bioremediation strategy.

1. Introduction

Bioremediation is considered the most cost-effective remediation technology (Tedesco et al. 2024). Bioremediation procedures, and especially in situ procedures, generally requires minimal equipment and effort since native or specifically designed microbial communities play a major role also favouring the ecosystems resilience (Yakimov et al. 2005; Genovese et al. 2014; Bagi et al. 2014). Bioremediation strategies can be divided according to the biological nature of the agent used and are generally referred to as bioaugmentation (the addition of hydrocarbons-degraders biomass strengthening the native potential) or biostimulation (the addition of nutrients or other additives in the environment to stimulate indigenous hydrocarbons-degraders) (Tyagi, da Fonseca, and de Carvalho 2011).

The two technologies of IDRABEL and EKOGRID represent advanced examples of bioaugmentation and biostimulation, respectively.

1.1. IDRABEL Remediation Process

IDRABEL SPRL[1] is a Belgian biotechnology company that develops, produces and markets biotechnological products allowing an in-situ biodegradation of all industrial and domestic organic pollution in wastewater, sludge and sediments. IDRABEL's technology is based on the unique bio- fixation method (Valdivia-Rivera et al. 2021), which allows to immobilize different microorganisms on natural mineral supports. Bio-fixation allows a longer lifespan of microorganisms, a better pollutant degradation efficiency and applications in unconfined environment like superficial water bodies and sediments. The mineral supports used are of natural origin (marine calcium carbonate and zeolites), this feature in addition to allowing higher degradation efficiency ensures that no synthetic material is used in decontamination

processes. IDRABEL has extensive experience in the application of microorganism for treating organic contamination in sediments. IDRABEL's technology has been commercially implemented (TRL9) for the degradation of organic matter in sediments, thus enabling the reduction of sediments height through a process called bio-dredging. IDRABEL has also applied its technology in sediments for the degradation of organic pollutants such as hydrocarbons and for the fixation of heavy metals applying it however in relatively closed environments such as ports, canals and ponds.

1 https://www.IDRABELbel.be/en/home-2/

1.2. EKOGRID Remediation Process

EKOGRID OY [2] is a Finnish company established 2009 and is the developer and sole owner of the IPR of the technology called **EKOGRID**™ TECHNOLOGY. This innovative electrochemical method or process utilizes the known electrokinetic reactions – electroosmosis, electrophoresis, and electromigration. Originally the method was used to control movement of water and/or its contents in porous media, such as soil. Today the main application is the use for soil and groundwater remediation. The Technology has been used successfully in almost twenty countries at all continents to treat oil-based Hydrocarbons (C5-C40), Crude oil tars (C40...>C200), Aromatic compounds (BTEX, PAH) and Chlorinated Hydrocarbons (e.g., TCE, TCP), but also many other toxic organic compounds, such as MTBE, ETBE, and Creosote. EKOGRID Technology is based on the controlled use of Electrokinetic Phenomena and Electrochemical reactions related to them (Gill et al. 2014; Lan et al. 2023). These reactions are triggered by applying short, and low (safe) output voltage (between 6 to 15V) DC pulses, which are led into the treatable soil matrix using ground

electrodes. The reactions generated in the soil matrix produce, e.g., free radicals and oxygen inside the pores of the matrix. The polarity of the output (the electrodes) is constantly reversed after each some milliseconds long DC pulse. This pulsing output is typical and unique for the Technology, and the key feature of the Innovation. The reaction products formed in the electrochemical processes play a key role in the remediation process. These processes are also referred as Electrokinetic Oxidation (Electrochemical oxidation) and Enhanced Bioremediation. The voltage pulses, typical for EKOGRID, enhance the processes by increasing the bioavailability. Meaning that all the reactants formed, hydrocarbons compounds, microbes, nutrients, water etc. are meeting each other in an effective way.
Generally, the aim is to activate natural bacteria present at the treatment zone.

In this project, we are studying if for the first time the integration of the electrokinetic systems with bioaugmentation of bio-fixed bacteria to observe a synergistic effect of the two technologies.

[2] https://www. EKOGRID.fi/

2. Objectives of this action

In this action, marine contaminated sediments selected in the action A1 were collected and after a thorough chemical characterization (**see deliverable B3.1**) used for baseline set-up, running mesocosms experiments, adapt the IDRABEL and EKOGRID technologies, monitoring the impact of the project at technical and scientific level. **The final goal of this action** was to combine the two different technologies in ex situ procedures to better define the on-site installation. The specific action aims are:

- Collect sediments from the contaminated areas, to run mesocosms in the dedicated IDRABEL premises.
- Adapt and combine the IDRABEL and EKOGRID technologies to the collected sediments in the most effective way in order to increase the remediation capability.
- Perform mesocosms tests on polluted sediments at IDRABEL and EKOGRID facilities.

Evaluate the decontamination effect of the two technologies combined.

SZN oversaw the collection of the sediments and repeated this task throughout the project to provide material for the activities. IDRABEL and EKOGRID, with the collaboration of the monitoring partner ISO e UNIVIPM, planned and executed the mesocosms. For each mesocosm a sampling strategy was defined and at the end of each run, samples we shipped to ISO that performed the chemical analysis and reported the results.

3. Preparatory activities

3.1. Sampling activities

In the preparatory action, two areas **(Figure 1)** were selected and characterized for their levels of contamination. Area H was significantly more polluted for both hydrocarbons and metals (**see Deliverable D3.1**). Moreover, contamination levels were higher in the deeper levels (>1 m of sediments' depth). For these reasons, for the mesocosms we decided to collect sediment from area H using Van Grab Samplers. The sediments corresponded to the first 50 cm of depth and were mechanically homogenized and stored in sterile plastic bags in packs of 1,5 kg on average. Before shipping, sediments were kept at 4°C.

Three sampling were performed:

- Sampling for preparatory mesocosms

Date: 14/07/2022

Sampling sites: H

About 70 kg of mixed sediments were sampled and sent to IDRABEL to start mesocosms activities.

- Sampling for mesocosms alpha

Date: 20/04/2023

Sampling sites: H

About 100 kg of mixed sediments were sampled and sent to IDRABEL to set-up 3 mesocosms runs in parallel (see description in paragraph 4).

- Sampling for mesocosms omega

Date: 14/09/2023

Sampling sites: H

About 100 kg of mixed sediments were sampled and sent to IDRABEL to set-up 3

mesocosms runs in parallel (see description in paragraph 4).

SAMPLING SITE COORDINATE:
Site H (High contamination):
40° 48,350’ N
14° 10,020’ E
Site L (lower contamination)
40° 48,306’ N
14° 10,131’ E

Figure 1: Aerial view and coordinates of the two selected area for SEDREMED activities.

3.2 Preparation of the IDRABEL product

IDRABEL products are composed of microorganisms biofixed on mineral support (>99%). In these preliminary studies IDRABEL performed different tests on supports and microorganisms in order to optimise the Bio-Vase for SEDREMED project.

3.2.1 Heavy Metal adsorption

Each mineral support tested has been shown to capture heavy metals. Some of them can accumulate up to 0,5 g of heavy metal per gram of raw material (Uddin 2017). IDRABEL tested the adsorption of 3 different heavy metals on several supports via filtration system: Ni, Pd and Cd which are common pollution elements in the environment. To determine the capabilities of the support to retain metal ions, it was employed a system that allows a metal solution to go through mineral support. This solution is placed at an upper position compared to the tube containing a mineral support sample. The scheme of the system is reported in **Figure 2.**

The quantification of the metals in the solution was performed using the DR3900

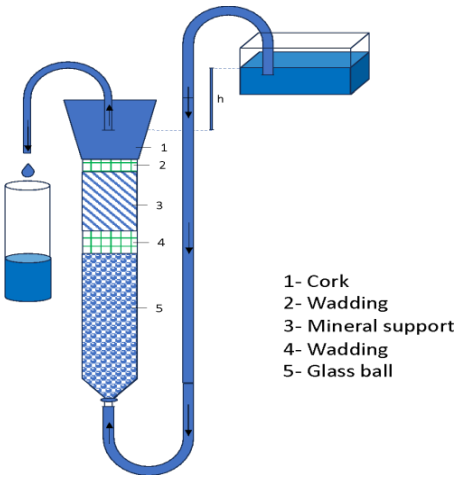


Figure 2: Scheme of the system utilized for the metal-retaining experiment.

Hach Lange Spectrophotometer (Lenntech, Nedherlands) using specific kit for each metal:

Cadium: LCK308 Cd 0.02-0.3 ppm Method: Cadion[3]

[3]https://www.lab-shop.com/environmental-testing-c95/test-kits-c104/hach-lange-lck308-cadmium-cuvette-test-0- 02-0-3-mg-l-cd-p509

Lead: LCK306 Pb 0.1-2 ppm Method: PAR [4]

[4]https://www.lab-shop.com/environmental-testing-c95/test-kits-c104/hach-lange-lck306-lead-cuvette-test-0-1-2-mg- l-pb-p531

The metal concentration is then analysed in the water before the filtration (Co) and after the filtration (C). In the graph shown in **figure 3**, it is reported the C/Co ratio of Cadmium depending on V/Vf (where V is the volume passed through the tube and Vf the volume of the tube). In this first test, two different mineral supports were analysed: "masse" and "metir".

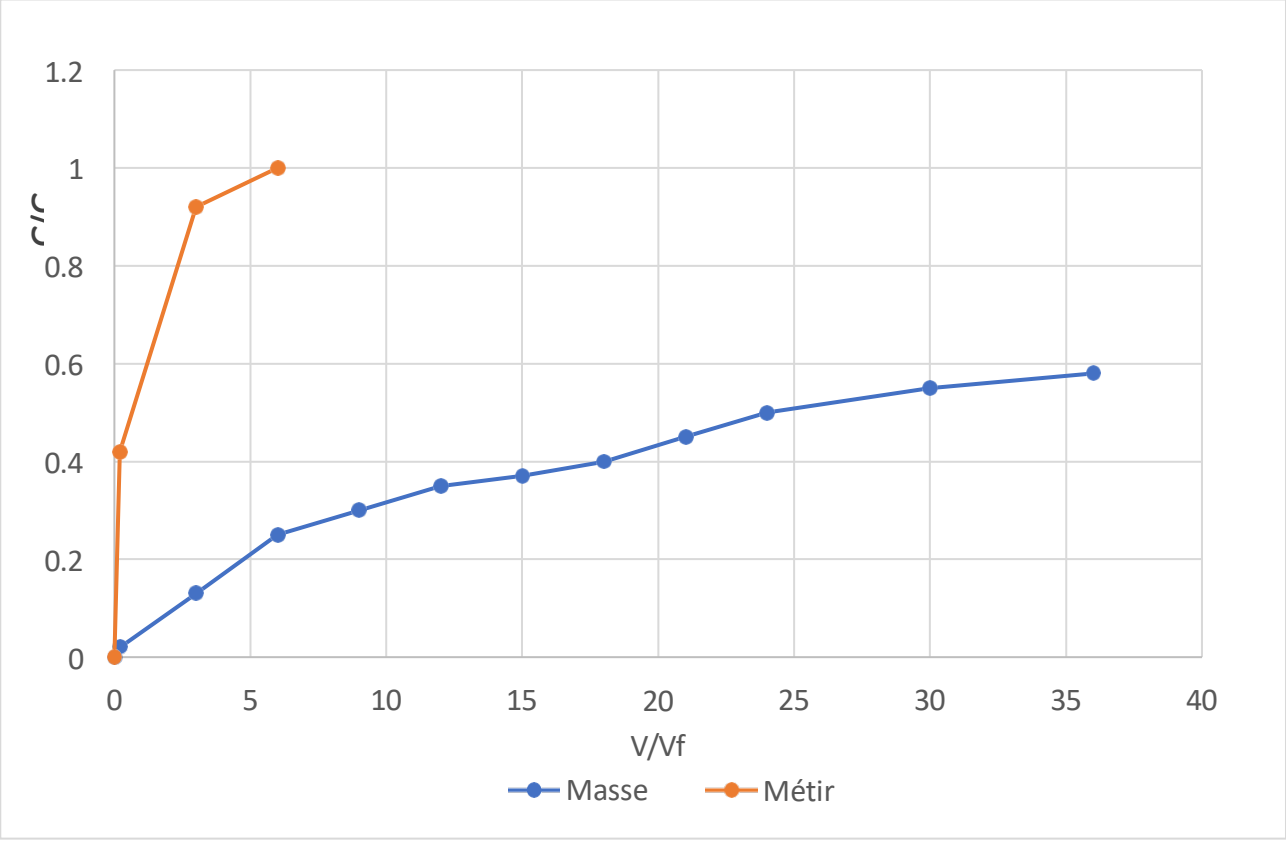


Figure 3: Evolution of the C/Co ratio for cadmium for two different supports: "Masse" and "metir".

As show in the graph, the support called "masse" composed principally by zeolite, has a significantly higher retention time than "metir" (a mix of mineral waste from coal mine). In fact, after 35 volumes the "masse" has still retained about 50% of the

Cadmium retained in the solutions, while when "metir" was used, after just 7 volumes of washing, no cadmium was retained on the support.

A similar test was performed to evaluate the retention of lead. In this case also a mixture of "metir" with bentonite mineral was used. The results are shown below in **figure 4.**

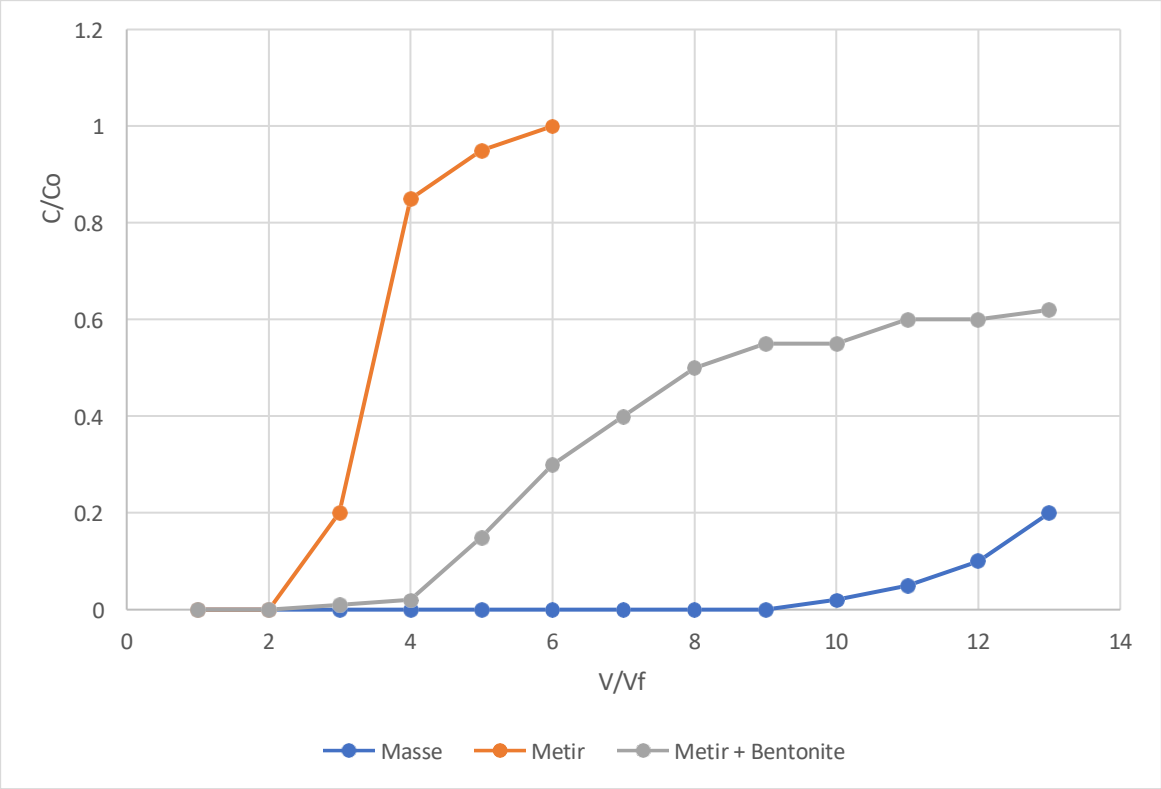


Figure 4: Evolution of the C/Co ratio for lead for two different support: "Masse" and "metir" and a combination of "metir" and bentonite.

Similarly, to the cadmium experiment, the "masse" shown a significant retention power compared to the other two conditions, retaining more than 80% of the lead after 13 volumes of washing. The combination of "metir" and "bentonite" was also able to retain 40% of the lead after the same amount of washing.

3.2.2 Hydrocarbon (HC) capture

To evaluate if the selected mineral supports are also able to capture hydrocarbons, several tests were performed. For a preliminary experiment, crude oil (Volume) was poured in 40 litres of salted water in aquarium tanks with (treatments) or without (control) the mineral support. Different mineral supports were thus added to the treatment tank. While a stable hydrocarbon layer formed above the salted water in the control tanks **(figure 5A)**, hydrocarbons were found to agglomerate around mineral particles in the treatment tanks **(figure 5B)** and also precipitate after few hours of incubation **(figure 5C)**. This suggests that the mineral supports tested (masse, metir, vulcanic stone and zeolite) are able to retain hydrocarbons. Best results were obtained by a mix of volcanic stone and zeolite.

Figure 5: HC capture from support. A) control tank with no supports; B) tank with zeolite; C) tank with a mixture of vulcanic stone and zeolite.

3.2.3 HC adsorption by the supports

The capability of zeolite to adsorb hydrocarbons was also tested. Initially adsorption of gasoline was performed on several supports. 100 grams for each mineral support were placed into a vessel and gasoline was added in excess. After one week of incubation the mineral support was placed on paper filters and rinsed in distilled water to remove excess hydrocarbons. The support was weighed before and after these operations. The weight before and after was reported in the graph below (**figure 6**) as % of increased weight. All the supports were able to retain a certain amount of HC, but only Zeolite and Volcanic stone were able to absorb more than 40% of their weight, with percentage of 64% and 45% respectively.

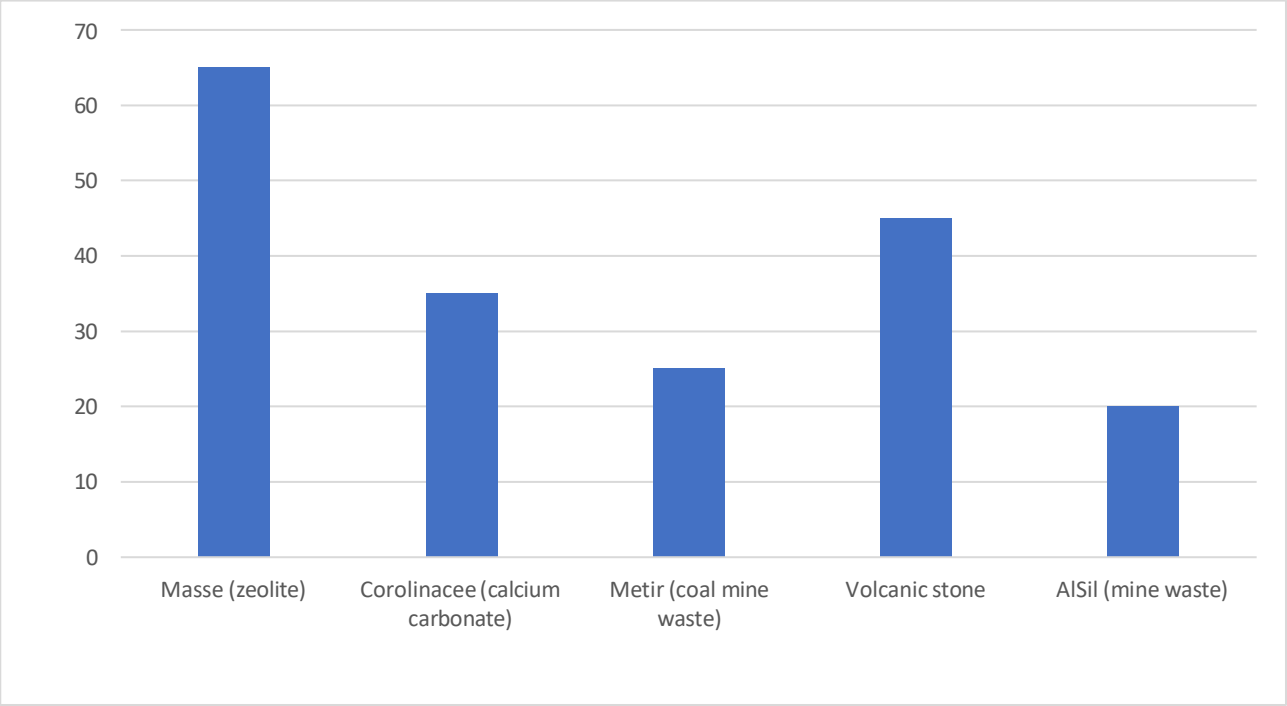


Figure 6: HC absorbtion capabilities of different support reported as percentage of increased weight.

Therefore, for the next steps Zeolite was selected as the major component for the incapsulation of microorganisms.

3.2.4 Selection of the microbiome

Activity of IDRABEL product vastly depends on the biofixed microorganisms. The final design of IDRABEL BIO-VASE SEDREMED was defined via several test aimed at verifying the capabilities of biofixed bacteria to survive in marine environment and in the presence of HC.

Strains Selection

Figure 7 explains the two different approaches for the generation of microbial consortia. The top- down approach starts from and environmental microbiomes and try to refine it to obtain an optimal consortium. The bottom-up approach rationally selects the microorganisms to utilize in a consortium and then screen and evaluated them for their utility. The approach chosen by IDRABEL falls into the second category. In the first phase, IDRABEL has recollected documentation of all the bacteria used in previous products, and from literature studies try to identify new strains compatible with the marine environment and capable of bioremediation, also checking for the level of biological safety of each strain.

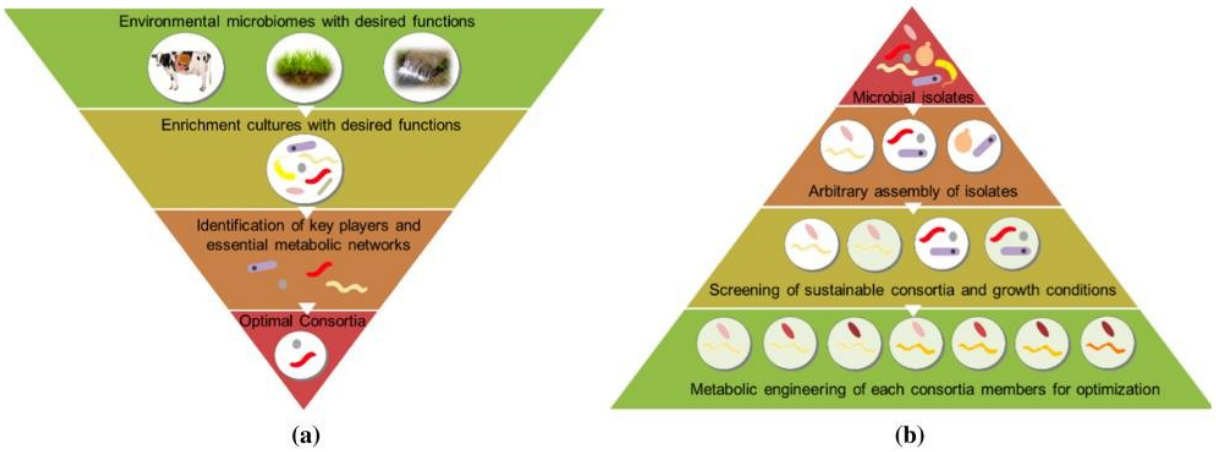


Figure 7: Top-down (a) and bottom-up (b) approaches for synthetic consortia construction (Che & Men, 2019).

This preliminary part led to the selection of several microbial strains and fungi that were used for other aspect of the project **(Table 1)**.

Table1: list of microorganisms used by IDRABEL for the biovase product.

Microorganism's name	Supplier
Pseudomonas strain mix	Greencell[5]
Bacillus amylolicefaciens	Agrostar[6]
Bacillus strains mix	IMBP[7]
Rhodococcus sp.	Artechno[8]
Bacillus licheniformis	Artechno[4]
Fungae Trichoderma artroviride	Artechno[4]

Strains Evaluation: salt concentration and hydrocarbons

Once chosen the bacteria, a first evaluation of their tolerance to the saline environment was performed.

For this evaluation 3 samples were prepared:

Masse is a mix of microorganisms (mainly bacillus + fungi + other bacteria) that we biofix on zeolite;

Pseudomonas strain mix from Greencell;

Bacillus amylolicefaciens from Agrostar;

These samples were evaluated for their growth against 4 different salinity concentrations: 0% - 2 % - 4 % - 6% (% w/V). The strains were inoculated at the initial OD_{600} concentration of 0.05 OD/mL in 10 mL microbiology tube containing LB media supplemented with the different concentrations of NaCl. The tubes were incubated at 30°C, and their growth was evaluated by the measurement of the OD_{600} concentration.

[5] https://www.greencell.tech/environnement

[6] http://agrostar.be/en/index.php

[7] http://www.imbp.be/

[8] http://agrostar.be/en/index.php

Results are summarized in **figure 8**, where we reported the OD_{600} values for each strain in the different conditions during the cultivation. Initial measurements were not reported in the graph as they were identical to all strains. It is possible to observe that the microbial growth decreases when the concentration of salt is higher. When the salt concentration is 6% the final OD_{600} value is 1/3 of the corresponding values at 0% of NaCl. However, the masse and the Greencell samples are able to retain more about 70% of growth at the concentration of 4%. Considering that sea concentration id between 3.3 and 3.6%, this probably indicated that the selected bacteria will be able to survive in marine environments.

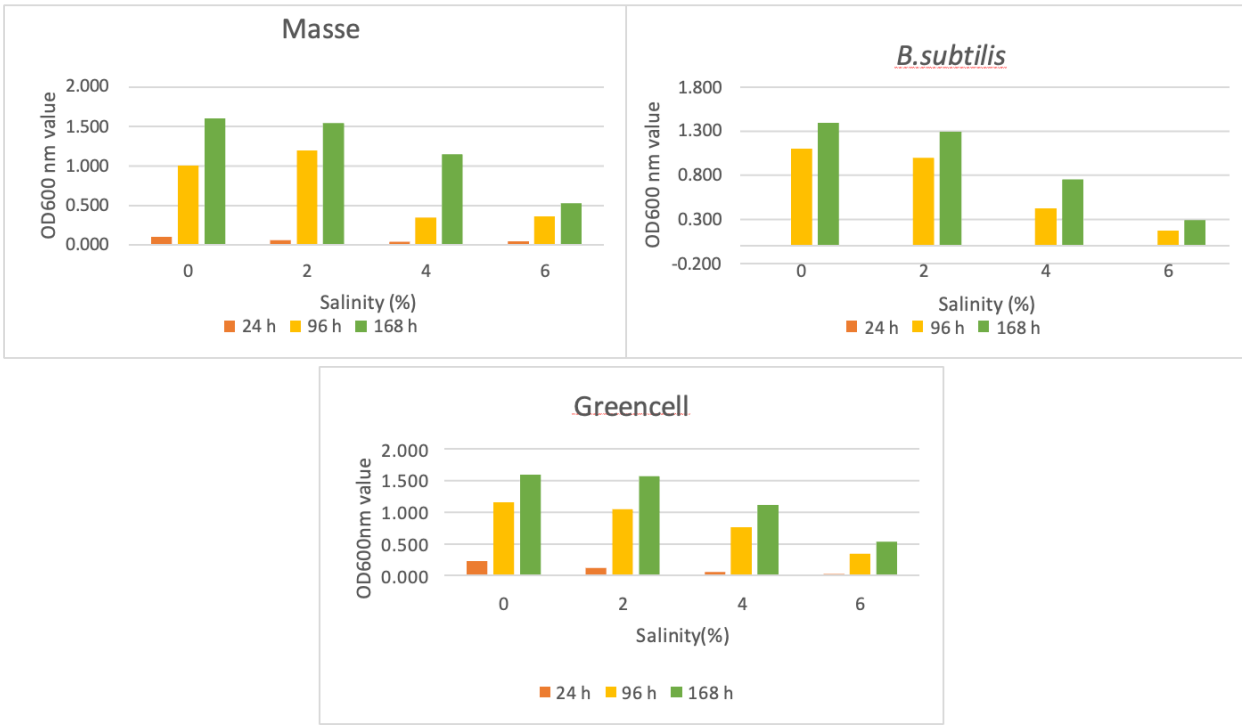


Figure 8: OD600 values as function of salinity and time for: Masse, B. subtilis, Greencell

After this phase, IDRABEL also performed an evaluation of the ability of one or more biofixed bacteria to grow in a saline environment and in the presence of hydrocarbons. The test was performed on LB media agar plates supplemented with 0.1% of gasoline and at different concentration of salt (from to 3% to 4%). As source of bacteria, 0.1 gr of masse was dissolved in water and then plated on the petri dish that were incubated at 30° for 48h. The visual analysis of the plates revealed that the microorganisms were able to grow in presence of hydrocarbons and salts. Salt concentration was indeed the variable that mostly affected the growth. In fact, at the concentration of 4% NaCl the size of the colony was sensible smaller compared with the colonies of 3% NaCl. However, the CFU numbers remains comparable. This indicate that the microorganisms, although affected by salt and hydrocarbons, are still viable and able to survive. After all this preliminary preparation, all the components for the SEDREMED BIOVASE were determined and are reported in **table 2**.

Table 2: final component of biovase product for SEDREMED mesocosm experiments.

Type	Family	What	Why
Mineral Supports	Chabasite	aluminosilicate of the zeolite family	to house bacteria and fix heavy metals
	Coal mine waste	mix of minerals as aluminosilicates	to fix heavy metals and other pollutants (comparable to activated carbon) multiple filtration function wide spectrum
	Pouzzolane	Volcanic Stone	for filtration properties, bacterial hosting and heavy metal capture (complementary to Chabasite)
	Calcium Carbona	Limestone	to buffer PH, provide C and Ca (specific microorganisms carrier)

	te		
Microorganism	Bacteria	*Rhodococcus erythropolis*	To treat HC
		Pseudomonas putida et *fluorescens*	To treat long chain organics
		Bacillus MIX	To treat short chain, by-products, organic compounds
		B. amyloliquefaciens Only for ANAERO BIOVASE **(see paragraph 4.3)**	Anoxic strain, soils treatments
	Fungi	Trichoderma artroviride	root growth, ability to colonise a compact soil, can be used as a vehicle for bacteria
Trace elements	Macro nutrients	Phosphate, maltodestrine, ammonium sulfate	Source of Carbon, sultur, nitrogen and phosphorus
	Micro nutrients	Oligo-elements such as Mg, Mn, Ca, K, Mo, Fe, vitamins	Co-factors of enzymes for improved growth

3.2. Preparation of EKOGRID technologies

The EKO Control Unit consists of AC/DC converter, which is running with 1-phase electric supply (240VAC) as shown in **figure 9**.

In the middle there is a microprocessor-controlled PCB (printed circuit board). With the software embedded the system generates programmable voltage output patterns. These are short 10 to 3000ms long constant voltage pulses, every other with reversed polarity. The output voltage level and the length of each specific pulse can be controlled using bespoke laptop software or by with SMS signalling remotely. The equipment is equipped with an IP56 cabinet can be used in very cold or hot environments, and even outside in rain or high humidity.

Electrode design: For the first tests we decided to use inert electrodes made of MMO

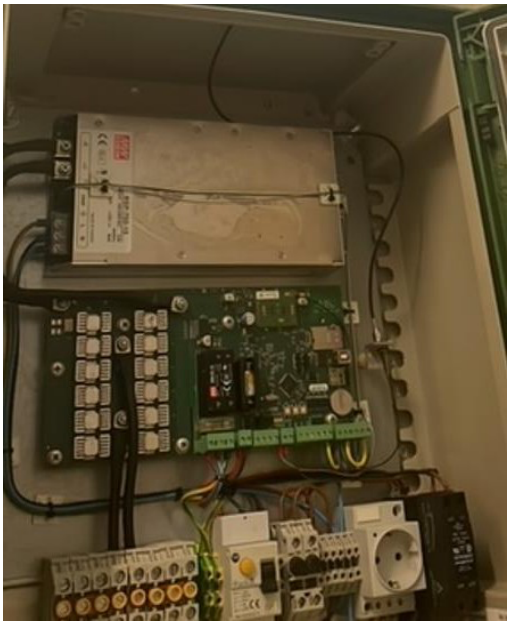


Figure 9: EKO control unit.

(mixed Metal Oxides) coated titanium (**figure 10**). For two reasons: the high salinity conditions are very corrosive for steel, and to maximise oxygen generation in the system.

Figure 10: EKO MMO electrodes for mesocosms experiments.

4. Mesocosms

4.1. Pilot mesocosm (February 2023)

The EKO unit was shipped to IDRABEL Lab in January 2023, and then IDRABEL started the set-up of a pilot mesocosm. EKOGRID recommended a minimum distance of 1 m between the electrodes. For this mesocosm artificial sediments were used: (Sand + bentonite) and salted water with a conductivity of 20 mS/cm, lower than sea water (to avoid any problem with chlorine).

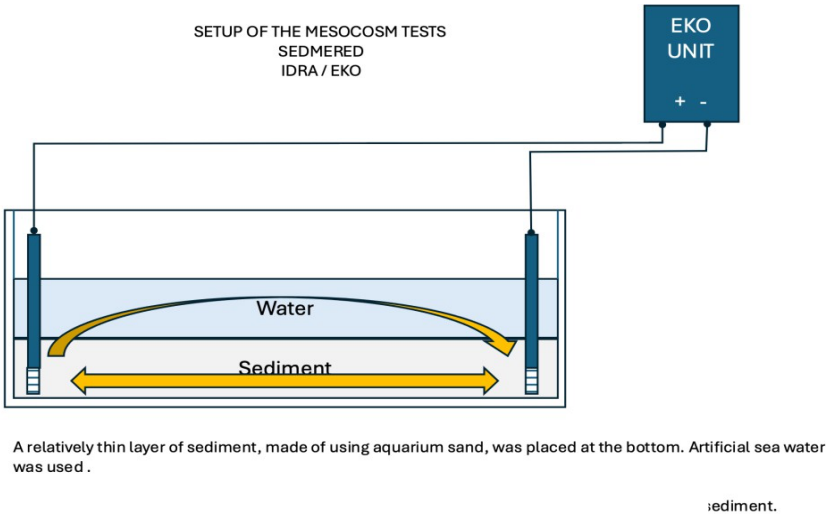


Figure 11: Scheme of the mesocosms test using IDRABEL and EKOGRID technology.

Electrical field must go through sediments, and it is very important to minimise contact with water. For this reason, plastic tubes with micro holes were and the electrodes were placed within these sealed tubes. For this first test the bacteria were injected in the middle of the mesocosm using an injection tube (**figure 12**).

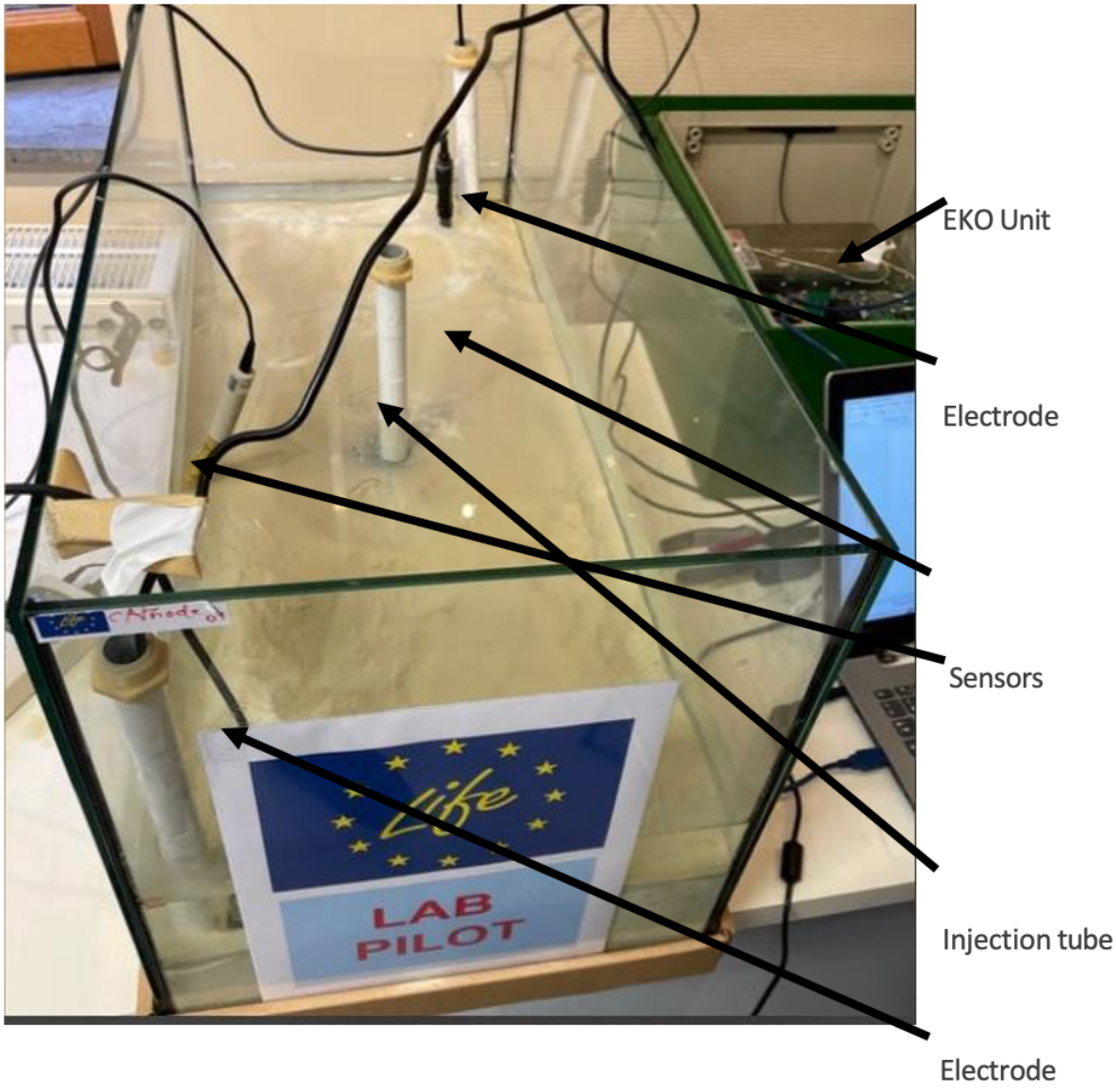


Figure 12: Set-up of the mesocosms blank test with all the components.

Blank test

Before doing any measurement, we had to verify if EKO (electro Kinetic Oxidation) was working. To measure the voltage and the current patterns a Fluke 125B Graphic multimeter[9] was utilized. The EKO unit applied a voltage of 10V and a pulsing length of 40 ms.

In **figure 13** we can see the output of a first attempt. Blue line shows the current value during the pulsing. The current was approx. 1A. But we can see a decline during the pulse period. This is a good mark indicating that there is a slight capacitor effect proving that some of the current is passing the sediment and can create some reactions in the sediment. Most likely this setup could generate some wanted reactions in the sediment.

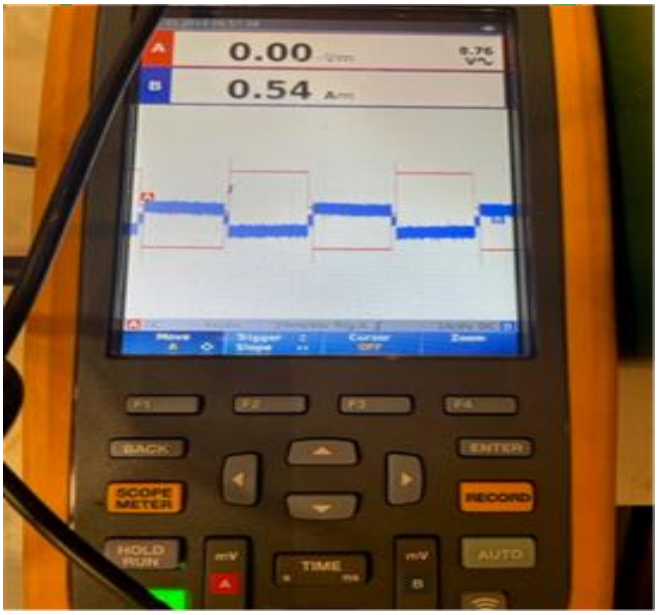


Figure 13: Output of the evolution current using a multimeter in the sediments when pulses are applied.

[9]https://www.fluke.com/en-us/product/electrical-testing/portable-oscilloscopes/120b?srsltid=AfmBOoqDbcvabi9awz9uNf_IaGF7aWrA0paqQ2C6Gu5l_9-JPnR4aL-L

IDRABEL placed sensors in artificial sediments to monitor the evolution of redox and pH close to the sensors and in the middle. The example in **figure 14** shows the evolution of this parameters in 24 hours using asymmetric "transportation pulse". Hereunder, on the left, evolution of pH and redox at electrode 01, and on the right, evolution of pH at electrode 02 and redox between electrodes in an experiment with a voltage of 10V and a pulsing lenght 50 ms with a pause between pulse of 3 seconds.

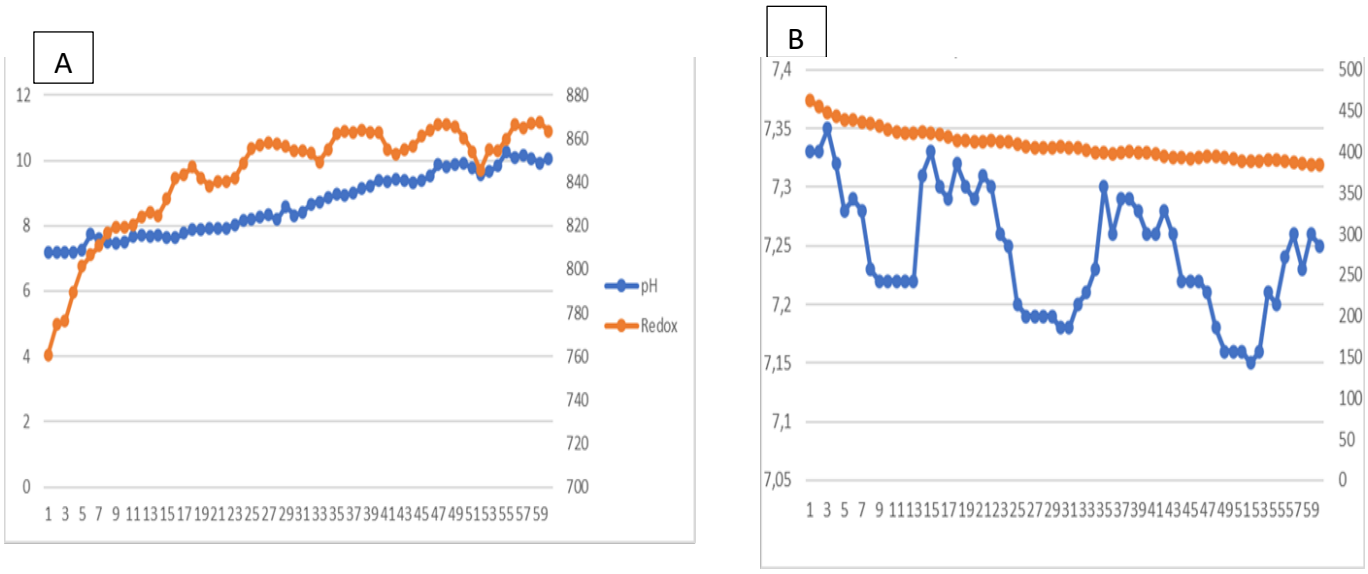


Figure 14: Evolution of pH and redox potential in sediments: A) close to the anode sensors; B) in the center of the mesocosm.

On the left it is possible to observe a rapid increase of pH value and of redox potential, while on the right the same values remain almost constant. This probably indicated that the microorganisms could not survive too close to the electrodes due to the harsh conditions generated.

Microbial survival test

To evaluate if the microorganisms would be able to survive in the condition of the mesocosms, few grams of BIO-VASE SEDREMED were injected in the central tube **(figure 15).**

At interval of days, few mL of pore water from sediments were taken around the injection tube and near electrodes. The water was serially diluted and plated on LB agar plates to evaluate the growth of the microorganisms.

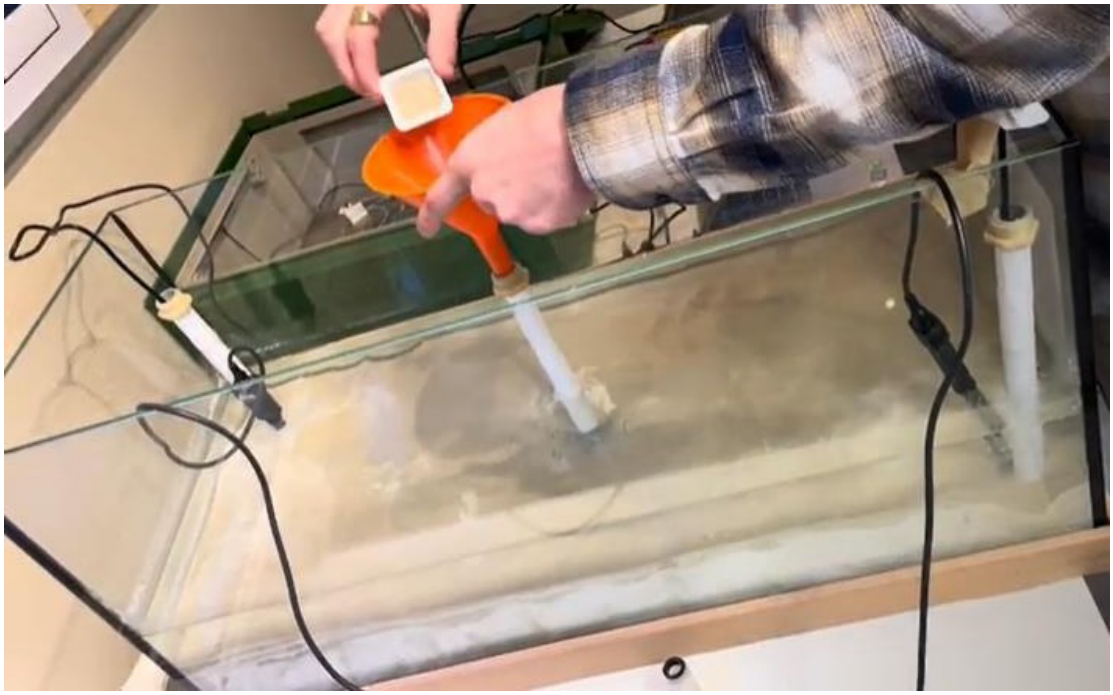


Figure 15: Evolution of pH and redox potential in sediments: A) close to the anode sensors; B) in the center of the mesocosm.

The results indicated absence of microbial activity close to the electrodes confirming the hypothesis when measuring pH and redox. However, in the central part of the sediment, bacterial colonization was observed indicating a favorable environment for the bacteria to grow.

4.2. Mesocosms planning

After the first preliminary set-up to evaluate the feasibility, two different sets of experiments were planned, each one made of three different mesocosms. In the first experiments (runs 1-2-3) we would evaluate the effect of the EKOGRID technology alone and in combination with the IDRABEL technology. In the second experiment (run 4-5-6) we would evaluate the effect of EKO-IDRABEL system in aerobic and partially anaerobic conditions. Both experiments will use an untreated mesocosm as reference. The second experiment was performed after a few months from the first one, in order to evaluate the effect and modify the conditions accordingly.

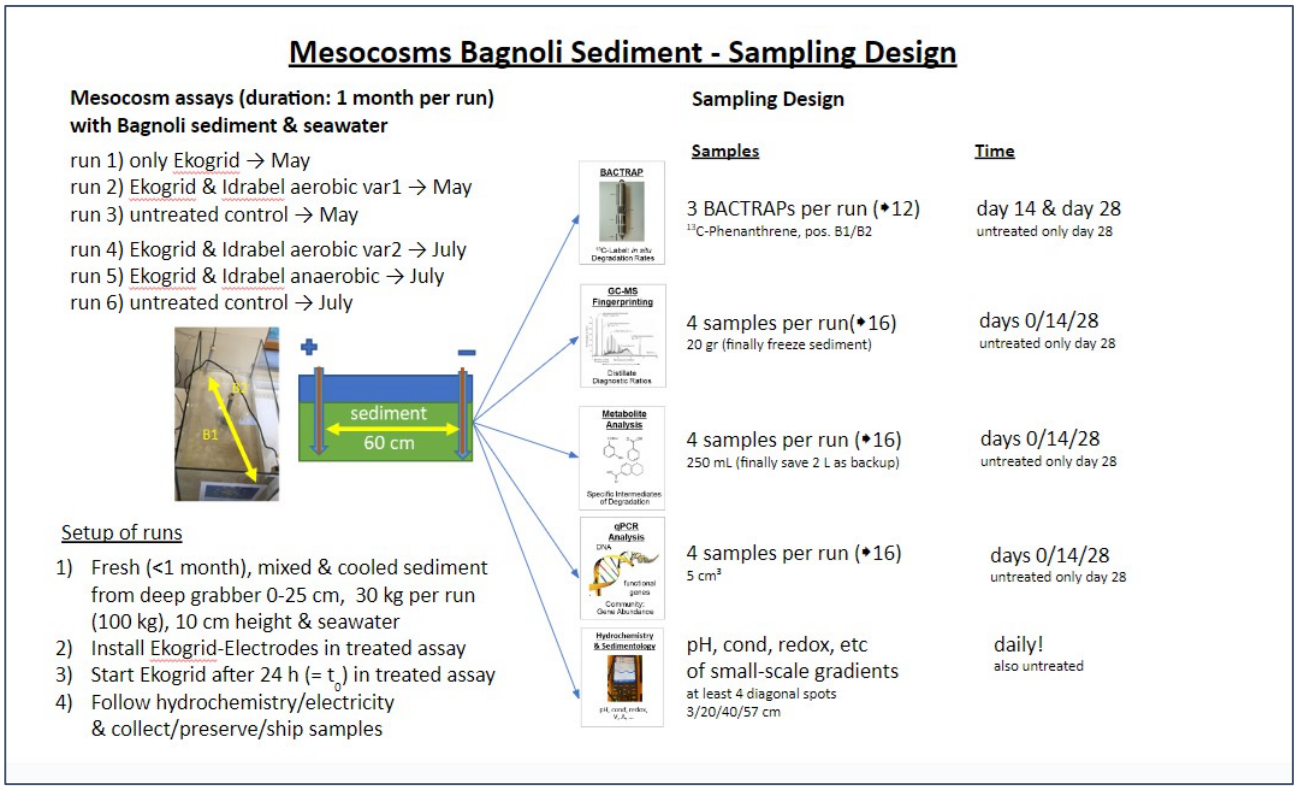


Figure 16: Preliminary scheme for mesocosms set-up and sampling.

An appropriate monitoring plan was also planned with 3 sampling at: installation, 14

days and 28 days (**figure 16**). Samples were collected for GC-MS analysis, metabolite analysis, and DNA analysis.

Bactrap labeled with C13-Phenantrene were also used with a sampling at day 14 and 28. Parameters such as: pH, conductivity, redox were monitored daily.

4.3. Mesocosm ALPHA (May 2023)

4.3.1. Material

Sediment reception

Bagnoli sediments arrived on 28/04/23 at IDRABEL premises. Parameters such as pH, redox, conductivity were measured to check homogeneity. Sediments shows a positive redox, which is not found in situ, meaning that during the transportation the sediments were oxygenated.

Set-up of the experiment

Experiments were carried out using aquarium tanks 80 cm long, 40 cm wide and 60 cm high. For this first run 3 replicate tanks were set up. Each tank contained 30 kg of sediment and 50 L of commercial sea water. Sediment was mechanically mixed and then divided into 3 equal parts of approximately 30 kg for each mesocosms. This resulted in a depth of the sediment layer of approximately of 10 cm. The scheme of the mesocosms is shown below in **figure 17.** Sea water is slowly added to avoid holes in the sediment layer.

The electrodes were again placed into two opposite corners. In the middle of the mesocosms a sample area was designed, a square with a side of 20 cm. Within this area were performed the measurements for pH, redox, conductivity, oxygen are taken, as well as the "swimming pool" test kit: TH and TA (hardness and alkalinity), total and free chlorine.

The EKO Unit consists of Electrical connections, 2 electrodes MMO coated with 2

plastic tubes with micro holes. Isodected has sent 6 bactraps (2 for each mesocosm) and glass bottle for sampling.

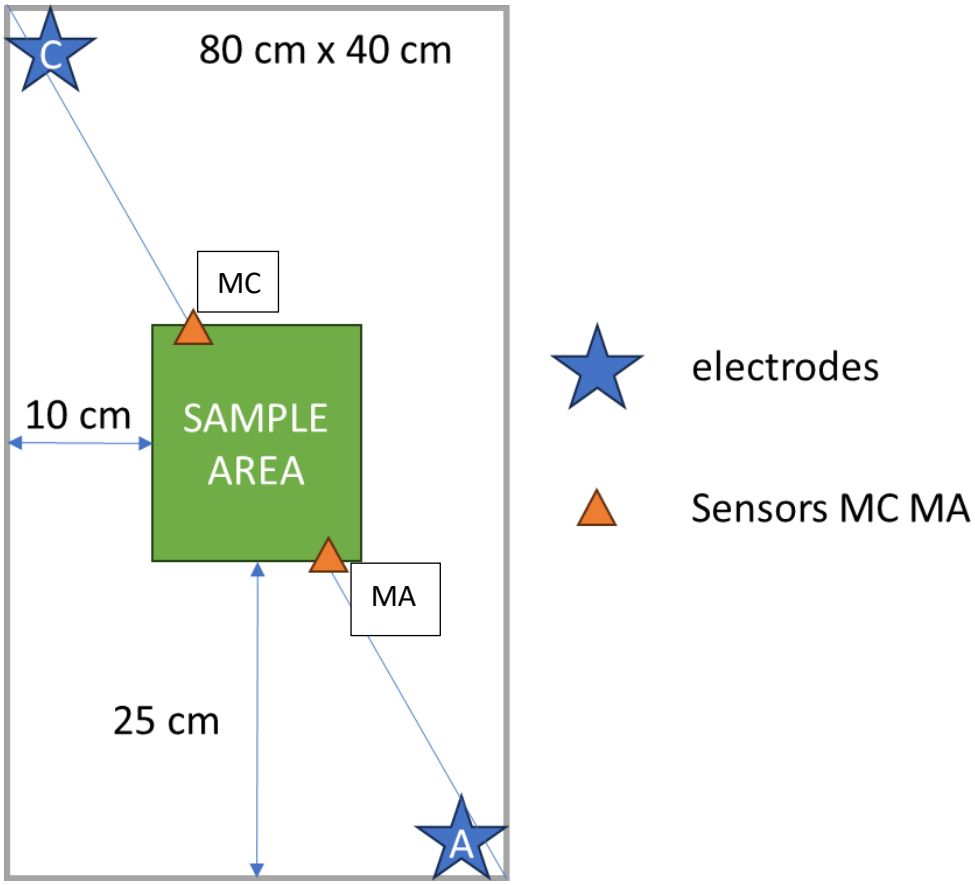


Figure 17: Mesocosms scheme and monitoring set-up. C: Cathode electrode; A: Anode electrode; MC: middle cathode; MA: middle anode.

For the EKO mesocosm (run 1) two electrodes are placed at 2 opposite corners at a distance of 1 m between each electrode is placed in plastic tubes and plastic rubber is used to protect electrical connections. The tubes have micro holes only under the sediment level so there is no direct contact between sea water and electrodes. For the EKO-IDRABEL mesocosm (run 2) the sediments were mixed with 500g of "BIOVASE SEDREMED" in a clean powder mixer, and then poured into the aquarium. The electrodes were also added as for the EKO mesocosms. The control mesocosm

(run 3) did not received any treatment or electrodes. After 16h of rest, 2 bactraps are placed in each sample area. For the sampling 250 ml of sediment were taken in the sample area with a spoon. 5 g sediment is taken in the sample area with a spoon. 250 ml of pore water is taken in the sample area with a pipet. Each sample was stored at -20°C.

4.3.2. Running of the mesocosm

EKO unit run

The operation parameter of EKO unit has been chosen to minimise chlorine production. Output from EKO unit: 10.5 V. Positive pulse for 250 mSec; no pulse for 3 mSec; negative pulse for 25 mSec; no pulse for 3 mSec. The unit worked 1 hour per day.

Daily measurement

pH, redox and conductivity sensors were placed in sediments at 4 different spots: A and C directly near electrodes, while MC and MA were situated on the diagonal at limit of sample area, MC at cathode side and MA at anode side. Before and after unit work, pH, redox and conductivity were measured in sediment at each 4 places in run 1 and 2.TH, TA, TCl, FCl, O2 were measured each day in water.

Sampling

Bactraps were removed on Day 14 and Day 28 of the experiment. Also, in day 14 and 28, 250 ml of sediment were collected, as well as 5 g sediment is taken in the sample area with a spoon. Moreover, 250 ml of pore water is taken in the sample area with a pipet. All the samples are stored at -20°C until expedition. At the end of the experiment, 1 kg of sediments for each run was also kept as -20°C as backup.

4.3.3. Preliminary results and modifications

Daily analysis of IDRABEL **(see ANNEX 1)** revealed that mesocosm 1 and 2 shown the formation of chlorine in the water. At the end of the sampling, chemical analysis was performed from ISO. The complete analysis of the mesocosms is reported in **Chapter 5**. However, from preliminary analysis, it was clear that the two runs with treatment did not how significant differences compared to controls. Two critical points were identified:

-the working time of the EKO unit, 1h per day (chosen to avoid the formation of chlorine) was very likely too little to cause significant effect.

- 4 weeks was likely a very short time to verify any effect of degradation. Therefore, for the next mesocosms it was decided to:

1) use steel electrodes, avoiding the problem of chlorine formation and therefore allowing the utilization of EKOGRID system for more time.

2) Increase the time of the experiment, evaluating results after 8 weeks instead of 4.

This shifted the timeframe for the second run that started with few months of delay to better prepare the experiments.

4.4. Mesocosm OMEGA (October-December 2023)

4.4.1. Material

Sediment reception

Sediments from Bagnoli Bay were delivered at IDRABEL premises on 27/09/2023. Parameters such as pH, redox, conductivity were measured to check homogeneity. Upon arrival, sediments exhibited a redox potential higher than that initially measured in situ, straight after sediment collection. This suggests that some oxygen

contamination occurred during sediment shipping.

Set-up of the experiment

Experiments were carried out using the same aquarium tanks of the first 3 runs. Each tank contained 30 kg of sediment and 50 liters of commercial sea water. Sediment was mechanically mixed and then divided in 3 equal parts of approximately 30 kg for each mesocosm. This resulted in a depth of the sediment layer of approximately 10 cm. The scheme of the set-up is identical to that of mesocosms alpha **(figure 17).**

The EKO Unit consists of Electrical connections and in this case, instead of MMO electrodes, steel electrodes were used. The electrodes were coated with 2 plastic tubes that were coated with rubber, to avoid the contact with the water phase, except for the final part **(figure 18)**.

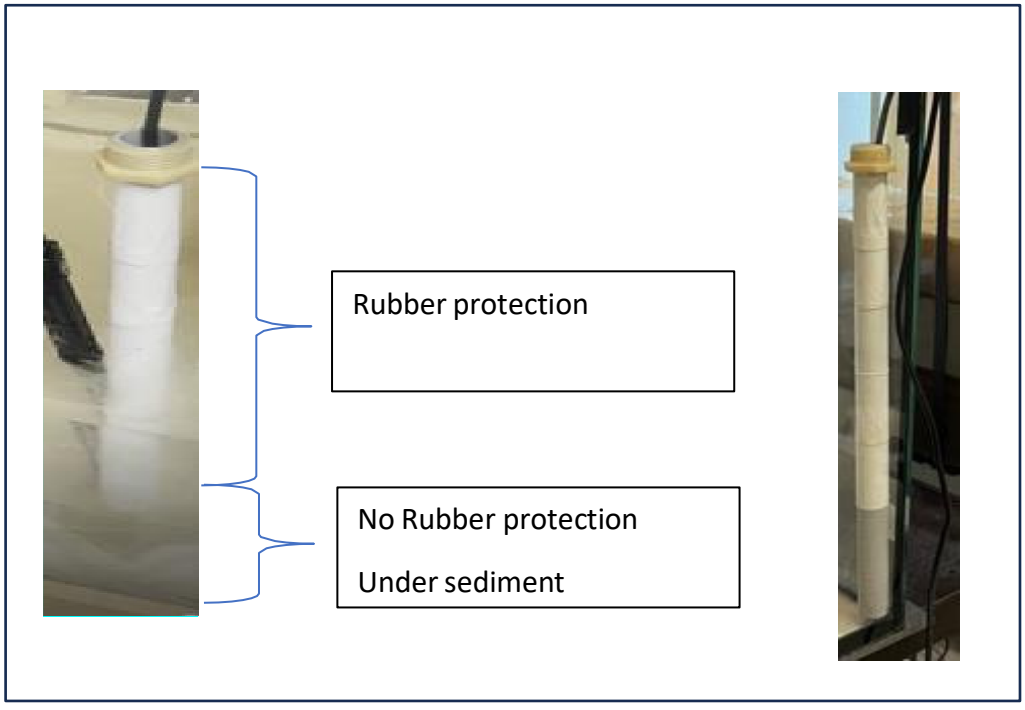


Figure 18: Coating of the steel electrodes to avoid direct contact with water.

30 kg of sediments are placed in each aquarium. A spoon has been used to have a homogenous layer of sediment in the aquarium that is about 10 cm high. Sea water

is slowly added to avoid holes in the sediment layer.

For the Aerobic mesocosm (run 4) the sediments were mixed with 500g of "BIOVASE SEDREMED" in a clean powder mixer, and then poured into the aquarium. Moreover, 5 "socks" with 50 g of "BIOVASE SEDREMED" each are installed in the sediment at centre of aquarium. The two electrodes are placed at 2 opposite corners at a distance of 1 m.

For the anaerobic mesocosm (run 5) the sediments were mixed with 250g of "BIOVASE ANAEROBIC" in a clean powder mixer, and then poured into the aquarium. Moreover, 5 "socks" with 50 g of "BIOVASE ANAEROBIC (Table 2)" each were installed in the sediment at centre of the aquarium. Moreover, A plastic layer is placed between water and sediment to limit oxygen transfers, and the Aquarium was closed with a lid to limit exchanges with the outside environment. The electrodes were also added as for the EKO mesocosms.

The control mesocosm (run 6) did not received any treatment or electrodes. After 16h of rest, 2 bactraps are placed in each sample area. For the sampling 250 ml of sediment were taken in the sample area with a spoon. 5 g of sediment were taken in the sample area with a spoon while 250 ml of pore water were taken in the sample area with a pipet. Each sample was stored at -20°C.

4.4.2. Running of the mesocosm

EKO unit run

For this run, using steel electrodes, the risk of chlorine formation was minimum. Output from EKOunit: 10.5 V. Positive pulse for 250 mSec; no pulse for 3 mSec; negative pulse for 25 mSec; no pulse for 3 mSec. The unit worked 22 hour per day.

Daily measurement

pH, redox and conductivity sensors were placed in sediments in 4 different spots: A and C directly near electrodes; MC and MA on the diagonal at limit of sample area while MC at cathode side and MA at anode side. EKO unit was stopped 2 hours every day to perform physicochemical measurements: pH, redox, conductivity, temperature, and dissolved oxygen (**see ANNEX 2&3)**. These data indicates that the chlorine concentration remained stable through the course of the experiment.

Sampling

After 14 and 28 days since the beginning of the experiments one bactrap was removed from each run. 250 ml of sediment is taken in the sample area with a spoon. 5 g sediment were taken in the sample area with a spoon. 250 ml of pore water is taken in the sample area with a pipet. All the samples are stored at -20°C until expedition. For run 4 and 5, one sock for run was removed after 0, 2, 4, 6 and 8 weeks.

5. Results from Advanced Monitoring Tools

Extensive monitoring activities have been carried out to assess the remediation efficiency of the EKOGRID technology (using asymmetric electric pulsing), alone or in combination with the IDRABEL product amendment, by mesocosm experiments carried out with Bagnoli sediments.

Major operating conditions of mesocosms are shown in **figure 19**. As described above, two runs (③ and ⑥) served as controls without any treatment to test the natural attenuation capacity of sediments. Others received either treatments from both technologies (②, ④, ⑤) or solely from EKOGRID (①). Several parameters were changed after experiencing the low performance of the primary mesocosms ① to ③.

In particular

electrode materials were switched from titanium to steel

daily application was prolonged from 1 hour (short) to 22 hours (long) duration of mesocosm operation was prolonged from 4 weeks to 8 weeks shielding of one mesocosm (⑤) from air at least to support anaerobic conditions.

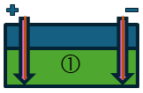
	EKOGRID 1h/d -	EKOGRID 1h/d BioVase 500 g	- -	EKOGRID 20h/d BioVase	EKOGRID 20h/d BioVase	- -
						
Electrodes:	titanium	titanium	titanium	steel	steel	steel
Pulses [msec]:	250(+)/25(-)	250(+)/25(-)	250(+)/25(-)	250(+)/25(-)	250(+)/25(-)	250(+)/25(-)
Voltage [V]:	10.5	10.5	10.5	10.5	10.5	10.5
Duration:	4 weeks	4 weeks	4 weeks	8 weeks	8 weeks	8 weeks
Redox:	aerobic	aerobic	aerobic	aerobic	"anaerobic"	aerobic
Seawater:	artificial	artificial	artificial	atlantic	atlantic	atlantic
Time:	May 2023	May 2023	May 2023	Nov/Dec 2023	Nov/Dec 2023	Nov/Dec 2023

Figure 19: Major operating conditions of the six mesocosms testing EKOGRID & IDRABEL technologies.

Similar to field monitoring tools, the following methods were applied to trace contaminant degradation and stimulation effects:

- GC-MS fingerprinting and diagnostic ratios of polyaromatic hydrocarbons (PAH)
- Metabolite analysis
- BACTRAPS.

The basic principles of these methods are explained in deliverable B3.1 "First Report with Monitoring Results".

5.1. GCMS Fingerprinting and Diagnostic Ratios

Sediment samples from mesocosms were analysed for PAH by ISO using GC-MS and SIM (single ion mode) at the starting time (t0) and the final date of operation. Short daily treatment and control assays showed no decrease of total PAH (**figure 20**). In contrast mesocosms ④ and ⑤ exhibited a slight decline of PAH after eight weeks of treatment. Though the decline isn't extensive due to analytic uncertainties and variation of initial concentrations it can be assessed as a notable indication for stimulated degradation of PAHs.

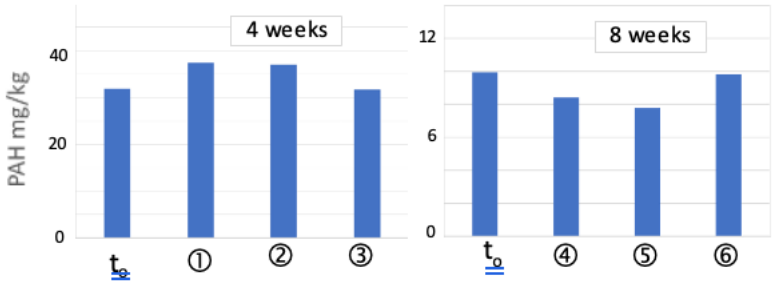


Figure 20: Amount of total PAHs determined mesocosms at time zero (to) and after 4 or 8 weeks.

The more specific patterns of seven polyaromatic hydrocarbons (**figure 21**) showed a slight decrease of concentrations for almost any of the detectable compounds in mesocosms ④ and ⑤, particularly for the higher concentrated phenanthrene, fluoranthene and pyrene. Thus, the stimulated degradation could be an overall effect instead of a compound-specific one.

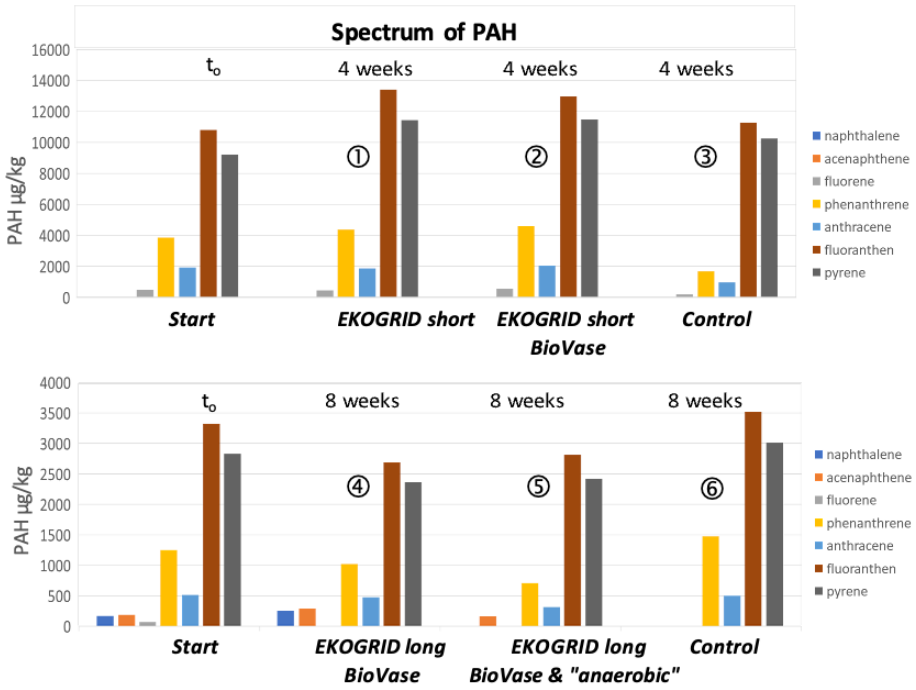


Figure 21: .Amount of specific PAHs in the mesocosms at starting time and after 4 or 8 weeks of operation.

Regarding diagnostic ratios **(table 3)**, however, there was no significant change observed in any of the mesocosms. Even more, only the ratio of phenanthrene and acenaphthene delivered suitable

values. Due to the slightly better microbial degradability of anthracene versus phenanthrene, an increase in this ratio would be expected in the case of stimulated microbial degradation. However, since concentrations in mesocosms 4 and 5 both fell slightly, no stimulated degradation can be inferred here. Nevertheless, it cannot be ruled out that there was a stimulation of degradation for both PAHs, even if only indicative. Thus, it would take a longer and more intense degradation process to achieve a considerable change of ratios. However, a remarkable difference of the phenanthrene/acenaphthene ratio can be stated for the sediments used for the primary and the latter mesocosms (0,5 vs. 2,5). These sediments have been collected from different spots at the Bagnoli coastline, where probably weathering or long-

time degradation had different impacts. Due to the unique PAH pattern (no significant alkylated PAHs), this method is somewhat limited for determining the stimulation of degradation.

Table 3. Diagnostic ratios (from peak heights) of specific polyaromatic compounds, indicating degradation by their increase ( ); nd = at least one compound was not detectable.

Mesocosm number	①	①	②	③	④	④	④	⑤	⑤	⑤	⑥	⑥
EKOGRID treatment intensity	short	short	short	-	long			long			-	
IDRABEL treatment	-	-	+	-	+			+			-	
Air access	+	+	+	+	(-)			+			+	
Treatment time (weeks)	0	4	4	4	0	4	8	0	4	8	0	8
Acenaphthene/Fluorene ↑	<0,1	<0,1	<0,1	<0,1	nd	nd	nd	1	nd	nd	nd	nd
Dibenzofurane/Fluorene ↑	<0,1	<0,1	<0,1	<0,1	nd	nd	nd	<0,1	nd	nd	nd	nd
(C2-Napht+C3-Naphth)/Napht ↑	nd	nd	nd	nd	nd	nd	<0,1	<0,1	<0,1	nd	<0,1	nd
Phenanthrene/Anthracene ↑	0,5	0,4	0,4	0,6	2,6	2,8	2,1	2,6	2,8	2,2	2,3	2,9
(C2-Dibenzothioph+ C3-Dibenzothioph)/(Dibenzothioph) ↑	<0,1	<0,1	<0,1	<0,1	nd	nd	nd	nd	nd	nd	nd	nd
((C2-Anthr+Phen+(C3-Anthr+Phen))/(Anthr+Phen) ↑	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1
Methylpyrene/Pyrene ↑	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1
Methylchrysene/Chrysene ↑	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1

5.2. Metabolite Analysis

Metabolite analysis represents a simple and quick method for providing evidence of in situ biodegradation (Morasch et al. 2011) and its potential enhancement due to the application of the selected SEDREMED technologies. Unfortunately, the samples for the final three mesocosms 4-6 were destroyed due to a transportation failure. The samples from mesocosms have been collected by suction porewater into a syringe inserted into the sediment. This is a rather undefined procedure, which probably collects also higher amounts of surface water. Moreover, it is likely that artificially blended Atlantic seawater was only very slowly charged with degradation products so that they could not be detected after 4 weeks, respectively.

Table 4. Metabolites from mesocosms to (others lacking due to transport failure); nd= below detection limits; blue frame: previously detected in field samples.

	4 weeks		
sample ID	①	②	③
unit	µg/l	µg/l	µg/l
Metabolites of aerobic BTEX biodegradation			
Catechol (1,2-dihydroxybenzene)	nd	nd	nd
Methylcatechol (Dihydroxytoluene)	nd	nd	nd
Ethylcatechol	nd	nd	nd
Benzyl alcohol	nd	nd	nd
Metabolites of aerobic Naphthalene biodegradation			
1-Naphthol (1-Hydroxynaphthalene)	nd	nd	nd
2-Naphthol (2-Hydroxynaphthalene)	nd	nd	nd
1,2 or 2,3-Dihydroxynaphthalene	nd	nd	nd
Metabolites of anaerobic BTEX biodegradation			
Benzylsuccinic acid	nd	nd	nd
(1-phenylethyl)benzylsuccinic acid	nd	nd	nd
(2 and/or 3 and/or 4)-Methylbenzylsuccinic acid	nd	nd	nd
Metabolites of anaerobic Naphthalene biodegradation			
(1 and/or 2)-Naphthoic acid	nd	nd	nd
5,6,7,8-tetrahydro-2-Naphthoic Acid	nd	nd	nd
Metabolites of anaerobic PAH biodegradation			
Naphthylmethylsuccinic acid	nd	nd	nd
Phenanthrene-4-carboxylic acid or 9-Anthracencaboxylic acid	nd	nd	nd
Flouren-9-carboxylic carboxylic acid	nd	nd	nd
Acenaphthene- 5 and/or 3 -carboxylic acid	nd	nd	nd
Metabolites aerobic & anaerobic mono- & PAH biodegradation			
Benzoic acid	**0,5**	nd	**+/-**
(2 and/or 3 and/or 4)-Hydroxybenzoic acid	nd	nd	nd
2,5-Dihydroxybenzoic acid (Gentisic acid)	nd	nd	nd
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	nd	nd	nd
o/m/p- Toluic acid (methylbenzoic acid)	nd	nd	nd
Phenol (probably co-contamination)	nd	nd	nd
o/m/p-Cresol (probably co-contamination)	nd	nd	nd
Metabolites of anaerobic alkane biodegradation			
Alkylsuccinic acids	nd	nd	nd

n.d. = not detected, below limit of detection

+ = detected, but quantification not possible due to peak overlay

+/- = detected, but below limit of quantification

These factors explain the absence of metabolites (even benzoate) in examined mesocosms (Table 4). In addition, no results are available for mesocosms ④ and ⑤, which are supposed to exhibit slightly stimulated degradation.

5.3. BACTRAPS

BACTRAPS are in situ microcosms containing surfaces amended with an adsorbed 13C-labeled contaminant. During their exposition in sediments, BACTRAPS can be colonized by pollutant- degrading microorganisms, which assimilate the 13C-label into their biomolecules such as amino acids (AA) and fatty acids (Bahr et al, 2015).

BACTRAPS labelled with 13C-phenanthrene have been recovered for all mesocosms except ⑤ (transport failure). For the primary three runs even two BACTRAPS have been analysed after 2 weeks and 4 weeks of exposition, respectively (**Figure 22, upper chart**). Probably, these periods were too short to receive remarkable signals of degradation. Though microbial colonization was proved by high amounts of amino acids, their 13C isotope content was only marginally exceeding the indicative benchmark for degradation (+2 ‰). Moreover, this benchmark was also outvalued for several amino acids from mesocosms without any treatment (③, ⑥). Thus, it has to be concluded that degradation in mesocosms was merely a matter of natural processes instead of technological stimulation. Logically, these processes were probably enhanced by air exposition of mesocosms, which favoured aerobic conditions also in the sediments. The air shielded mesocosm, which had been foreseen as a reference, could not be examined, unfortunately. Nevertheless, also technological treatments might have contributed to degradation, particularly in the long-term EKOGRID application in mesocosm ④.

Summing up, BACTRAPS have proven to be a very sensitive (yet highly selective) monitoring tool in mesocosms. They were able to trace natural attenuation, although it seemed to be very weak. On the other hand, they showed no clear evidence for stimulated degradation, which is in contrast to slightly decreasing PAH concentration measurements.

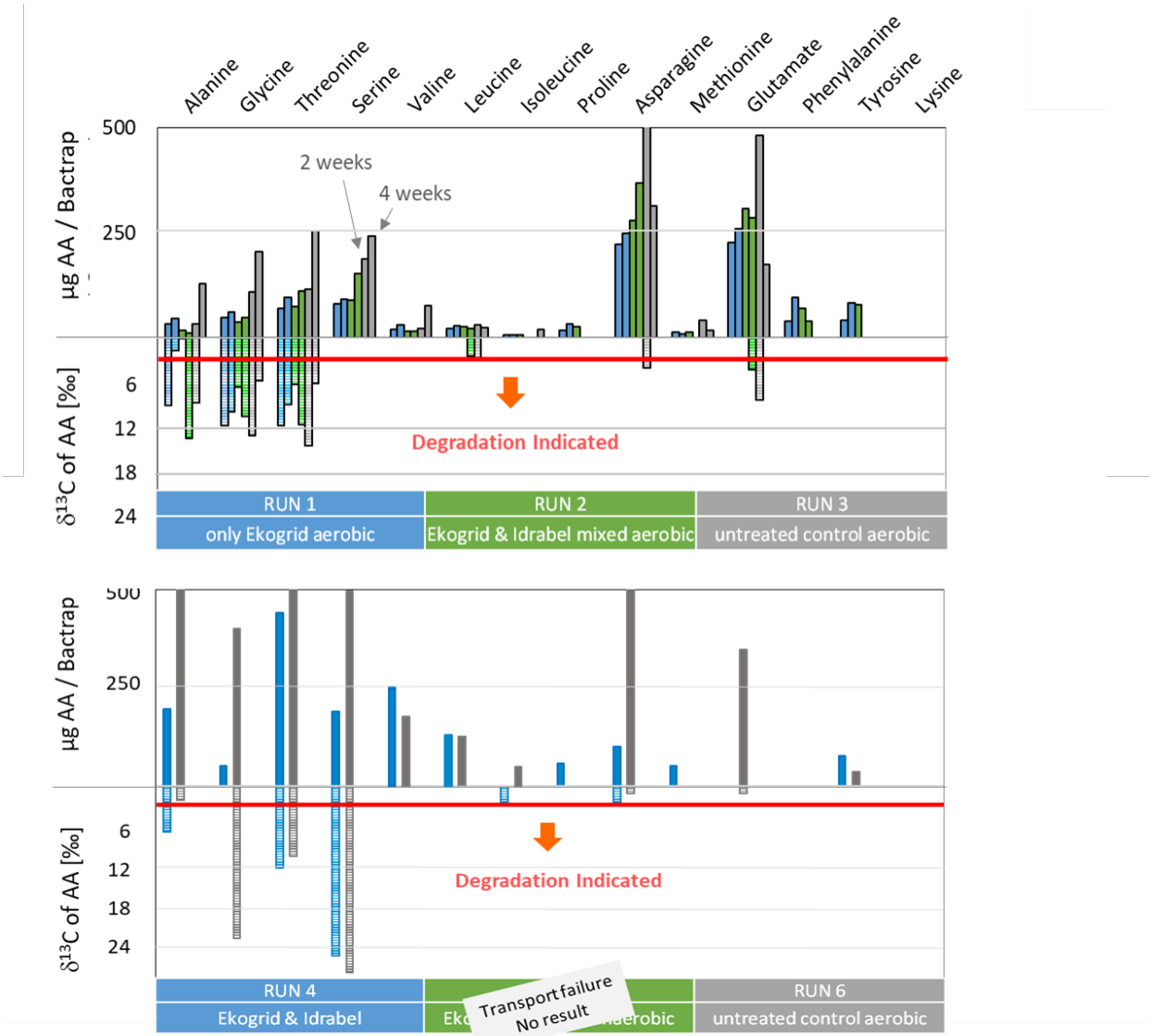


Figure 22: Columns upwards: Amount of microbial colonization (denoted as amino acid concentration AA) in BACTRAPS (loaded with 13C-phenanthrene) exposed in six mesocosms (above for 2 or 4 weeks; below for 8 weeks). Columns downwards: Isotope values of respective.

6. Monitoring Conclusions

In a nutshell, only weak evidence for stimulated degradation of pollutants in mesocosms was provided with the applied monitoring methods. At least, the decrease of total PAH in mesocosms continuously treated by the EKOGRID technology showed some evidence for enhanced attenuation after eight weeks of operation. Moreover, several BACTRAPS indicated the appearance of natural attenuation.

However, the implemented mesocosms seemed to exhibit significant artifacts, when compared to the natural environment such as infiltration of oxic seawater by air access (not comparable to deep sediment layers) destruction of sediment matrix by mixing local destruction of microbes by chlorine formation (close to titanium electrodes) alteration of natural conditions due to transport effects and long-term operation lack of turbidity naturally created by waves room temperature instead of seawater temperature.

Despite of these drawbacks, the mesocosm applications delivered remarkable experiences and improvements of the integrated technologies. Particularly, EKOGRID electrodes and daily application were adapted in order to achieve better remediation effects.

Considering the practical limitations, the setup of laboratory mesocosms might not have met the requirements for an effective technological assessment within the SEDREMED context. Finally, the pilot field test will show also the feasibility of the mesocosm approach.

7. References

- Bagi, A., D. M. Pampanin, A. Lanzén, T. Bilstad, and R. Kommedal. 2014. 'Naphthalene biodegradation in temperate and arctic marine microcosms', *Biodegradation*, 25: 111-25.
- Genovese, M., F. Crisafi, R. Denaro, S. Cappello, D. Russo, R. Calogero, S. Santisi, M. Catalfamo, A. Modica, F. Smedile, L. Genovese, P. N. Golyshin, L. Giuliano, and M. M. Yakimov. 2014. 'Effective bioremediation strategy for rapid in situ cleanup of anoxic marine sediments in mesocosm oil spill simulation', Front Microbiol, 5: 162.
- Gill, R. T., M. J. Harbottle, J. W. N. Smith, and S. F. Thornton. 2014. 'Electrokinetic-enhanced bioremediation of organic contaminants: A review of processes and environmental applications', *Chemosphere*, 107: 31-42.
- Lan, Jun, Fang Wen, Yongxiang Ren, Guangli Liu, Yi Jiang, Zimeng Wang, and Xiuping Zhu. 2023. 'An overview of bioelectrokinetic and bioelectrochemical remediation of petroleum- contaminated soils', *Environmental Science and Ecotechnology*, 16: 100278.
- Morasch, Barbara, Daniel Hunkeler, Jakob Zopfi, Brice Temime, and Patrick Höhener. 2011. 'Intrinsic biodegradation potential of aromatic hydrocarbons in an alluvial aquifer – Potentials and limits of signature metabolite analysis and two stable isotope-based techniques', *Water Research*, 45: 4459-69.
- Tedesco, Pietro, Sergio Balzano, Daniela Coppola, Fortunato Palma Esposito, Donatella de Pascale, and Renata Denaro. 2024. 'Bioremediation for the recovery of oil polluted marine environment,

opportunities and challenges approaching the Blue Growth', *Marine Pollution Bulletin*, 200: 116157.

- Tyagi, M., M. M. da Fonseca, and C. C. de Carvalho. 2011. 'Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes', *Biodegradation*, 22: 231-41.
- Uddin, Mohammad Kashif. 2017. 'A review on the adsorption of heavy metals by clay minerals, with special focus on the past decade', *Chemical Engineering Journal*, 308: 438-62.
- Valdivia-Rivera, Sergio, Teresa Ayora-Talavera, Manuel Alejandro Lizardi-Jiménez, Ulises García- Cruz, Juan Carlos Cuevas-Bernardino, and Neith Pacheco. 2021. 'Encapsulation of microorganisms for bioremediation: Techniques and carriers', *Reviews in Environmental Science and Bio/Technology*, 20: 815-38.
- Yakimov, M. M., R. Denaro, M. Genovese, S. Cappello, G. D'Auria, T. N. Chernikova, K. N. Timmis, P. N. Golyshin, and L. Giluliano. 2005. 'Natural microbial diversity in superficial sediments of Milazzo Harbor (Sicily) and community successions during microcosm enrichment with various hydrocarbons', *Environ Microbiol*, 7: 1426-41.

8. Annex list

- Mesocosms alpha raw data https://doi.org/10.5281/zenodo.13919371
- Mesocosms omega raw data https://doi.org/10.5281/zenodo.13919386
- Mesocosms omega raw data https://doi.org/10.5281/zenodo.13919409