

# Soil microcosm set up for a bioremediation study

[Annalisa Casale, Francesca Bosco, Fulvia Chiampo, Diego Franco, Barbara Ruffino, Alberto Godio, Paras Ranjan Pujari]

**Abstract**—In this work the biostimulation strategy was investigated in soil microcosms to enhance indigenous microorganism activity in presence of diesel oil pollutant. Two mineral salt media, one specific for bacteria and the other specific for fungi, and different glucose concentrations were tested. From respirometric analyses (i.e. CO<sub>2</sub> production) and biomass dry weight results, it was observed that mineral salt medium favoring bacterial growth was more effective in promoting microbial activity in contaminated soil, also in the presence of diesel oil as sole carbon source.

**Keywords**—soil, bioremediation, biostimulation, microcosms, diesel oil.

## I. Introduction

Bioremediation has recently attracted the attention of the scientific world for the soil remediation of polluted sites. It is widely considered an environmental-friendly method and needs simpler equipment and low-cost techniques, with respect to chemical and physical methods [1]. Bioremediation does not require high energy consumption, the method is suitable for large areas and in most cases leads to the complete removal of the contaminant [2].

Degradation of pollutants mediated by microorganisms can occur naturally (natural attenuation), but this requires usually a very long time. For this reason, different approaches were developed for the enhancement of microbial growth and activity. The most important strategies are biostimulation and bioaugmentation.

Biostimulation consists in the improvement of environment conditions from a nutritional point of view. It involves the addition of essential nutrients, such as nitrogen, phosphorous, carbon or oxygen, with the aim of stimulating the activity of microorganisms capable of remediation of contaminant. An investigation on the effect of the addition of different nutrients on microbial activity is very important for the optimization of the process and the cost reduction.

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The main advantage of biostimulation is that microorganisms already adapted to the site conditions and well distributed into the soil are exploited for bioremediation.

When the microbial population is not active enough (even after nutrient addition), or is not capable of degrading contaminant species, the bioaugmentation technique could be applied. It involves the supplement of a microbial population with the aim of improving the speed and obtaining a complete degradation of different and complex pollutants. A careful choice and adaptation of microorganisms is essential for the success of the bioaugmentation strategy.

The results of physical and biological characterization of a specific sandy soil are herein discussed; particularly, we focused on the laboratory experiments on several microcosms in order to test different environmental conditions. The effect of different mineral salt media and glucose addition were investigated. We detected the microbial activity by measuring the CO<sub>2</sub> produced in microcosms.

## II. Experimental

### A. Soil

The soil used in this study was excavated from 3 m under the surface, brought to the laboratory and sieved through a 2-mm mesh sieve to remove stones.

Soil was characterized by Water Content Reflectometry (WCR) inserting a sensor into the soil for a height of 30 cm and measuring the dielectrical permittivity of the soil. The volumetric water content ( $\vartheta$ ) can be derived from observed dielectrical permittivity according to the equation of Topp et al.[3](1):

$$\vartheta = 4.3 \cdot 10^{-6} \cdot \varepsilon^3 - 5.5 \cdot 10^{-4} \cdot \varepsilon^2 + 2.92 \cdot 10^{-2} \cdot \varepsilon - 5.3 \cdot 10^{-2} \quad (1)$$

where  $\varepsilon$  is the relative electrical permittivity measured by WCR. The tests was carried out in order to test the reliability of the approach to estimate the water content and the porosity of the sandy soil; moreover an infiltration test has been performed in order to evaluate the permeability of the soil. The test was performed in a Plexiglas column (soil height = 0.35 m; column diameter = 0.15 m), filled with sandy soil; the infiltration of water was carried out from the bottom upwards, applying a constant hydraulic head (about 1 m). The volume of about 0.007 m<sup>3</sup> of soil was saturated in about 20 minutes. The infiltration was monitored by continuous measurements of the dielectric permittivity. Response of the sample in dry and saturated condition has been analysed.

Following the method EN 1936:2006 (E), picnometry was exploited for the definition of real density of the soil.

Porosity was measured by means of infiltration tests in a column and calculated as the ratio of added water volume and material volume in the column.

## B. Bioremediation treatments

### 1) Soil microcosms

Several microcosms were prepared in order to test different environmental conditions. We placed 200 g of soil in sealed glass jars (1-L volume). Two replicates were tested for each condition. Different mineral salt media (MSM) were added to the soil microcosms separately:

- Czapek medium (CZ) [4]: 3 g/L NaNO<sub>3</sub>; 1 g/L K<sub>2</sub>HPO<sub>4</sub>; 0.2 g/L MgSO<sub>4</sub>; 0.2 g/L KCl; 0.01 g/L FeSO<sub>4</sub>.
- Mineral salt medium specific for bacteria (MSMB) [5]: 3 g/L NH<sub>4</sub>NO<sub>3</sub>; 0.5 g/L KH<sub>2</sub>PO<sub>4</sub>; 0.5 g/L K<sub>2</sub>HPO<sub>4</sub>\*H<sub>2</sub>O; 0.008 g/L MgSO<sub>4</sub>\*7H<sub>2</sub>O; 0.002 g/L CuSO<sub>4</sub>\*4H<sub>2</sub>O; 0.002 g/L MnSO<sub>4</sub>\*H<sub>2</sub>O; 0.002 g/L FeSO<sub>4</sub>\*7H<sub>2</sub>O; 0.002 g/L CaCl<sub>2</sub>\*2H<sub>2</sub>O. pH 7.5.
- Mineral salt medium specific for fungi (MSMF) [6]: 3 g/L Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>O; 3 g/L KH<sub>2</sub>PO<sub>4</sub>; 0.5 g/L NaCl; 1g/L NH<sub>4</sub>Cl; 0.5 g/L MgSO<sub>4</sub>\*7H<sub>2</sub>O; traces of CaCl<sub>2</sub>; FeCl<sub>3</sub>\*H<sub>2</sub>O. pH 5.5.

The effect of glucose addition in the medium (20 and 30 g/L) was tested to investigate the influence of the concentration of an easily exploitable carbon source.

Microcosms with CZ were monitored for a period of 30 days. Microcosms with MSMB and MSMF were monitored for 156 days.

Microcosms with MSMB and MSMF were contaminated with commercial diesel oil at 7.5% w/w soil. Sterilized soil was used for abiotic control microcosms and not contaminated soil was used for biotic controls.

During the monitoring period, all of the microcosms were opened every 3-4 days and mixed in order to promote the soil oxygenation. Moisture content was maintained at about 16% w/w with periodic addition of water.

### 2) Liquid cultures

Liquid cultures were prepared in Erlenmeyer flasks (500 mL) with cotton caps with 100 mL of medium. Different medium compositions were used:

- MSMB or MSMF
- Different concentration of glucose (0, 20 or 30 g/L) for both media.

1 g of soil, taken from MSMF or MSMB soil microcosms, was added as inoculum. In all the cultures 1% w/w of diesel oil was added. The cultures were incubated for 38 days in static conditions at ambient temperature.

### 3) Analytical techniques

The evaluation of the microbial activity was carried out by the quantification of the CO<sub>2</sub> (mg) produced in microcosms with the modified Isermeyer method [7]. The CO<sub>2</sub> produced

during the microbial activity was absorbed by a 1.5 M NaOH solution. Every 3-4 days, the NaOH solution was removed and titrated with HCl 1.5 M solution. The amount of carbon dioxide produced (mg CO<sub>2</sub>) was obtained by the equation (2):

$$\text{mg CO}_2 = (V_0 - V) * f \quad (2)$$

where V<sub>0</sub> and V are the volumes (mL) of HCl used to titrate the blank and the sample, respectively; and f=22\*M (22=CO<sub>2</sub> equivalent weight; M=molar concentration of HCl solution).

Value of pH and moisture content were also monitored in soil microcosms during the incubation period. The measurement of pH was carried out by taking a soil sample from microcosms and dispersing it in distilled water (1 g soil in 2,5 mL of water). The dispersion was stirred for 30 min and then the pH of water was measured after sedimentation of soil. Moisture content was monitored by weighing soil samples before and after complete drying in oven at 105°C.

In liquid cultures the biomass was recovered by means of centrifugation of the medium (4100 rpm for 15 min) followed by filtration (1.2 μm glass fiber filter). The biomass was then dried in oven at 105°C until constant weight and weighted on an analytical balance (accuracy ± 10<sup>-4</sup>g).

## III. Results and discussion

The optimization of bioremediation conditions for a specific soil was conducted after a physical characterization of the soil.

For the bioremediation process the biostimulation strategy was considered; the nutrient species for an effective stimulation of indigenous microorganism activity were defined.

### A. Soil characterization

The results obtained from soil characterization tests are reported in Table I.

The water content in dry condition was estimated by applying equation (1) from the dielectrical permittivity data; the soil porosity is derived by dielectrical permittivity measurement of the samples in saturated conditions.

TABLE I. SOIL PHYSICAL PROPERTIES

Property	Value
Water content in dry condition	1.3-1.5 % v/v
Real density	2700 kg/m <sup>3</sup>
Porosity	40-42 % v/v
Dielectrical permittivity of sandy soil (dry)	2.5-3
Dielectrical permittivity of saturated sandy soil	25-30
Dielectrical permittivity of water (at 20 °C)	78-79
Dielectrical permittivity of diesel oil	2.1-2.2

## B. Bioremediation set up

### 1) Indigenous microorganism activity in the soil

Initially, the water content was corrected to 16% w/w and microcosms were monitored for 9 days. Since there was no respirometric activity, Czapek medium was added at day 9 and 22 to stimulate microbial activity. Microcosms were monitored for a total of 31 days. Respirometric analysis results are reported in Fig.1.

After every addition of nutrients there was an increase in CO<sub>2</sub> production that showed that probably the soil was very poor in essential nutrients and that a stimulation was necessary for the activity of indigenous microbial population in aerobic conditions.

### 2) Identification of proper mineral salt medium for biostimulation

With the purpose of defining the most suitable mineral salt medium for the microbial growth and the diesel oil degradation, both soil microcosms and liquid cultures were investigated.

#### a) Soil microcosms

Cumulative CO<sub>2</sub> values at the end of the investigation period (156 days) are reported in Fig. 2.

It can be observed that, in all the microcosms contaminated with diesel oil (dashed bars), the production of CO<sub>2</sub> was higher than that of the corresponding ones without contamination (solid bars). This probably means that the inhibition of microbial activity due to the contaminant did not occur. On the contrary, the microbial population was probably exploiting and mineralizing the carbon derived from diesel oil.

As far as the medium composition is concerned, with 30 g/L of glucose, MSMF allowed to obtain a higher respiration than MSMB (3107 mg total CO<sub>2</sub> and 2853 mg total CO<sub>2</sub>, respectively). With 20 g/L of glucose the situation was reversed: in microcosms with MSMB the respiration was higher (2295 mg total CO<sub>2</sub>) than that obtained with MSMF (1714 mg total CO<sub>2</sub>).

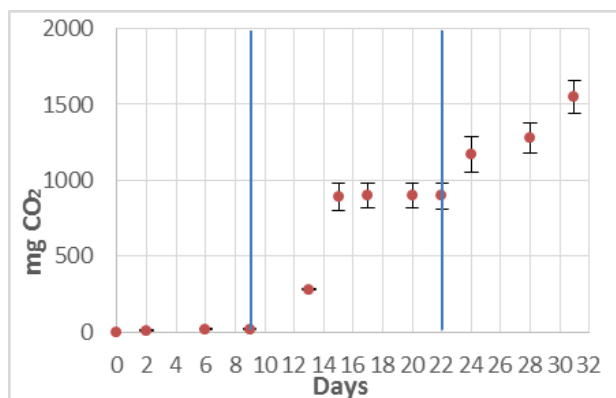


Figure 1. Cumulative CO<sub>2</sub> produced in microcosms with addition of Czapek medium. Vertical bold lines indicate the days of addition.

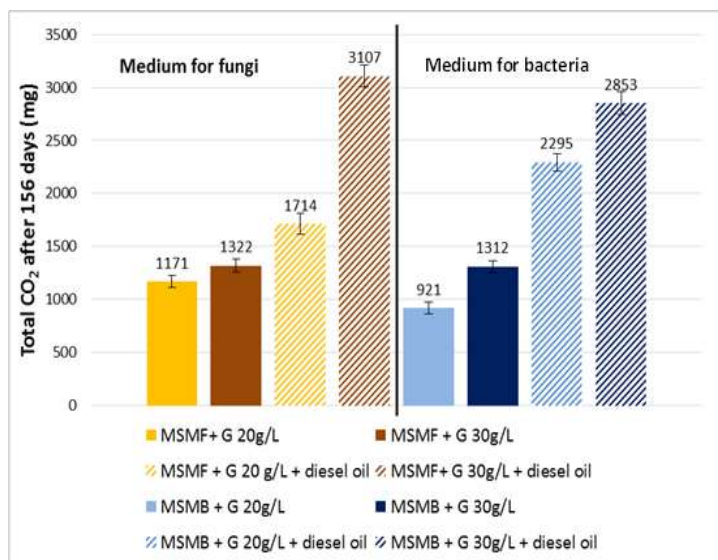


Figure 2. Cumulative CO<sub>2</sub> production (day 156) of soil microcosms with different medium compositions

pH of soil was not subjected to significant changes during the incubation period. It was in the range of 7.0-8.3, which is in the optimum range of 6÷8 for bioremediation applications [8].

#### b) Liquid cultures

The results from soil microcosms were not conclusive since there was not an MSM that allowed to obtain higher results of CO<sub>2</sub> production with both 20 g/L and 30 g/L of glucose. For this reason, liquid microcosms were prepared in order to observe differences in the biomass growth in the presence of contaminant and of different initial glucose concentrations. Diesel oil was added in all the liquid cultures. Values of biomass concentrations at the end of the incubation period (38 days) are reported in Table II.

As far as MSMF is concerned, there are no significant differences in biomass growth between the two higher concentration of glucose: 2,83 g/L for 20 g/L of glucose and 2,80 g/L for 30g/L of glucose. A lower amount of biomass was produced with only diesel oil as carbon source (1,33 g/L for MSMF).

With MSMB there is a greater distinction among the cultures with the two different glucose concentrations. The highest biomass concentration was obtained with 30 g/L of glucose (4,95 g/L of biomass), followed by the cultures with 20 g/L of glucose (3,53 g/L of biomass) and then the ones with no glucose (1,51 g/L of biomass).

TABLE II. BIOMASS CONCENTRATIONS IN LIQUID CULTURES

Medium composition	Biomass (g/L)
MSMF + 2%G + diesel oil	2,83 ± 0,26
MSMF + 3%G + diesel oil	2,80 ± 0,18
MSMB + 2%G + diesel oil	3,53 ± 1,90
MSMB + 3%G + diesel oil	4,95 ± 1,77
MSMF + diesel oil	1,33 ± 0,10
MSMB + diesel oil	1,51 ± 0,95

## References

In all the cases, there was a noticeable biomass growth even with only diesel oil as sole carbon source and the final biomass concentrations were higher with the MSMB than with MSMF. Probably, bacterial species can exploit more easily diesel oil as carbon source than fungi species. This was also reported in literature by other authors [9].

## IV. Conclusions

In this work, a biostimulation approach was set up for the remediation of diesel oil in soil.

The soil was firstly physically characterized. Then soil microcosms were prepared with the aim of investigating the most effective composition of added mineral salt medium. Two different mineral salt media, one specific for bacteria and one specific for fungi, were tested and two different glucose concentrations were considered.

Results showed that the medium favoring bacterial species (MSMB) was more effective in microbial stimulation, as observed in respirometric analyses and biomass concentration measurements. As far as the glucose concentration is concerned, a higher microbial activity was observed in the presence of the highest glucose concentration (30 g/L). At the same time, biomass was able to grow also in absence of glucose, exploiting diesel oil as sole carbon source. For this microbial population the presence of an easily accessible carbon source could not be essential for a successful bioremediation process.

These results will be used for the set-up of future bioremediation tests on this soil, where the bioaugmentation technique will be applied, too.

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