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# Speciation of the Removed Pollutants in Bioremediation of Hydrocarbon-Contaminated Soil

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**Abstract:** The biological removal of a mixture of soil contaminants, namely, hydrocarbons, is not equally efficient for each compound. Some pollutants can be metabolized by the microbial consortium but also generated again as by-products from the removal of others. At the end of the runs, notwithstanding the high integral removal, single compounds can still be present with a relevant concentration. This paper presents the results achieved in a study of the aerobic degradation of diesel oil in three mesocosms carried out for several months with the same operative conditions. They differed in biological management: Natural Attenuation (NA), Biostimulated without inoculation (BS), and Biostimulated with Inoculation (BS + IN). At the end of the runs, the pollution removal was calculated by measuring the residual diesel oil, both as an average in the total amount of soil and only at the bottom of each column. The overall removal was around 2%, 66%, and 72% for NA, BS and BS + IN, reduced to 0%, 48%, and 47%, respectively, if measured only at the bottom. For the biostimulated mesocosms, the speciation of the hydrocarbons was carried out to assess their concentration. The findings evidence the need to delve deeper into this issue and assess the speciation of contaminants.

**Keywords:** soil bioremediation; chemical speciation; gas chromatography analysis; diesel oil; pollution removal

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## 1. Introduction

Bioremediation is one of the efficient techniques used to remove biodegradable pollutants from soils, especially when aerobic degradation is adopted. It is easy to carry out and has a low cost, but these issues are counterbalanced by the long duration of the process and the need to know the optimal operative conditions to remove the highest amount of pollutants, different for each pollutant and microbial consortium. This information is obtained with experimental runs [1,2]. Usually, the main parameters to be optimized are the water content and the ratio of carbon to nitrogen (C/N) [3,4]. Other conditions, for example, the condition of soil saturation or the pollutant solubility in water, have relevance due to their influence on removal efficiency and kinetics [5,6].

The removal efficiency is usually assessed and calculated as an integral value, especially when a mixture of congeners, such as diesel oil, is present.

In a previous paper on a study in microcosms with 200 g of soil [7], the speciation of hydrocarbons evidenced their differential removal. The findings showed the changes in the pollution composition during the runs due to different metabolization rates for each compound. The overall removal efficiency was around 65%, while this value calculated for hydrocarbon clusters ranged between 53% and 88%.

In the current paper, bioremediation of soil polluted with diesel oil was studied in three systems at the mesocosm scale.

The findings of the geophysical monitoring and overall removal efficiency were published in a previous paper [8]. Concerning the geophysical properties, the results showed that the inoculated system had the fastest change rate of the electromagnetic properties, probably due to the enhanced microbial activity. This is in line with the hypothesis of depletion of the salts added for the process (decrease in the electrical conductivity) and growth of the microbial community (decrease in the electrical permittivity). These two hypotheses have not been experimentally demonstrated, yet.

In the present manuscript, the results achieved from the speciation of the residual hydrocarbons present at the end of each run in the biostimulated systems (BS and BS + IN) are presented.

In both mesocosms (BS and BS + IN), the removal efficiency by clusters achieved for the total amount of soil and just on the bottom was different, with a higher value for the total amount of soil for all the clusters. The results evidence the need to delve deeper into this issue when the process is applied onsite and the legislative limits to the concentration of specific pollutants must be respected.

## 2. Materials and Methods

### *2.1. Experimental Set-Up*

This study was carried out in three Plexiglas columns. Each column, with a diameter equal to 19 cm, contained 20 cm of soil, corresponding to 8.4 kg of soil. According to the USDA classification, the soil is loam-sand. The grain size of the soil was in the range between 0.15 mm and 2 mm. Moreover, a value of organic carbon content with mass equal to 1.2% and pH equal to 6.8 was measured.

The soil was artificially polluted with commercial diesel oil by manually mixing soil and diesel oil. Moreover, the first mesocosm was hydrated with tap water to simulate natural attenuation (NA). The others were hydrated by adding a nutrient solution suitable to biostimulate the bacterial growth and reach the optimal value of carbon to nitrogen ratio (C/N), as per the results of a previous study carried out in microcosms [9].

The biostimulated mesocosms had, respectively, no inoculum (BS) and inoculum deriving from a soil rich in indigenous hydrocarbon-degrading microorganisms (BS + IN) deriving from previous runs carried out at a smaller scale, on the same type of soil and with the same diesel oil. Each column was equipped with a time-domain reflectometry (TDR) probe to monitor the electrical conductivity and electrical permittivity of the soil. TDR sends a low-voltage pulse into the probe; the pulse propagates along two metallic rods and the impedance change at the end of the rods provides a reflection of the pulse. TDR measures the time between the release and return of the pulse from the reflection. By measuring the time and knowing the length of the rods, the propagation velocity of the soil is calculated, and the electrical permittivity is then computed. The electrical conductivity is estimated by the drop in the pulse amplitude (voltage) along the rods. The length of the rods was 0.12 m. Each probe (spacing between the rods = 32 mm) was completely immersed in the soil. Figure 1 shows the studied mesocosms.

**Figure 1.** Experimental setup of the three mesocosms with the sensors for monitoring electrical conductivity and permittivity and the connection with the datalogger and the laptop.

Table 1 reports the main physical and chemical parameters of the tested mesocosms.

**Table 1.** Main parameters of the mesocosms.

| Parameter/Mesocosm | Natural Attenuation (NA) | Biostimulation (BS) | Biostimulation + Inoculum (BS + IN) |
|---|---|---|---|
| Soil mass (kg) | 8.4 | 8.4 | 8.4 |
| Diameter of soil column (cm) | 19 | 19 | 19 |
| Height of soil (cm) | 20 | 20 | 20 |
| Porosity ($Volume_{pores} \cdot Volume_{tot}^{-1}$) | 0.45 | 0.45 | 0.45 |
| Quantity of inoculum (kg) | - | - | 0.65 |
| Inoculum/soil ratio ($kg_{inoculum} \cdot kg_{soil}^{-1}$) | - | - | 0.08 |
| Initial oil-degrading microorganisms ($CFU \cdot kg_{soil}^{-1}$) | - | - | $6.59 \times 10^7$ |
| Diesel oil content ($kg_{oil} \cdot kg_{soil}^{-1}$) | 0.05 | 0.05 | 0.05 |
| Water saturation ($Volume_{water} \cdot Volume_{pores}^{-1}$) | 0.33 | 0.33 | 0.33 |
| Moisture content ($kg_{water} \cdot kg_{soil}^{-1}$) | 0.10 | 0.10 | 0.10 |
| Carbon to nitrogen ratio ($g_C \cdot g_N^{-1}$) | - | 135 | 135 |
| Electrical conductivity of the nutrient solution ($S \cdot m^{-1}$) | 0.054 | 1.45 | 1.45 |
| Run duration (d) | 193 | 228 | 228 |

The run with the NA mesocosm was stopped after 193 days, having seen that the monitored geophysical parameters did not change anymore, demonstrating the absence of biological activity, while the test on the biostimulated mesocosms was prolonged to 228 days. At the end of each run, soil samples were collected according to the Italian Standards for Soil Sampling [10], as representative of the average amount of soil and of the bottom of the mesocosm.

### 2.2. Microcosm Properties

In all the mesocosms, the bioremediation process was studied using uncontaminated sandy soil with particle size distribution in the range of 0.15–2 mm.

Each mesocosm was manually polluted with commercial diesel oil (concentration = 0.05 $kg_{oil} \cdot kg_{soil}^{-1}$). Water was added to give the right water content to the microbial consortium (0.1 $kg_{water} \cdot kg_{soil}^{-1}$).

The biostimulation of BS and BS + IN mesocosms was carried out with a water solution containing salts suitable for stimulating bacterial growth. The solution composition is given in Table 2.

**Table 2.** Composition of the aqueous solution to stimulate bacterial growth.

| Salt | Concentration ($g \cdot L^{-1}$) |
|---|---|
| $(NH_4)_2HPO_4$ | 11.62 |
| $NH_4NO_3$ | 75.97 |
| $KH_2PO_4$ | 0.5 |
| $K_2HPO_4 \cdot H_2O$ | 0.5 |
| $MgSO_4 \cdot 7H_2O$ | 0.008 |
| $CuSO_4 \cdot 4H_2O$ | 0.002 |
| $MnSO_4 \cdot H_2O$ | 0.002 |
| $FeSO_4 \cdot 7H_2O$ | 0.002 |
| $CaCl_2 \cdot 2H_2O$ | 0.002 |

### 2.3. Diesel Oil Extraction and Gas Chromatography Analysis

The diesel oil was extracted from each mesocosm by applying the EPA method 3546 (moisture 15–30% by mass) [11], based on microwave heating. In this methodology, 2 g of soil and 30 mL of solvent (solution acetone/*n*-hexane with a ratio of 1:1 by volume)

were mixed, adding 2 g of anhydrous sodium sulphate to dry the soil. Then, the sample was heated at 110 °C and power of 1100 W for 15 min by microwaves; this temperature and power of 1100 W were kept for 10 min, and then the sample was cooled for 20 min. After the extraction, the sample was filtered through a 0.45 µm filter. This procedure was replicated, thus obtaining two extracts.

The diesel oil concentration was quantified by a gas chromatograph and by adopting the EPA method 8015 [12]. The gas chromatograph was equipped with a flame ionization detector and DB-5 fused silica capillary column, operated with helium as the carrier. The injector and detector were maintained at 220 °C and 250 °C, respectively. The following temperature program was used: initial temperature of 50 °C, ramp to 320 °C at 8 °C·min$^{-1}$, and a constant temperature of 320 °C for 40 min.

The characteristic peaks of the diesel oil hydrocarbons were contained between 6 and 31 min. The compounds that characterize the diesel oil were identified by standard kits, namely:

- C7–C30 saturated *n*-alkanes, 1000 µg·mL$^{-1}$ of each component in hexane;
- PAHs (acenaphthylene, anthracene, benzo(α)anthracene, fluorene, phenanthrene, pyrene), 500 mg·L$^{-1}$ of each component in acetone.

In each analysis, the retention times of the peaks were compared to the standard ones, and the *n*-alkanes from C8 to C24 and the six PAHs above-listed were identified.

Table 3 shows their list and their retention time; particularly, all the compounds exhibit retention times in the range between 6 and 31 min.

**Table 3.** Identified hydrocarbons and their retention time.

| Compound | Retention Time (min) |
|---|---|
| *n*-C8 | 6.3 |
| Acenaphthylene | 6.5 |
| *n*-C9 | 7.6 |
| *n*-C10 | 8.3 |
| *n*-C11 | 10.3 |
| *n*-C12 | 12.3 |
| *n*-C13 | 14.3 |
| *n*-C14 | 16.0 |
| Fluorene | 17.1 |
| *n*-C15 | 17.8 |
| *n*-C16 | 19.4 |
| Phenanthrene | 20.2 |
| *n*-C17 | 20.9 |
| *n*-C18 | 21.0 |
| Anthracene | 22.4 |
| Pyrene | 22.5 |
| *n*-C19 | 23.8 |
| *n*-C20 | 25.1 |
| *n*-C21 | 26.4 |
| *n*-C22 | 27.6 |
| Benzo(α)anthracene | 28.8 |
| *n*-C23 | 29.9 |
| *n*-C24 | 31.0 |

A calibration line of the gas chromatograph carried out with the commercial diesel oil used in the mesocosms was exploited to measure the residual diesel oil concentration.

For each extract, two gas chromatography analyses were carried out.

Figure 2 shows the chromatogram of the tested commercial diesel oil.

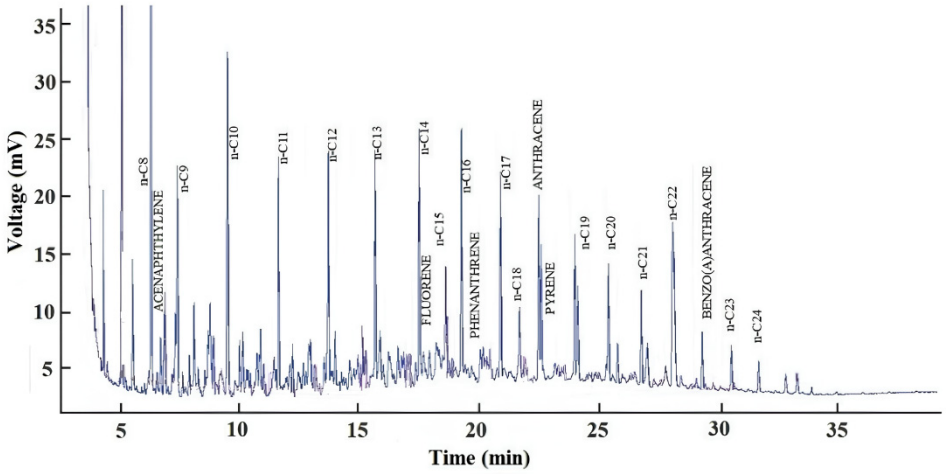


**Figure 2.** Chromatogram of the commercial diesel oil used in the study.

Diesel oil does not have a sharp composition. However, it is well known that it contains 65–70% by mass of saturated hydrocarbons and 30–35% by mass of aromatic ones.

Making use of the analytical results and the literature data [13,14], the composition of commercial diesel oil was defined by the number of carbon atoms and considering the three main kinds of hydrocarbons, namely, *n*-alkanes, branched and cycloalkanes, and aromatics. Table 4 reports the data.

**Table 4.** Composition of the commercial diesel oil used in this study.

| No. of Carbon Atoms | Concentration (% by Mass) | | |
|---|---|---|---|
| | ***n*-Alkanes** | **Branched- and Cyclo-Alkanes** | **Aromatics** |
| C8 | 100 | 0 | 0 |
| C9 | 65 | 0 | 35 |
| C10 | 53 | 18 | 30 |
| C11 | 42 | 47 | 11 |
| C12 | 12 | 80 | 8 |
| C13 | 29 | 14 | 57 |
| C14 | 13 | 66 | 21 |
| C15 | 23 | 73 | 4 |
| C16 | 59 | 0 | 41 |
| C17 | 55 | 31 | 14 |
| C18 | 44 | 22 | 34 |
| C19 | 59 | 32 | 9 |
| C20 | 37 | 47 | 16 |
| C21 | 67 | 33 | 0 |
| C22 | 100 | 0 | 0 |
| C23 | 36 | 25 | 39 |
| C24 | 100 | 0 | 0 |

### *2.4. Removal Efficiency*

The pollution removal efficiency, η%, can be calculated as follows:

$$\eta\% = (C_{IN} - C_{FIN})/C_{IN} \cdot 100 \quad (1)$$

where $C_{IN}$ and $C_{FIN}$ are the pollutant concentrations at the start and end of the test, respectively.

## 3. Results

### 3.1. Residual Concentration of the Diesel Oil Hydrocarbons

The results for the mesocosms at the start and end of the run, as average values on the total mesocosm soil, are reported in Table 5.

**Table 5.** Chemical composition ($g \cdot kg^{-1}$ of soil) of diesel oil by the number of carbon atoms at the beginning and end of the run for the tested mesocosms (average values on the total mesocosm).

| No. of Carbon Atoms | Composition ($g \cdot kg^{-1}$ of Soil) | | | |
|---|---|---|---|---|
| | At t = 0 Days | At t = 193 Days NA Mesocosm | At t = 228 Days BS Mesocosm | At t = 228 Days BS + IN Mesocosm |
| C8 | 0.8 | 1.7 | 0.3 | 0.4 |
| C9 | 1.7 | 1.5 | 0.2 | 0.1 |
| C10 | 4.0 | 1.4 | 0.4 | 0.4 |
| C11 | 5.3 | 4.5 | 1.4 | 1.2 |
| C12 | 4.9 | 4.1 | 1.9 | 1.5 |
| C13 | 5.6 | 4.6 | 2.6 | 2.0 |
| C14 | 7.3 | 6.8 | 2.2 | 1.9 |
| C15 | 3.9 | 4.0 | 1.2 | 0.9 |
| C16 | 4.6 | 5.5 | 0.9 | 0.5 |
| C17 | 2.3 | 2.7 | 1.3 | 1.3 |
| C18 | 1.7 | 1.3 | 1.4 | 0.9 |
| C19 | 2.5 | 2.0 | 0.9 | 0.4 |
| C20 | 3.3 | 2.3 | 0.9 | 1.2 |
| C21 | 1.0 | 2.8 | 0.4 | 0.5 |
| C22 | 0.7 | 2.5 | 0.3 | 0.1 |
| C23 | 0.6 | 1.0 | 0.3 | 0.3 |
| C24 | 0.2 | 0.4 | 0.2 | 0.2 |

At a glance, the very limited removal of diesel oil hydrocarbons for the NA mesocosm as supported by the geophysical monitoring is evident [8], whereas in the others, the values show degradation of the pollutants, even if to a different extent by the number of carbon atoms.

To better manage the data, the composition of the residual diesel oil was expressed by clusters of isomeric alkanes and aromatics. To this aim, it was decided to cluster the alkanes and aromatic according to the following ranges:

- C8–C11;
- C12–C15;
- C16–C19;
- C20–C24;
- Aromatics.

For the NA mesocosm, this analysis was not carried out due to its near-zero removal efficiency.

As for the removal efficiency, speciation was carried out on two kinds of soil samples: samples collected by mixing the whole mesocosm, and samples taken just at the bottom.

Composition by cluster is also interesting from the applicative point of view when the legislation on soil pollution imposes concentration limits linked to the number of carbon atoms. The current Italian legislation is an example of this, even if not unique. The concentration limits present in the current legislation are given in Table 6 [15].

**Table 6.** The maximum concentration of hydrocarbons given by the Italian legislation.

| | Maximum Allowed Concentration | |
|---|---|---|
| | Site for Public Use ($g \cdot kg^{-1}$ of Soil) | Site for Industrial and Commercial Use ($g \cdot kg^{-1}$ of Soil) |
| Light hydrocarbons (C $\leq$ 12) | 0.010 | 0.25 |
| Heavy hydrocarbons (C > 12) | 0.050 | 0.75 |
| Pyrene | 0.0050 | 0.050 |
| Benzo(α)anthracene | 0.00050 | 0.010 |

Tables 7 and 8 show the chemical composition of diesel oil by clusters for the biostimulated mesocosm, analyzed on the total soil and at the bottom.

**Table 7.** Chemical composition ($g \cdot kg^{-1}$ of soil) of diesel oil by clusters, at the beginning and end of the run for the BS mesocosm.

| Cluster | Composition ($g \cdot kg^{-1}$ of Soil) | | |
|---|---|---|---|
| | At t = 0 Days | At t = 228 Days Total Mesocosm | At t = 228 Days Bottom |
| C8–C11 | 9.4 | 2.2 ± 0.05 | 5.3 ± 0.46 |
| C12–C15 | 16.4 | 6.3 ±0.27 | 9.2 ± 0.51 |
| C16–C19 | 8.0 | 4.0 ± 0.47 | 5.3 ± 0.62 |
| C20–C24 | 4.9 | 2.2 ± 0.73 | 3.2 ± 0.35 |
| Aromatics | 11.4 | 2.1 ± 0.26 | 2.8 ± 0.01 |

**Table 8.** Chemical composition ($g \cdot kg^{-1}$ of soil) of diesel oil by clusters, at the beginning and end of the run for the BS + IN mesocosm.

| Cluster | Composition ($g \cdot kg^{-1}$ of Soil) | | |
|---|---|---|---|
| | At t = 0 Days | At t = 228 Days Total Mesocosm | At t = 228 Days Bottom |
| C8–C11 | 9.4 | 2.0 ± 0.02 | 5.6 ± 0.12 |
| C12–C15 | 16.4 | 4.9 ± 0.20 | 8.7 ± 0.06 |
| C16–C19 | 8.0 | 2.8 ± 0.24 | 4.7 ± 0.60 |
| C20–C24 | 4.9 | 2.4 ± 0.52 | 4.7 ± 1.94 |
| Aromatics | 11.4 | 1.7 ± 0.01 | 2.3 ± 0.05 |

In both cases, except for the aromatics, the concentration of residual diesel oil on the bottom is always much higher than the value of the total mesocosms.

Moreover, comparing our findings with the maximum allowed limits given by the current Italian legislation, the biological process did not sufficiently remove the pollution to leave the site suitable for reuse; this is to say, additional treatment for soil clean-up is compulsory.

### *3.2. Diesel Oil Removal Efficiency*

The integral quantification of pollution removal is given by Equation (1) in Section 2.4, and the results for the tested mesocosms are given in Table 9.

**Table 9.** Diesel oil removal efficiency (%) for the tested mesocosms.

| Mesocosm | Total | Bottom |
|---|---|---|
| NA (after 193 days) | 2 | 0 |
| BS (after 228 days) | 66 | 48 |
| BS + IN (after 228 days) | 72 | 47 |

The biostimulated mesocosms gave similar results, both for the total column and for the bottom. In other words, the inoculum was not so active in increasing the overall removal efficiency, even if it could have enhanced the kinetics in the first weeks, as could be interpreted from the faster-observed change in geophysical parameters [8]. Concerning the removal at the bottom, it was probably less efficient due to ineffective oxygen distribution.

Removal efficiency can also be calculated by clusters, also by applying Equation (1), to obtain more detailed results of the bioremediation process.

The results achieved for the BS and BS + IN mesocosms are shown in Figures 3 and 4, respectively.

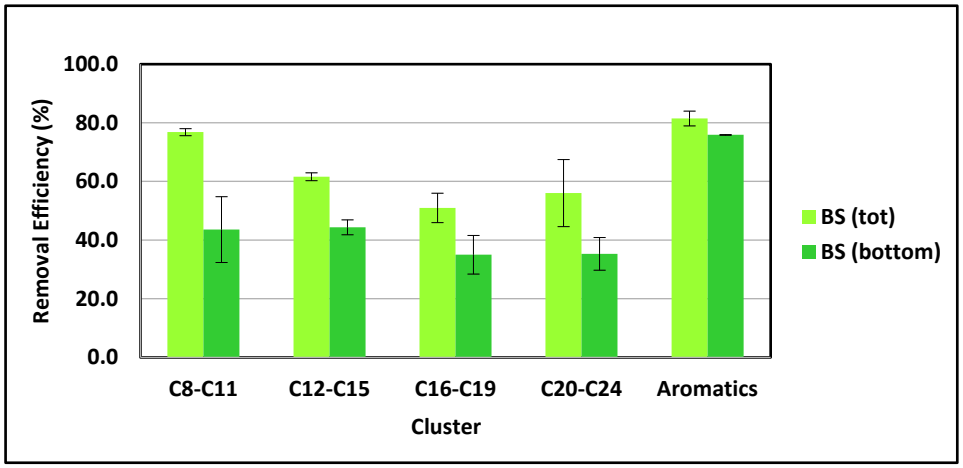


**Figure 3.** Removal efficiency by clusters of BS mesocosm.

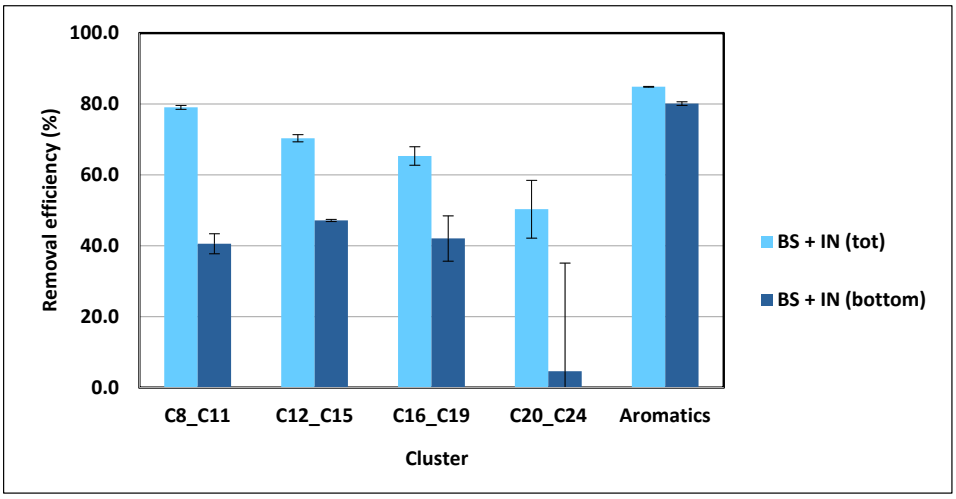


**Figure 4.** Removal efficiency by clusters of BS + IN mesocosm.

As expected, the removal efficiency reflects the findings for the pollutant residual concentration, with the values for the bottom always being lower than the average removal for the total amount of soil. The inoculated mesocosm (BS + IN) has a slightly better performance, even if the difference is not high.

## 4. Discussion

Looking at Figures 3 and 4, it is worth noting the behavior of clusters C16–C19 and C20–C24, which show values lower than those of the other clusters for both mesocosms and both assessments (average on the total and bottom). This result could be due to two contemporary and contrary effects:

1. The removal of alkanes belonging to these cluster;
2. The generation of these alkanes due to the degradation of other hydrocarbons.

As expected, the biostimulated mesocosms efficiently removed diesel oil from polluted soil, by 66% and 72%, respectively, with a slightly higher value for the inoculated system (BS + IN), whereas the NA test had almost no removal (around 2%). These findings are in line with the data achieved in microcosms where the tests of diesel oil biodegradation were carried out with the same soil and similar operative conditions [9].

Brzeszcz et al. [16] studied the removal of aliphatic and polycyclic aromatic hydrocarbons (PAHs) in microcosms of 500 g of polluted soil for 60 days. They used two consortia, namely, one with mixed identified microorganisms (C1) and one with identified hydrocarbon-degrading strains (C2). They also tested a control microcosm where only

natural attenuation was applied and one with biostimulation. The results for the removal of aliphatic compounds were as follows:

- 70% in the microcosm C1;
- 87% in the microcosm C2;
- 1% in the natural attenuation;
- 35% with biostimulation.

Correspondingly, for the PAH removal, the results were as follows:

- 65% in the microcosm C1;
- 85% in the microcosm C2;
- 0% in the natural attenuation;
- 24% with biostimulation.

Comparing these data to ours, good agreement of the findings achieved in the tests with mixed microorganisms can be observed, i.e., 66% (BS) and 70% (C1).

Regarding aromatics and PAHs, the comparison is less sharp since our result refers to aromatics (PAHs are part of aromatics): in the mesocosms, we achieved the removal of about 80% for both mesocosms, while Brzeszcz et al. removed 85% of their PAHs in the microcosm C2. With a gross comparison, the lower values achieved for the mesocosms can be also ascribed to the different management during the runs, especially the absence of aeration, whereas in the microcosms, the soil was periodically aerated.

Provided that the consortia were not analyzed by strains in our mesocosms, altogether, the findings for removal efficiency are satisfactory, especially considering the scale of the systems (8.4 kg of soil).

The speciation of hydrocarbons, carried out as the average value on the total amount of soil and the bottom, allowed for a more detailed picture of the removal efficiency given by clusters of compounds. The results reflect the integral ones. However, the value for aromatics needs a comment: among the clusters, it is always the highest for both mesocosms. Zeng et al. [17] studied the effects of temperature on the biodegradation of PAHs in microcosms (5 g of soil) for 60 days. They found that the optimal temperature was in the range of 20–28 °C. They also showed that at higher temperatures, as drawbacks, co-metabolism starts and the main PAH degraders deactivate, with a decrease in PAH removal. This seems in line with the results achieved in our tests, which were carried out at temperatures over 20 °C during the first 80 days of the runs, as monitored by the geophysical probe. This can support the high values obtained in both mesocosms, including the removal at the bottom of each mesocosm. In other words, also at the bottom of the mesocosms, the conditions for the process were more suitable at the start of the runs than towards the end when the depletion of nutrients and oxygen could have occurred.

Ma et al. [18] studied the removal of petroleum from sludge-polluted soil by different consortia and with different strategies of inoculation in biopiles of about 2500 kg. The starting concentration was around 124 $g \cdot kg^{-1}$ of soil and the runs lasted 60 days.

Their best efficiency was over 90% as an overall value with a sequential inoculation of fungal–bacterial consortia. Looking at their findings achieved with batch inoculation, it can be seen that:

- In the first case, the removal of aliphatic compounds was comparable to ours of alkanes, whereas the removal of the aromatics was lower than 60% (our values always reach 80%);
- In the second case, where the bacterium was the same, but the fungus was changed, the removal of aromatic compounds was over 80%, showing the relevance of the biological activity of the applied fungus.

These findings demonstrate that, when the strategy of inoculation is chosen, an accurate control of the adopted consortium could improve the efficiency of removal.

However, the comparison with our results also demonstrates the efficacy of consortia which contain microorganisms acclimated to diesel oil or petroleum hydrocarbons for the removal of these pollutants, also evidenced by other authors [16,17,19,20].

In their study on the strategies for remediation of soils contaminated with petroleum [20], Cui et al. also reported the relevance of monitoring soil enzyme activities. One of these activities is the hydrolysis of fluorescein diacetate (FDA) to fluorescein, which reflects microbial activity [21,22]. The quantity of fluorescein produced by hydrolysis in the mesocosms at the end of the runs [8] confirmed this enzymatic activity, especially for the BS + IN mesocosm: after 228 days, the fluorescein production was 2.2 $mg \cdot kg^{-1}$ of soil against the value 0.9 mg/kg of soil for the BS mesocosm.

At last, the microbial colonization in each mesocosm was shown by Scanning Electron Microscope images taken at different points and reported in a previous paper [8], as a further demonstration of microbial activity, even if at different extents. In the same images, the presence of biofilm can be supposed, even if no specific analyses were carried out to support this hypothesis.

To this end, nowadays, the biofilm role is known and verified in many systems [23], including the removal of long-chain alkanes [24]. One of its roles is the protection of microorganisms under extreme environmental conditions, such as oligotrophic conditions, where the quantity of nutrients is scarce. In the present study, at the end of the run (228 days), the biostimulated mesocosms had a depletion of micronutrients, as evidenced by the geophysical monitoring, and this occurred at the mesocosm bottom to a greater extent. At the same time, in both cases, the pollution removal efficiency was more than satisfactory, showing that the microbial consortia were active, and this could have occurred due to the protection of microorganisms by biofilm.

Deeper investigations are required to confirm the presence of biofilms as a "tool" involved positively in the biodegradation process. For this purpose, the analytical task could also make use of geophysical measurements (e.g., X-ray tomography with high resolving power is suggested to scan the pore–solid interaction) as a complementary system.

## 5. Conclusions

The analysis and speciation of the diesel oil compounds removed in aerobic mesocosms showed the following:

- A good removal efficiency could be achieved as the overall value in the total amount of soil;
- Lower values were obtained when the analysis of the residual diesel oil was carried out only at the bottom of the mesocosms;
- The speciation of the compounds showed different values, with the aromatics always presenting the highest removal efficiency, considering the alkanes (by clusters of the number of carbon atoms) and aromatics (altogether);
- The selective removal in biodegradation needs timely consideration when the process is applied at a real scale and legislative limits for specific compounds must be respected.

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