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Sustainable Recovery of an Agricultural Area Impacted by an Oil Spill Using Enhanced Phytoremediation

Marco Vocciante [1,*], Elisabetta Franchi [2], Danilo Fusini [2], Francesca Pedron [3], Meri Barbafieri [3], Gianniantonio Petruzzelli [3] and Andrea P. Reverberi [1]

[1] Department of Chemistry and Industrial Chemistry, University of Genova, Via Dodecaneso 31, 16146 Genova, Italy; andrea.reverberi@unige.it
[2] R&D Environmental & Biological Laboratories, Eni S.p.A., Via Maritano 26, 20097 San Donato Milanese, Italy; elisabetta.franchi@eni.com (E.F.); danilo.fusini@eni.com (D.F.)
[3] Institute of Research on Terrestrial Ecosystems, National Council of Research, Via Moruzzi 1, 56124 Pisa, Italy; francesca.pedron@cnr.it (F.P.); meri.barbafieri@cnr.it (M.B.); gianniantonio.petruzzelli@cnr.it (G.P.)
* Correspondence: marco.vocciante@unige.it

Abstract: In this paper, a viability study is conducted for a bioremediation intervention in a cultivated area contaminated by a pipeline oil spill. In this context, green technologies such as bioremediation and phytoremediation could represent an optimal solution for reducing pollution without deteriorating soil quality. The phytoremediation test was conducted at the microcosm scale using three plant species (*Zea mays*, *Lupinus albus* and *Medicago sativa*) and at the mesocosm scale (*Zea mays*), also evaluating the application of plant growth-promoting bacteria (PGPB). The results showed that the selected plants, being able to grow satisfactorily, are able to lessen the presence of hydrocarbons in the soil. An increase of 15–18% in the degradation of the C > 12 fractions in vegetated soils was observed, confirming the effect of plants on the biodegradation of hydrocarbons in the soil. Moreover, a further improvement was recorded after adding PGPB, resulting in fresh biomass production being up to 50% higher than the controls and the degradation of the C > 12 fraction increasing by up to an additional 10%. Particular attention was also paid to pyrene, considered an indicator of PAH contamination. At the end of the experimentation in vegetated soils, pyrene removal reached values above 50%. By favoring plant growth, the addition of PGPB resulted in a further up to 20% reduction in the content of the contaminant in the soil. The primary role of the plants in soil contaminated by petroleum derivatives was to accelerate the degradation of contaminants through the stimulation of microbial activity. Therefore, the cooperation between plants and microorganisms can be concretely used as a nature-based solution in a sustainable and economical way.

Keywords: hydrocarbon biodegradation; phytoremediation; nature-based solutions; plant growth-promoting bacteria; ecosystem restoration; carbon neutrality

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1. Introduction

When the environment is contaminated by liquid petroleum derivatives, whether intentionally or not, this act is denoted as an "oil spill". Even on a small scale (e.g., failures of pipelines), such events can cause severe environmental damage due to the displacement of contaminants, with transboundary effects [1].

Among the various approaches developed to deal with oil spills, sorbents are quite popular because they act in two ways: passively, exerting a containment effect, and actively, for the physical recovery of oil contamination from both land and water [2,3]. In fact, adsorption is known for its simplicity and efficacy in treating aqueous solutions contaminated with a wide range of pollutants [4–6].

Due to additional advantages such as the possibility of oil recovery and the absence of secondary pollution [7], the use of adsorbents is widespread in the case of oil spills involving water bodies, while for terrestrial oil spills, other physical, chemical, and biological

approaches are preferred, including in situ burning and bioremediation [8,9]. However, an aspect that is often not considered is that remediation activities do have an impact on the environment because the chemical products or processes involved may consume both materials and energy or lead to the undesired production of toxic byproducts (secondary contamination) [10].

An environmentally friendly and sustainable alternative consists of remediation technologies based on natural-based solutions (NBS), which allow either the elimination or reduction in the level of contaminants to achieve the desired security levels while looking for a minimal environmental footprint. Among NBS approaches, phytoremediation is crucial [10,11]. In this approach, the handling of organic and inorganic contaminants in the target matrix is carried out by plant species via extraction, degradation and stabilization, the actual methodology being dependent on the pollutant.

The low cost and simplicity of operation combined with the obvious environmental benefits [12] fuel interest in these phytotechnologies, also in combination with other green strategies to further reduce the overall carbon footprint [13,14]. Also included in this discourse are green roofs [15] and green-based materials derived from agricultural waste [16], which allow for an improvement of the energetic and environmental performance of urbanized areas by combining constructed wetland techniques with other approaches in order to alleviate the increasing effects of climate changes [17–19].

In addressing contamination by potentially toxic metals, phytoextraction—where roots adsorb metals which are subsequentially accumulated and translocated in different plant tissues—is considered a non-invasive, environmentally friendly, and economical approach [20,21]. Since plants can only take up species dissolved in the soil moisture [22], strategies to facilitate this process are constantly being investigated, such as chelating/mobilizing agents to increase the bioavailability of metal ions [23,24] or the use of plant growth-promoting bacteria (PGPB) such as *Bacillus flexus* [25] and *Kocuria rhizophila* [26], especially those who live in the rhizosphere (PGPR) [27,28], to support the plant in its biological activities, as well as promoting metals' mobility/bioavailability in the soil in some cases [29–31].

In the case of oil spills, petroleum derivatives such as alkanes, monocyclic aromatic hydrocarbons (e.g., BTEX), and polycyclic aromatic hydrocarbons (PAHs) impact soil quality due to important alterations to various physicochemical and biological characteristics. Even if present in traces, such compounds can harm soil microorganisms, reducing their activity and causing the saturation of the soil matrix, preventing root functionality and compromising plants subsistence [32]. PAHs, in particular, are of great concern both for the environment and human health, mainly due to the strong toxic properties they possess and the possibility of bioaccumulation [33,34], to the point that some of them are considered "priority pollutants" due to their carcinogenicity by the U.S. Environmental Protection Agency [35].

In general, the natural attenuation process takes place, that is, biodegradation mediated mainly by the native soil microbial community, which rapidly changes in both the type and quality of species through selective pressure operated by the specific contaminants [36]. However, each soil microorganism does not express all the required enzymes needed to oxidize hydrocarbons entirely, so only symbiotic relationships with other microorganisms (consortia) allow their effective potential to be fully exploited [37,38].

Unfortunately, PAHs are not easily degraded naturally and persist for long periods in soil, especially when they have a high molecular weight. Because most PAHs stay in the surface layers of soil explored by plant roots [39], these compounds pose a considerable threat to human health due to uptake from the soil by plant roots, compounded by atmospheric deposition through leaf stomata [40].

In addition to soil characteristics and plant species, plants uptake depends on PAHs' properties, such as the octanol–water partition coefficient (Kow) [41]. Highly lipophilic substances with log Kow > 5 can adsorb tightly on root surfaces. In contrast, hydrophilic substances with log Kow < 5 may be taken up by roots and translocated into other areas

of the plant, such as the shoots. This especially concerns water-soluble chemicals such as pyrene (log Kow = 4.88) [42].

The risk of contaminants entering the food chain makes contamination in agricultural areas of particular concern. At the same time, it is also necessary to reduce the soil degradation resulting from the remediation of this ecosystem as much as possible.

In this context, phytoremediation technologies play an essential role. The use of plants is particularly suitable for remediating soils aimed at agricultural production [43]. In addition to the attractive remediation features already mentioned, from low cost to a reduced environmental impact [12], these technologies do not interfere with other activities occurring outside of contaminated areas.

After an oil spill, plants provide optimal conditions for the proliferation of microorganisms capable of oxidizing hydrocarbons. This results in a greater drop in hydrocarbon concentration in vegetated soils compared to non-vegetated soils, confirming that rhizodegradation (oxidizing activity provided by rhizosphere microorganisms) is the main factor in the decomposition of hydrocarbon compounds [44,45]. Depending on soil characteristics, further contributions in reducing contamination may come from uptake by certain plant species, resulting in the degradation of contaminants into harmless substances or their immobilization through accumulation by roots [46].

Considering the above, while the release of root exudates by the plant is crucial in decreasing the concentration of PAHs—through increased populations of hydrocarbon-oxidizing microorganisms—for biodegradation to actually occur, it is important to exploit appropriate rhizodegradation processes [44]. However, PGPB are very specific in their action and their efficacy, which are influenced by many factors related to complex soil and plant interactions, making the selection of potentially effective consortia nontrivial [47].

This study deals with an extensive laboratory-scale experimental campaign conducted on soil from an agricultural area contaminated by a diesel oil spill due to the break-in of a pipeline near the field. The purpose was to evaluate the feasibility of phytoremediation by assessing the effectiveness of three plant species: *Zea mays*, *Lupinus albus* and *Medicago sativa*. This specific choice stems from the fact that they are plants commonly used in agriculture and are also already cultivated in the specific area studied, so they have obvious advantages in terms of their adaptability to the soil and climatic conditions. The efficacy of adding ad hoc selected PGPB in strongly promoting the biodegradation of PAHs by plants was also investigated to obtain an economic and ecological solution to fully restore the area ecosystem.

2. Materials and Methods

2.1. Site Description

The cultivated field of interest covers approximately 10,000 m^2 and is located in northern Italy. A nearby oil pipeline break-in caused diesel fuel to spill into the soil, resulting in hydrocarbon contamination. Surface (up to 1 m) and deep (1 to 2 m) soil samples were prepared, taking soil at selected investigation points (Figure S1) for laboratory (25 kg c.a.) and chemical (1 kg c.a.) analysis. The soil homogenization and preliminary sieving to 2 cm were conducted immediately in the field.

2.2. Soil Characterization

A chemical characterization of the soil samples taken was performed to assess the actual contamination levels. Table S1 reports the concentrations of the principal hydrocarbon contaminants detected by means of GC-MS analysis. This includes the hydrocarbon fractions $C \leq 12$ and $C > 12$ and the amounts of aromatics grouped according to Italian legislative decree D. Lgs.152/06. In particular, significant amounts of $C > 12$ are observed, between approximately 1900 and 5100 mg kg^{-1} (Table 1). The values for volatile hydrocarbons (VOCs) as measured directly during sampling are also reported.

Table 1. Soil texture and hydrocarbon concentrations for the four soils (labeled from A to D) undergoing phytoremediation tests, as obtained by appropriately grouping the sampled soils (except for S3). Data reported as the mean of replicates with the related standard deviation.

	Depth (m)	C ≤ 12 (mg kg^{-1})	C > 12 (mg kg^{-1})	Aromatics (mg kg^{-1})	Sand (%)	Clay (%)	Silt (%)
A (BH3 + CO8 + PZ8)	0–1	79.3 ± 3.7	3800 ± 97	1.41 ± 0.14	86.1	5.1	8.8
B (CO5 + PZ9 + PZ7 + C13 + C41)	0–1	50.6 ± 2.4	1820 ± 48	0.89 ± 0.09	88.6	4.3	7.1
C (BH3 + PZ7 + C13 + C41)	1–2	79.5 ± 3.5	3475 ± 85	2.89 ± 0.25	88.1	4.1	7.8
D (CO5 + PZ9 + CO8 + PZ8)	1–2	113.8 ± 5.1	4800 ± 115	5.55 ± 0.48	92.6	1.8	5.6

Sample S3, adjacent to the barrier, had significantly higher concentrations of hydrocarbons than the other samples. Therefore, this sample was selected for different treatments using a "train" of bioremediation technologies not discussed in this contribution. A detailed description can be found in [47]. The remaining soils were divided into four samples according to contaminant concentration and sampling depth, organizing the inquiry so that the samples analyzed were as informative as possible, considering that experimentation with plants is always subject to high variability due to the biological aspect. A physical characterization [48] of the as-obtained samples was also conducted (Table 1).

2.3. Hydrocarbon Oxidizing Bacteria Isolation and Characterization

For the isolation of hydrocarbon-oxidizing bacteria, one gram of the contaminated soil was obtained by mixing all the samples collected, as reported in Table S1. Then, this obtained sample was treated with 200 mL of a trace element solution [47], adding a MEM vitamin solution (Merck®, Darmstadt, Germany) and 5% diesel as the sole carbon source. This additional amount of diesel fuel added to soil already contaminated with hydrocarbons maintained the high selective pressure and promoted the subsequent selection of hydrocarbon-oxidizing strains in the soil. The resulting suspension was incubated in four 250 mL Erlenmeyer flasks, each containing 50 mL of liquid, at 30 °C under stirring for three full days. Then, to increase the enrichment and select the hydrocarbon-oxidizing microorganisms, the suspensions were diluted in fresh medium and regrown by repeating the step twice. The third growth serial dilutions (10^5–10^7) in sterile water were prepared and seeded on LB (Luria Bertani) and R2A plates.

After about five days, numerous phenotypically different colonies were visible. This result is consistent with the selection performed using diesel as the sole carbon source and with the recent contamination of the site. After several passages on the plate, 20 pure colonies were obtained, from which the genomic DNA was extracted using the Maxwell 16 system (Promega, Madison, WI, USA). The extracted DNA was used as a template to amplify the 16S rRNA fragments, as described in [49]. The 16S rRNA fragments were sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher™, Waltham, MA, USA) and the Seq Studio Genetic Analyzer Automated DNA Sequencer (ThermoFisher™, Waltham, MA, USA).

The SeqMan application (v.11.2.1) of Lasergene (DNASTAR®, Madison, WI, USA) software was used to assemble the resulting nucleotide sequences. Homology comparison, on the other hand, was performed with the basic local alignment search tool (BLAST analysis) by connecting to the server of the National Center for Biotechnology (NCBI) [50]. The consultation was carried out on 18 February 2022.

2.4. In Vitro Evaluation of PGP Properties

Only two isolated strains (*Kocuria rhizophila* and *Bacillus weidmanii*) were found to belong to Biohazard Level 1. For this reason, only these were used as subjects for a set of in vitro tests to comprehend their plant growth-promotion capabilities [51,52]. The capability of producing exopolysaccharides (EPS) was assessed as described in [53], the production of siderophore molecules was assessed as reported in [54], the synthesis of

3-indole acetic acid (IAA) was assessed as described in [55] and the synthesis of ammonia was assessed based on the indications in [56]. The proteolytic activity (degradation of casein) was evaluated via the method adopted in [57], and the ability to solubilize inorganic phosphate was determined as described in [58]. Finally, the last test was designed to check biofilm formation ability. The samples were inoculated in glass test tubes with LB medium and incubated at 30 °C for one week without shaking. A visible pellicle at the interface between the culture medium and air suggested the potential ability of in vivo biofilm production [49]. Both strains have shown the ability to produce IAA, siderophores and ammonia. The *Kocuria rhizophila* strain could also solubilize inorganic P, and the *Bacillus weidmanii* strain also formed biofilm.

The two strains were grown separately in LB medium for 48 h to prepare the inoculum. Then, the cultures were centrifuged (9000 rpm, 20 min), resuspended using a solution containing 1% sodium glutamate and 7% sucrose (after discarding the supernatant), and then combined. The resulting medium was divided into aliquots, frozen for 16 h, and then freeze-dried for storage [47].

2.5. Microcosm Tests

The microcosm-scale experimental campaign was carried out following a fully randomized strategy with the following characteristics:

- Four soil types: A, B, C and D (as described in Table 1), with 500 g of contaminated soil per pot;
- Three plant species: *Medicago sativa* (alfalfa)—0.8 g seeds, *Zea mays* (corn)—5 seeds, *Lupinus albus* (lupine)—6 seeds;
- Three different tests per plant and soil type: a control (CT) using neighboring uncontaminated agricultural land with similar characteristics, a "base" test using contaminated soil, and an additional test by adding PGPB inoculum to the contaminated soil.

Control tests were run in duplicate, while 5 replicates were conducted for the other tests, for a total of 144 microcosms. The tests occurred inside a climatic chamber (CCL300BH-AS S.p.A., Perugia, Italy) to allow full control of the environment. The night and day cycle was set as follows: 24 °C with a light on (photon flux density of 130 μmol m^{-2} s^{-1}) for 14 h, followed by a 10 h period with no light at 19 °C. For irrigation purposes, tap water was administered daily with no addition of fertilizer due to the good concentration of nutrients (mainly N, P, and K) already present in the soil. Inocula were added at a concentration of 10^8 CFU (colony forming units). After 30 days of experimentation, plants were collected and, after the separation of roots and shoots, the fresh biomass (FW) was measured through gravimetrical methods [47]. A GC-MS analysis was conducted following the protocol described in EPA 3545A:2007 and EPA 8270E:2017 to determine the presence and concentration of PAHs in both soil and plants. The PAH concentration in soils was measured at the beginning of experimentation and after plant harvest.

2.6. Mesocosm Tests

Following the microcosm growth tests, a greenhouse (mesocosm) experiment was also conducted with the plant species *Z. mays* to assess the effectiveness of plants while biodegrading under conditions more similar to a potential real-world application. For this test, a larger amount of soil was used, specifically 5 kg of soil (contaminated or control) per mesocosm, in which 14 seeds of *Z. mays* per pot were sown. The experiment, replicated five times, followed the same setup as the microcosm trial, but plants were allowed to grow for longer. Water was added depending on plant needs, making sure to add water to non-vegetated soils to mimic natural attenuation processes. Approximately 60 days after planting, the plants were harvested, and the C > 12 hydrocarbons concentration was analyzed as already described in vegetated and non-vegetated soils. Due to the increased complexity of the trials and the uncertainty regarding the effect of the inocula, at present, it was chosen not to extend such experimentation to mesocosms, so the use of PGPB was limited to microcosms.

2.7. GC-MS Analysis of Samples

A Buchi Speed Extractor E-916 (BÜCHI Labortechnik AG, Flawil, Switzerland) was used to perform the extraction. This machine operates using 20 mL steel cells; each cell was filled with a bed of diatomaceous earth and 10 g of sample diluted with quartz. A mixture of hexane/dichloromethane 9:1 v/v was chosen as the extraction solvent. To perform the extraction, each cell was held at 50 °C and 50 bar for 5 min, washed with fresh solvent for 3 min and washed again with nitrogen for three more minutes. The whole procedure was repeated a total of 10 times for each cell. The resulting extract was concentrated to below 10 mL using a 40 °C bath under nitrogen flow. Pure hexane was used to wash the vials and increase the volume to 10 mL.

An Agilent 7820A GC using an MSD5977E mass selection detector operating in split mode was employed to analyze the extracts. The chosen column was an Agilent HP5 MS 30 m, 0.25 mm, 0.25 mm column. The method was as follows:

- Thermal gradient of 40 °C (isothermal for 2 min) with a ramp of 7 °C/min up to 270 °C;
- Tamp of 15 °C/min up to 320 °C;
- A 320 °C isotherm for 10 min;
- "SCAN" mode (mass from 50 to 600).

A 5-point calibration curve with a concentration between 5 and 100 ppm was used for quantification. A 200 ppm control point was performed to check linearity (not used for analysis).

2.8. Statistical Analysis

All statistical analyses were performed using STATISTICA version 6.0 (StatSoft Inc., Tulsa, OK, USA, 2017).

3. Results and Discussion

3.1. Properties of the PGPB Inoculum

Kocuria rhizophila (GenBank Accession No. OM 743439) and *Bacillus wiedmannii* (GenBank Accession No. OM 743440) strains have demonstrated the possession of three of the most interesting plant growth-promoting (PGP) properties. In particular, both strains have demonstrated the ability to produce IAA, siderophores and ammonia. The *K. rhizophila* strain was also capable of inorganic phosphate solubilization, while the *B. weidmanii* strain formed biofilm in vitro. Hussain et al. [26] observed that *K. rhizophila*, associated with citric acid, increased *Glycine max* L. plant biomass by approximately 38.73% compared to uninoculated plants. Furthermore, the *Kocuria rhizophila* Y1 strain isolated from the rhizosphere of *Zea mays* has been shown to solubilize phosphate and produce IAA [59–61]. Inoculating *Zea mays* with 10% salinity increased biomass production, photosynthetic capacity, antioxidant levels and chlorophyll accumulation compared to uninoculated plants [60]. Lastly, in a recent study [62], isolation and selection of Pb and Cd-tolerant PGPB from the roots of *Helianthus petiolaris* were performed. One of these strains (*Bacillus wiedmannii* ST29), inoculated into *Helianthus annuus* plants, reduced the bioaccumulation of Cd by 40%.

3.2. Plant Response at Microcosm Scale

Biomass data are shown in Table S2 and Figure 1. As can be seen, there are no significant variations in growth when the different contaminated soils are considered. For each species, the values of biomass produced also seem to depend little on the sampling depth (see samples description in Table 1). In general, plants sown in the contaminated soil did not demonstrate any visible signs of stress or phytotoxicity. In the case of microcosms not treated with PGPB, however, there is a tendency for lower biomass production than growth on uncontaminated soil (CT) taken as a reference. This is especially evident in the aerial part, with an average growth reduction of about 33% for *M. sativa*, 38% for *L. albus*, and 40% for *Z. mays*.

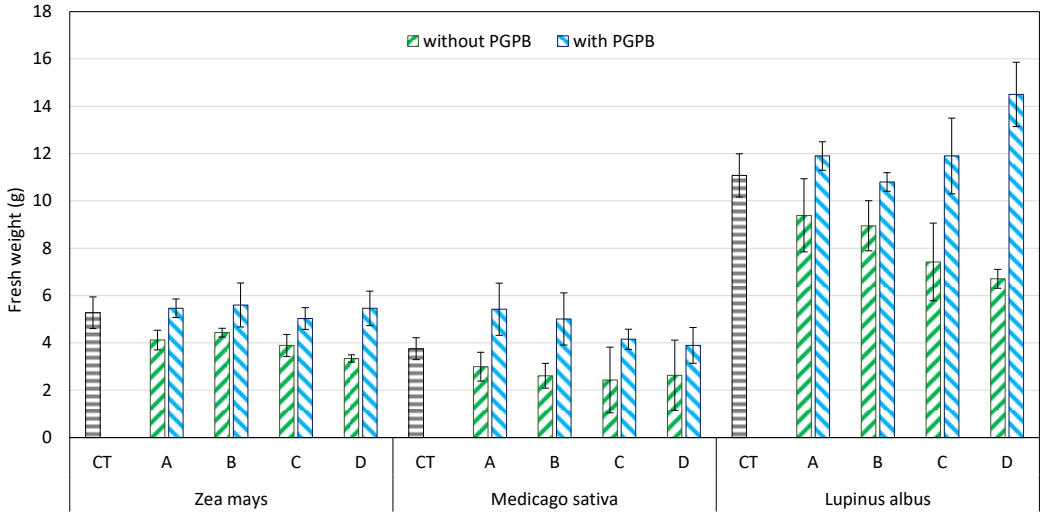


Figure 1. Fresh weight (g) of shoots of plants grown on different soils (A–D) compared to control (CT) during microcosm tests. Data reported as the mean of replicates with the related standard deviation. (+) indicates the addition of PGPB.

On the other hand, the addition of PGPB shows a significant positive effect, which succeeds in overcoming plant growth difficulties, as confirmed by the production of fresh biomass at levels similar to or even higher than the control. This increase is up to 50% higher than in the respective case without PGPB, with results particularly evident in the aerial part of *L. albus* in soil D and *M. sativa* in soils A and B (Figure 1). This is because PGPB create an environment that allows the plant to withstand the pollution-driven abiotic stress by increasing the bioavailability of essential nutrients such as iron, nitrogen, and phosphorus, or by producing phytohormones like indoleacetic acid (IAA). This results in better plant health and, ultimately, higher biomass production, which is one of the core pieces of data needed to identify a successful phytoremediation intervention [14].

The hydrocarbon content in plants (Table 2) was evaluated for both the C > 12 fraction and the C $\leq$ 12 fraction (of minor significance).

Table 2. Concentration (mg kg^{-1}d.m.) of hydrocarbons (C $\leq$ 12 and C > 12) contained in shoots of plants grown on different soils (A–D) compared to control (CT) during microcosm tests. Data reported as the mean of replicates with the related standard deviation. (+) indicates the addition of PGPB.

	Zea mays		***Medicago sativa***		***Lupinus albus***	
	C $\leq$ 12	**C > 12**	**C $\leq$ 12**	**C > 12**	**C $\leq$ 12**	**C > 12**
A	0.91 ± 0.16	215 ± 100	4.10 ± 0.9	931 ± 217	0.83 ± 0.28	227 ± 51.6
A+	1.07 ± 0.49	207 ± 35.3	4.30 ± 1.59	988 ± 144	1.16 ± 0.53	240 ± 42.1
B	0.98 ± 0.18	185 ± 117	3.40 ± 0.7	675 ± 149	1.10 ± 0.25	167 ± 85.9
B+	1.07 ± 0.18	162 ± 53.6	2.50 ± 0.46	664 ± 81.5	0.91 ± 0.30	144 ± 51.6
C	0.90 ± 0.22	228 ± 133	4.60 ± 1.0	1050 ± 340	0.87 ± 0.46	273 ± 45
C+	0.92 ± 0.21	238 ± 47.0	4.40 ± 1.28	1073 ± 239	0.95 ± 0.49	260 ± 97.0
D	0.85 ± 0.10	183 ± 111	4.30 ± 1.0	1271 ± 381	0.84 ± 0.33	264 ± 68.6
D+	1.01 ± 0.14	180 ± 39.7	5.10 ± 0.35	1266 ± 302	0.88 ± 0.27	274 ± 49.1
CT	n.d.	122 ± 67.0	1.23 ± 0.81	253 ± 136	4.00 ± 1.56	265 ± 75

To assess the origin of hydrocarbons in plants, plants were also grown (in duplicate) on control soil from organic farming, which was characterized by very low values of hydrocarbons, showing considerable variability in the concentration of these compounds.

Since corn is the most widely grown plant in the area, special attention was paid in checking the behavior of this species, so as also to protect the food chain from the potential

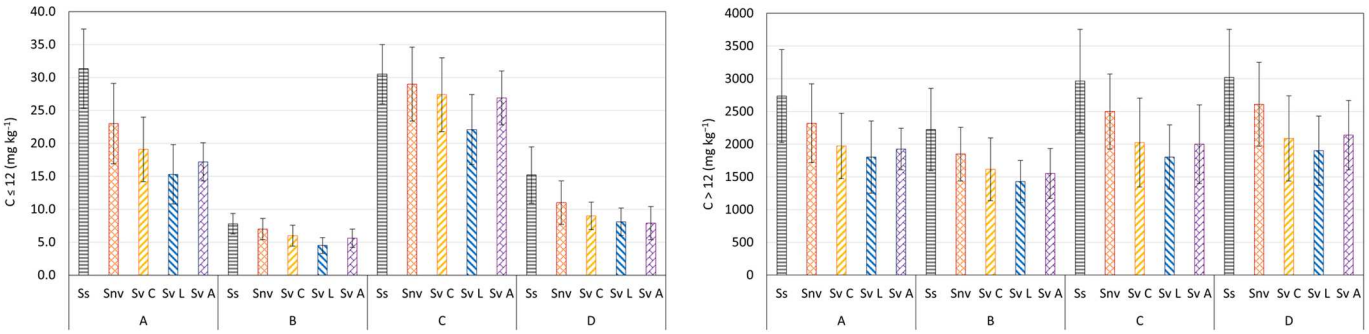
spread of the contamination. Considering as a reference the natural value of biogenic origin obtained, which in the case of *Z. mays* is 122 ± 67.0 mg kg^{-1} of C > 12 (control soil in Table 2), it is noted that higher concentrations in plants were detected in the presence of contaminated soil (in some cases over 300 mg kg^{-1}), which could indicate a passage of the hydrocarbons into the plant. However, this aspect is not particularly evident when considering the average value of all microcosms, given the significant variability in the individual concentrations found. Even for *M. sativa*, an increase is noted that would seem quite significant with respect to the biogenic content, although the inherent variability of the biological system should always be considered. With regard to *L. albus*, by contrast, the values found in plants grown on the contaminated soil fall substantially within the range of the concentrations found in the control plants grown on organic farming soils.

Also, in the tests with PGPB, the hydrocarbon concentration values were generally not significantly different from those found in plants grown on control soils. This seems to confirm that the uptake of these compounds from the soil, if any, is very small and that the essential action of plants in the phytoremediation process is that of rhizodegradation, which is further promoted by the presence of PGPB [63]. The only exception seems to be *M. sativa*, which reconfirms a more pronounced difference in hydrocarbon content than plants grown in the control soil. However, the considerable data variability invites some caution in interpreting the results. In fact, as will be shown in the next section, examining the residual amounts of hydrocarbons in the soil after plant growth does not reveal particularly different reductions compared to the other two plant species considered.

3.3. Contaminants Biodegradation at Microcosm Scale

Concerning the soil, it should be considered that this test takes place under conditions of "still dynamic" contamination, leading to a natural reduction in the level of hydrocarbons resulting from the oil spill. This is a markedly different condition than in a site where those substances have reached a kind of equilibrium with all soil components, both biotic and abiotic (i.e., degradation processes with much slower kinetics).

For this reason, the behavior of hydrocarbons C ≤ 12 and C > 12 in non-vegetated soil (Snv) was also evaluated to control the action of different plant species (Sv) in the microcosm test performed compared to the initial values detected in each soil (Ss) (Figure 2).



(**a**) (**b**)

Figure 2. Variation of C ≤ 12 (**a**) and C > 12 (**b**) hydrocarbon content in the soil (mg kg^{-1}d.m.) for different soils (A–D) during microcosm tests. Data reported as the mean of replicates with the related standard deviation. Ss = starting soil, Snv = non-vegetated soils; Sv = vegetated soil; C, L and A denote *Z. mays* (Corn), *L. albus* (Lupine) and *M. sativa* (Alfalfa), respectively.

Looking at the trends in Figure 2 and considering the average concentration values, a downward trend of these compounds in the microcosm test period emerges (Snv), which seems to be further favored by the presence of plants (Sv). Although the results obtained are not always statistically significant due to the considerable variability of the analytical data, this observation can be quantified as an increase in degradation of about 15–18% in

planted soils in comparison to bare soils, confirming the generally positive effect of the presence of plants in reducing hydrocarbons in soil.

This trend of reducing hydrocarbon concentrations in soils shows further accentuation in the presence of PGPB. A direct comparison is shown in Figure 3, where the percentage reduction in the level of the C > 12 fraction in soils during microcosm tests with and without PGPB is reported.

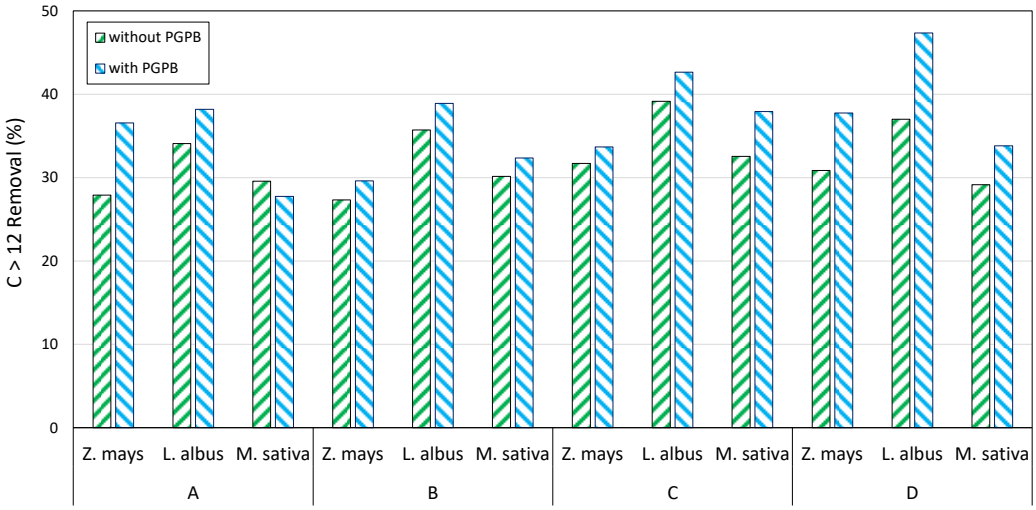


Figure 3. Comparison of the percentage reduction in the mean values of C > 12 in vegetated soil for different soils (A–D) from microcosm tests with and without PGPB. The reduction is intended as relative to the control values of the corresponding soil type, obtained as (Ss − Sv)/Ss × 100.

Although the percentage of reduction in the concentration of C > 12 does not vary significantly with the addition of PGPB, Figure 3 suggests a general improvement, quantifiable as 5 to 10 percent of C > 12 further eliminated from the soil compared to the initial concentration.

The results obtained are generally in line with what was expected. Although the plant can aid soil remediation via the absorption of contaminants and accumulation at the root level [42], the main action that plants perform in the presence of hydrocarbons is the stimulation and promotion of microorganisms capable of oxidizing hydrocarbon through the production of root exudates [44]. The direct activity of hydrocarbon-oxidizing bacteria, in addition to reducing contamination levels, promotes plant growth under stressed conditions. Thus, the interaction between plants and microorganisms is crucial in removing soil contaminants [26].

3.4. PAHs Evolution in Plants and Soils

The reduced biomass observed in Figure 1 in normal conditions (without PGPB) is also influenced by the presence of PAHs in the soil [64]. However, a clear match between the reduction in biomass yield and a boost in PAH concentration in the plants used was not found, since PAHs in plants are usually below the limit of quantification. Rare exceptions are sporadic values of a few μg kg^{-1} of pyrene detected in the aerial part of *M. sativa* and *Z. mays* [63].

Referring specifically to pyrene, which, as mentioned, is generally used as a reference for PAHs, the very low uptake observed agrees with previous studies reporting an often-negligible uptake due to its limited presence in available forms, i.e., dissolved in the liquid phase of the soil [65]. However, the concentrations of these PAHs in plants were not directly related to the lowering of their concentrations in the soil because of plant growth, which is the most important aspect of assessing the effectiveness of phytoremediation. The related data are shown in Table 3.

Table 3. Concentration of PAHs in the soils (μg kg^{-1}d.m.) under initial conditions (Ss) and at the end of the microcosm test for non-vegetated soils (Snv) and after the growth of investigated plants (Sv). The addition of PGPB is indicated with (+). Mean values are shown for each soil, and different superscript letters in each row indicate significant differences using one-way ANOVA ($p < 0.05$). nd = not detected. Adapted from [63].

	A								**B**							
			Z. mays		*L. albus*		*M. sativa*				*Z. mays*		*L. albus*		*M. sativa*	
	Ss	Snv	Sv C	Sv C+	Sv L	Sv L+	Sv A	Sv A+	Ss	Snv	Sv C	Sv C+	Sv L	Sv L+	Sv A	Sv A+
Benzo(a)pyrene	0.74 ^{c}	0.70 ^{b}	nd	nd	nd	nd	0.65 ^{ab}	0.58 ^{a}	0.38 ^{a}	0.74 ^{b}	nd	nd	nd	nd	nd	nd
Benzo(b)fluoranthene	3.1	nd	nd	nd	nd	nd	nd	nd	2.1	nd	nd	nd	nd	nd	nd	nd
Benzo(k)fluoranthene	1.7	nd	nd	nd	nd	nd	nd	nd	1.0	nd	nd	nd	nd	nd	nd	nd
Benzo(g,h,i)perylene	8.6 ^{e}	7.2 ^{d}	6.2 ^{c}	5.7 ^{c}	4.2 ^{b}	3.1 ^{a}	6.5 ^{c}	5.5 ^{c}	5.4 ^{b}	6.8 ^{c}	5.2 ^{b}	5.1 ^{b}	3.4 ^{a}	2.9 ^{a}	5.2 ^{b}	5.0 ^{b}
Dibenzo(a,i)pyrene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Chrysene	28.0 ^{c}	21.0 ^{b}	17.8 ^{a}	16.4 ^{a}	16.5 ^{a}	15.4 ^{a}	18.5 ^{a}	16.8 ^{a}	26 ^{d}	22 ^{c}	18.1 ^{b}	14.8 ^{a}	15.4 ^{a}	13.9 ^{a}	20.0 ^{b}	15.0 ^{a}
Indeno(1,2,3-c,d)pyrene	0.81 ^{a}	0.82 ^{a}	nd	nd	nd	nd	nd	nd	0.45 ^{a}	0.43 ^{a}	nd	nd	nd	nd	nd	nd
Pyrene	1500 ^{e}	890 ^{d}	725 ^{c}	650 ^{b}	683 ^{b}	536 ^{a}	720 ^{b}	564 ^{a}	1100 ^{e}	800 ^{d}	681 ^{c}	610 ^{b}	630 ^{b}	546 ^{a}	632 ^{b}	535 ^{a}
	C								**D**							
			Z. mays		*L. albus*		*M. sativa*				*Z. mays*		*L. albus*		*M. sativa*	
	Ss	Snv	Sv C	Sv C+	Sv L	Sv L+	Sv A	Sv A+	Ss	Snv	Sv C	Sv C+	Sv L	Sv L+	Sv A	Sv A+
Benzo(a)pyrene	0.66 ^{a}	1.2 ^{b}	nd	nd	nd	nd	0.81 ^{b}	0.5 ^{a}	0.78 ^{a}	0.81 ^{a}	nd	nd	nd	nd	0.68	0.55
Benzo(b)fluoranthene	3.3	nd	nd	nd	nd	nd	nd	nd	2.7	nd	nd	nd	nd	nd	nd	nd
Benzo(k)fluoranthene	1.6	nd	nd	nd	nd	nd	nd	nd	1.7	nd	nd	nd	nd	nd	nd	nd
Benzo(g,h,i)perylene	10.0 ^{b}	13.0 ^{d}	9.5 ^{b}	8.5 ^{ab}	8.1 ^{a}	7.6 ^{a}	9.4 ^{b}	7.8 ^{a}	11.1 ^{b}	10 ^{b}	8.3 ^{a}	7.1 ^{a}	8.0 ^{a}	6.8 ^{a}	10.1 ^{b}	8.1 ^{a}
Dibenzo(a,i)pyrene	0.62	nd	nd	nd	nd	nd	nd	nd	0.66	nd	nd	nd	nd	nd	nd	nd
Chrysene	52.0 ^{d}	29.1 ^{c}	23.4 ^{b}	20.4 ^{a}	20.1 ^{a}	17.2 ^{a}	30.5 ^{c}	20.4 ^{a}	53.2 ^{e}	42 ^{d}	35.1 ^{c}	23.8 ^{a}	30.4 ^{b}	18.6 ^{a}	39.6 ^{c}	21.4 ^{a}
Indeno(1,2,3-c,d)pyrene	0.81 ^{a}	0.92 ^{a}	nd	nd	nd	nd	nd	nd	0.84 ^{a}	0.79 ^{a}	nd	nd	nd	nd	nd	nd
Pyrene	1700 ^{e}	780 ^{d}	598 ^{b}	530 ^{a}	562 ^{b}	509 ^{a}	694 ^{c}	588 ^{b}	1600 ^{c}	633 ^{b}	511 ^{a}	500 ^{a}	495 ^{a}	464 ^{a}	651 ^{b}	573 ^{a}

The data in Table 3 show a reduction in the presence of PAHs in the soil at the end of the experiment, magnified by the plants' contribution compared to barren soil. Indeed, plants produce a wide spectrum of root exudates, which support the increase in the level of microorganisms in the rhizosphere by providing nutrients; this effectively quickens the microbial degradation of PAHs in the soil.

Nutrients such as nitrogen, phosphorus, potassium (NPK) and even electron acceptors like oxygen are often added to hydrocarbon-polluted sites to promote the biodegradation activity of aerobic bacteria. However, many nutrients are naturally provided by the root exudates, activating a powerful synergy between plants and rhizosphere bacteria (PGPB), which can decrease the toxicity of organic contaminants, thus promoting plant growth [66–68]. Furthermore, this decrease can be amplified wherever PGPB are also added. This is in line with what has already been discussed: in the presence of contaminants, PGPB inoculation resulted in improved plant growth attested by fresh biomass production.

Specifically, different behaviors can be observed depending on the contaminant and its initial concentration. In some cases, a substantially complete removal is achieved even in the absence of plants, simply via natural soil processes (benzo (b) fluoranthene and benzo (k) fluoranthene). In other cases, degradation occurs only in vegetated soils (benzo (a) pyrene), or it occurs appreciably only after the addition of PGPB (benzo (g, h, i) perylene). However, it is not possible to identify more effective strains for general use, as soil conditions and the type of contamination can affect their effectiveness [66]. Luckily, the strains isolated in this trial proved applicable for the intended purpose. More evident results have been obtained with chrysene, demonstrating the same decreasing tendency in planted soil, particularly when adding PGPB [66].

Similar behavior was also found for pyrene, which is of particular interest both because it is the hydrocarbon present in the highest concentration and because it is treated as a PAH contamination indicator in soils and as a clue regarding the contamination origin [69].

Figure 4 shows the degradation of pyrene in vegetated (Sv) and non-vegetated (Snv) soils compared with the initial values (Ss).

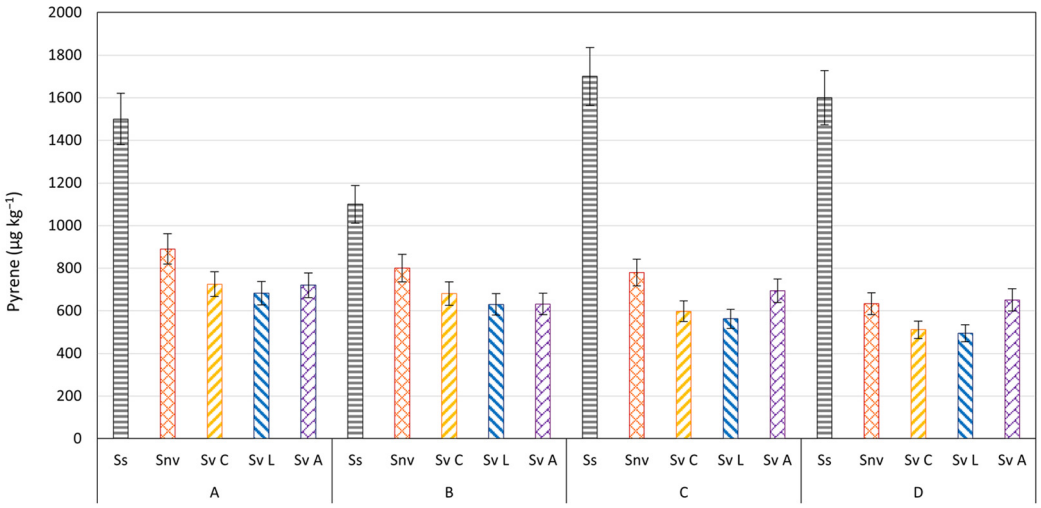


Figure 4. Concentration of pyrene in the soil (µg kg^{-1}d.m.) at initial conditions (Ss) and at the end of the microcosm test for non-vegetated soils (Snv) and after the growth of investigated plants (Sv) for different soils (A–D) during microcosm tests. Data reported as the mean of replicates with the related standard deviation.

As mentioned, adding PGPB further improves the observed levels of pyrene degradation. A direct comparison of removal percentages compared to the related initial values is shown in Figure 5.

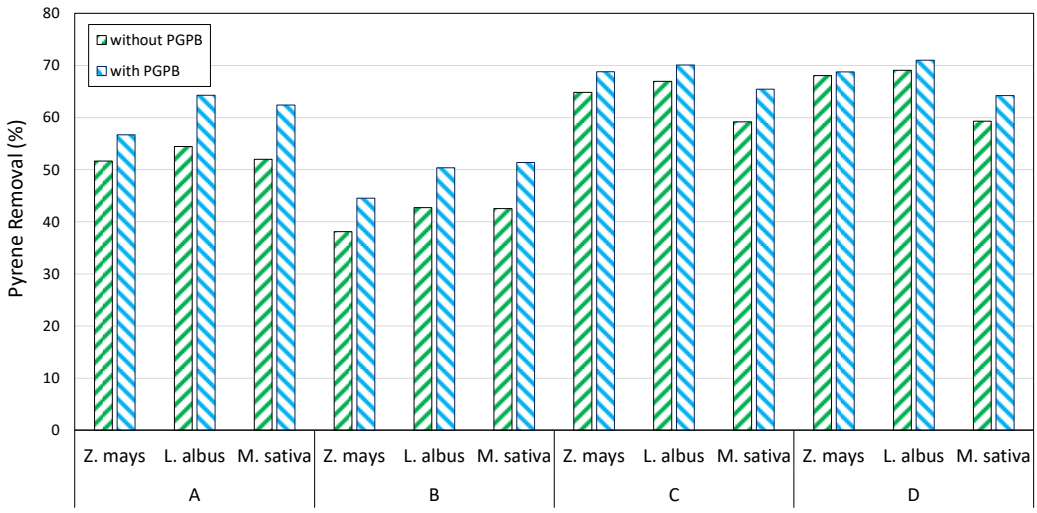


Figure 5. Comparison of the percentage reduction in the mean values of pyrene in vegetated soil for different soils (A–D) from microcosm tests with and without PGPB. The reduction is intended as relative to the initial values of the corresponding soil type, obtained as (Ss − Sv)/Ss × 100.

The results show that rhizodegradation processes promote the degradation of PAHs, leading to an effective reduction in their concentrations in soil, and this effect is amplified by the inoculation of the strains isolated as part of this experimentation. This hypothesis is supported by the low concentrations of pyrene and PAHs in plants. These complex species are not extracted from the soil but transformed by microorganisms into lighter compounds, even more effectively near the areas close to the roots. This also justifies the enhancement observed with the addition of PGPB, which, as mentioned, increases microbial activity in the rhizosphere by promoting plant development, ultimately resulting in enhanced degradation. Overall, the percentage of reduction in PAHs' concentration ranged from a minimum of about 40% to more than 70% depending on the soil and plant species, assuming pyrene as

an indicator (Figure 5), with the contribution made by the addition of PGPB resulting in an additional 5–10% removal from the respective initial concentration.

Thus, contamination with organic chemicals can be effectively reduced by the combined activities of specifically selected plants and microorganisms that utilize organic compounds as a primary carbon source. In addition, relying solely on biological activity, the approach illustrated is particularly suitable to remediate land intended for agricultural use. However, an important consideration is that this great reduction in contaminant concentrations is possible due to the peculiar condition of macrocosm tests, as they allow roots to explore the polluted soil fully; such a condition is often unrealistic when working in the field.

3.5. Mesocosm Tests

The average values of the fresh biomass of the root and aerial part of *Z. mays* related to mesocosm tests are given in Table 4.

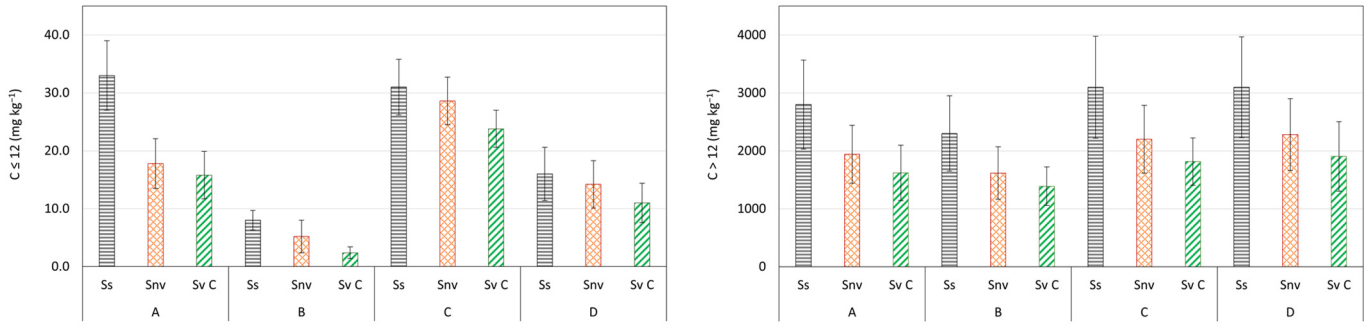
Table 4. Fresh weight (g) and concentration (mg kg^{-1}) of hydrocarbons (C ≤ 12 and C > 12) contained in *Z. mays* plants grown in different soils (A–D) during mesocosm tests. Data reported as the mean of replicates with the related standard deviation.

	Zea mays			
	Shoots	**Roots**	**C ≤ 12**	**C > 12**
A	31.3 ± 1.43	8.65 ± 1.32	1.16 ± 0.56	250 ± 53.0
B	33.8 ± 1.72	9.15 ± 1.22	1.06 ± 0.58	202 ± 70.0
C	31.4 ± 1.55	8.50 ± 1.25	2.4 ± 0.89	300 ± 52.0
D	30.7 ± 1.32	8.71 ± 1.82	2.3 ± 0.76	320 ± 60.0
CT	83.8 ± 2.10	11.1 ± 1.70	---	---

As the experimentation scale increases, the contamination effect is observed more. Plants grown on the contaminated soils produced less biomass, reducing by more than 20 percent for the root system and more than 60 percent for the aerial part compared to the control.

The concentrations of hydrocarbons in *Z. mays* plants for the mesocosm tests are also shown in Table 4. It should be noted that PAHs were always below the limit of quantification for both the aerial and root parts in all samples.

Figure 6 shows the concentration trends of C ≤ 12 and C > 12 in the soil at initial conditions (beginning of the mesocosm test Ss) and at the end of mesocosm test in the non-vegetated (Snv) and vegetated case (Sv C). It is worth pointing out that the observable deviations in this further experimentation from the microcosm Ss and Snv values shown in Figure 4 are within the estimated experimental uncertainty and are, therefore, consistent.



(**a**) (**b**)

Figure 6. Variation of C ≤ 12 (**a**) and C > 12 (**b**) hydrocarbon content in the soil (mg kg^{-1}d.m.) for different soils (A–D) during mesocosm tests. Data reported as the mean of replicates with the related standard deviation. Ss = starting soil, Snv = non-vegetated soils; Sv = vegetated soil; C denote *Z. mays* (Corn).

Figure 6 confirms a trend of decreasing concentration values related to natural soil attenuation mechanisms (Snv) as time passes, but this trend is accentuated using plants (Sv C). Indeed, considering C > 12 hydrocarbons (the most relevant portion of the contamination), a 15–18% increase in degradation from non-vegetated to vegetated soils is observed. However, it should be noted that given the considerable variability in the analyses, the results are not always statistically significant.

The resulting concentration of PAHs compared to the beginning (Ss) of the mesocosm experimentation is presented in Table S3.

Also, in the specific case of pyrene (Figure 7), as with other PAHs (Table S3), the trend observed in the mesocosm tests is similar to that found in the microcosm, although the effect of plants is proportionally less pronounced due to contamination-related distress, which was found to be more pronounced than in the microcosm. Therefore, it is likely that the appropriate selection of PGPB can significantly improve the degradation yield at the mesocosm scale.

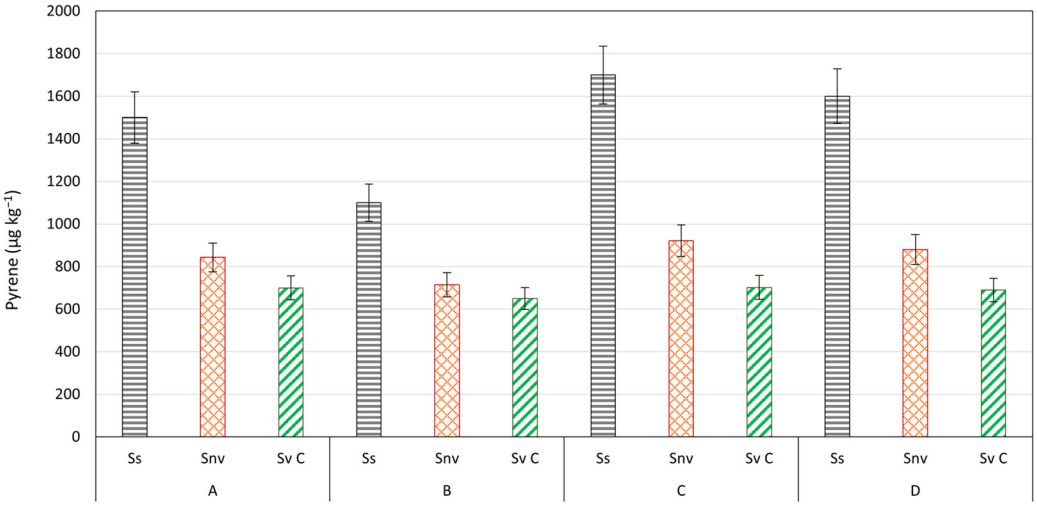


Figure 7. Concentration of pyrene in the soil (µg kg^{-1}d.m.) at initial conditions (Ss) and at the end of the mesocosm test for non-vegetated soils (Snv) and after the growth of *Z. mays* (Sv C) for different soils (A–D) during mesocosm tests. Data reported as the mean of replicates with the related standard deviation.

In general, it is confirmed that relying just on the biological synergy of chosen plants and PGPB is possible, especially for treating soils intended for agricultural use. In addition, compared to traditional remediation techniques such as landfill disposal, it is a cost-effective and low-impact approach, which results in a lessening of CO_2 equivalent emissions by whole orders of magnitude [12]. Furthermore, it is possible to dispose of the produced biomass through waste-to-energy solutions such as electricity production through incineration or bio-oil production through pyrolysis, increasing overall efficiency and further reducing the remediation environmental impact by roughly 30% [12].

4. Conclusions

A feasibility study with different kinds of plants has evaluated the possible use of phytoremediation for agricultural soils contaminated by oil spills. Green technologies represent the best possible solution for remediation without damaging soil properties in these soils, whose quality is strictly related to the food chain and human health. The results show that phytoremediation can lower the concentration of hydrocarbons in the soil due to the ability to promote degrading microbial activity in the contaminated soil. The data also highlight the potential efficiency of selected PGPB to support and improve the biodegradation activity of the rhizospheric microbial population. Phytoremediation in agricultural areas offers the possibility to reduce contamination in an environmentally and economically sustainable way, also characterized by high social acceptability. The

results obtained in the study are strictly site-specific. However, the data strongly support the coordinated use of both plants and microorganisms to concretely use NBS as winning strategies to remediate recalcitrant organic compounds, such as petroleum hydrocarbons, maintaining or promoting soil quality. Indeed, an increase in the degradation was observed in vegetated soils with selected PGPB addition compared to natural attenuation soil phenomena, quantified in the up to around 30% removal of the C > 12 fractions, and the up to 70% removal of pyrene, considered an indicator of PAH contamination.

Furthermore, the biomass produced after plant harvest can be exploited by waste-to-energy solutions, allowing greater benefit for the environment.

Based on the results obtained, a field test preparatory to the full-scale reclamation is underway, in which, in addition to corn and sunflower, other local species (castor and sorghum) will be tested to gather more information and increase the chances of successful intervention.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14020582/s1, Figure S1: Spatial distribution of the sampling points, pipeline track and break-in point; Table S1: Concentration of the main hydrocarbon groups detected in the soil samples considered and the depth at which the samples were taken; Table S2: Fresh weight (g) of plants (shoots and roots) grown during microcosm tests; Table S3: Concentration of PAHs in the soils (μg kg^{-1}) at initial conditions (Ss) and at the end of mesocosm test for non-vegetated soils (Snv) and after growth of *Z. mays* (Sv).

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