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Potential Enhancement of the *In-Situ* Bioremediation of Contaminated Sites through the Isolation and Screening of Bacterial Strains in Natural Hydrocarbon Springs

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Abstract: Petroleum hydrocarbon contamination (PHC) is an issue of major concern worldwide. These compounds represent the most common environmental pollutants and their cleaning up is mandatory. The main goal of this research was to analyze microbial communities in a site in southern Italy characterized by the presence of hydrocarbons of natural origin by using a multidisciplinary approach based on microbiological, geological and hydrological investigations. Bacterial communities of two springs, the surrounding soils, and groundwater were studied through a combination of molecular and culture-dependent methodologies to explore the biodiversity at the study site, to isolate microorganisms with degradative abilities, and to assess their potential to develop effective strategies to restore the environmental quality. Next-generation sequencing revealed the dominance of species of the *Proteobacteria* phylum but also the presence of other autochthonous hydrocarbon-oxidizing microorganisms affiliated to other phyla (e.g., species of the genera *Flavobacterium* and *Gordonia*). The traditional cultivation-based approach led to the isolation and identification of 11 aerobic hydrocarbon-oxidizing proteobacteria, some of which were able to grow with phenanthrene as the sole carbon source. Seven out of the 11 isolated bacterial strains produced emulsion with diesel fuel (most of them showing emulsifying capacity values greater than 50%) with a high stability after 24 h and, in some cases, after 48 h. These results pave the way for further investigations finalized at (1) exploiting both the degradation ability of the bacterial isolates and/or microbial consortia to remediate hydrocarbon-contaminated sites and (2) the capability to produce molecules with a promoting effect for oil polluted matrices restoration.

Keywords: hydrocarbon springs; groundwater; bioremediation; microbiological-hydrogeological investigations; next-generation sequencing (NGS)

1. Introduction

Hydrocarbon pollution is a widespread phenomenon that affects human health and the environment, including air, water, and soil [1]. Hydrocarbons of petroleum origin, despite being essential energy resources and one of the raw materials needed for different types of industries [2], are classified as priority pollutants [3]. Many of them, such as Polycyclic Aromatic Hydrocarbons

(PAHs), are recalcitrant and highly dangerous, as they can be hemotoxic, carcinogenic and teratogenic [3–7]. Accordingly, over the decades, the awareness to protect the environment has increased, especially now that pollution is recognized as one of the most severe and urgent issues society has to face. The sources of environmental contamination caused by hydrocarbons are different, such as accidents in the transport of fuel by ships and tankers, leaks from underground tanks and service stations, oil extraction and processing operations, release of oily waste generated by industries that use oil in the production of plastics, solvents, pharmaceuticals and cosmetics [8,9]. The cleaning up of these contaminants from the environment is mandatory and can be reached by using physical-chemical or biological strategies [1,10]. Biological approaches have shown several advantages compared to traditional physical-chemical treatments, being more cost effective and allowing for the complete mineralization of the organic pollutant [10,11]. For this reason, bioremediation has been widely studied and is an environmentally friendly technology used for the removal of hydrocarbons in both terrestrial and aquatic ecosystems [12]. The bioremediation of contaminated sites by organic compounds is based on the stimulation of the catabolic activity of microorganisms capable of using polluting organic contaminants as a source of carbon and energy. So far, bioremediation is performed through different practices such as biostimulation (the addition of macro- and/or micronutrients to enhance indigenous biomass growth and pollutant degradation), bioaugmentation (inoculation with pollutant-degrading microorganisms) or combined biostimulation and bioaugmentation [13].

The microbial community in a given ecosystem is crucial for the biodegradation of pollutants to occur [14]. In fact, bacterial adaptation, defined as an evolutionary process in which shifts in the microbial community composition or abundances take place in response to changes in environmental conditions and contaminant content, can improve the biodegradation rate of a chemical.

On the other hand, the existence of a microbial potential does not always lead to in situ biodegradation since many limitations, such as insufficient biomass, utilization of a wide range of substrates, competitive inhibition, or catabolite repression, can all inhibit the process [14].

From this perspective, the analysis and characterization of microorganisms involved in biodegradation processes are of the utmost importance for the ultimate success of bioremediation. Furthermore, it is essential not only to dwell upon microbial communities but also have knowledge on the geological, hydrogeological and geochemical characteristics of the environment in which they live because several other factors may influence their activities [5,15].

The main aim of the present work was to analyze microbial communities in soil, spring and groundwaters from a study site in southern Italy characterized by the presence of hydrocarbons of natural origin, through a combination of culture-dependent and molecular methods. In addition, the comprehension of the geological and hydrogeological features of the aquifer system was fundamental to identify the active circuits that continuously feed the studied soil, springs and groundwaters, all year round, and could influence the composition of the bacterial communities. Some of the bacterial isolates were identified and screened for their ability to grow in the presence of different pollutants and emulsifying capacity, to assess their potential as candidates in biotechnological applications to treat and recover the polluted environmental matrices.

2. Materials and Methods

2.1. Study Area

The study area (Tramutola, Agri Valley, Southern Italy; Figures 1 and S1) is characterized by some natural outcrops of hydrocarbons, whose existence was already known from the end of the 19th century [16]. The Agri Valley is an intramountain valley, with a North-West/South-East orientation, a length of 30 km and an average width of 12 km. This study is focused on a small portion of the valley that includes two springs (S1 and S2) with natural hydrocarbon outcrops close to wells drilled in the early 20th century by the AGIP Company (at present ENI, Milano, Italy) for gas and oil extraction. Among these wells, the artesian well P_{art} was included in this research. The springs S1 and S2 (Figure S2), as well as the P_{art} well, are located along an E-W fault where the Apulian carbonate

platform and Rio Cavolo Unit (Oligocene) [17] crop out (Figure 2). The Rio Cavolo Unit is made up of clays, micaceous limestone and rare marly layers. The P_{art} well (404 m deep) intercepted oil and gas at different depths [18,19].

Different hydrogeological series complexes crop out at the study site [20]: (i) carbonate rocks belonging to the Mesozoic carbonate platform series complexes, characterized by very high permeability due to a well-developed fracture network and karst conduits; (ii) sedimentary rocks belonging to the syn-orogenic turbidite series complexes and the outer and the inner basins' series complexes, characterized by a permeability ranging from very low to low due to a mixed pore-fracture network. Along the Cavolo stream and the whole Agri Valley, heterogeneous alluvial sediments crop out, whose hydraulic conductivity ranges between less than 1×10^{-8} to 2×10^{-2} m/s [21].

The hydrocarbon springs S1 and S2 flow out along a fault zone which enhances fluid upflow and allows the mixing of hydrocarbons and gas (mainly CH_4 and H_2S) [22] with the local groundwater, which is primarily fed by the nearby carbonate aquifer (Figure 2) [19]. The groundwater intercepted by the P_{art} well is fed by a more prolonged pathway in a relatively deep aquifer made of Scisti Silicei (Figure 3) [19]. This deep groundwater naturally flows eastwards, towards the alluvial aquifer of the Agri Valley.

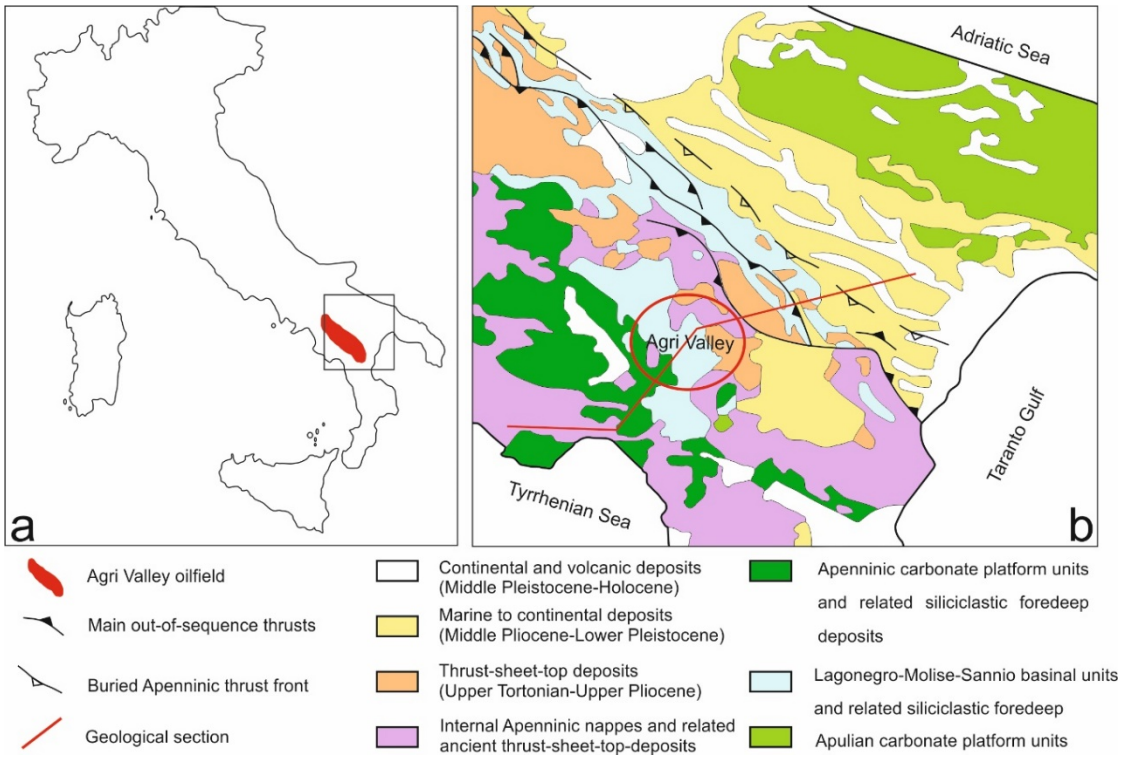

Figure 1. (**a**) Localization of the study area. (**b**) Schematic structural-geological map of the southern Apennines from Patacca et al. [23], modified.

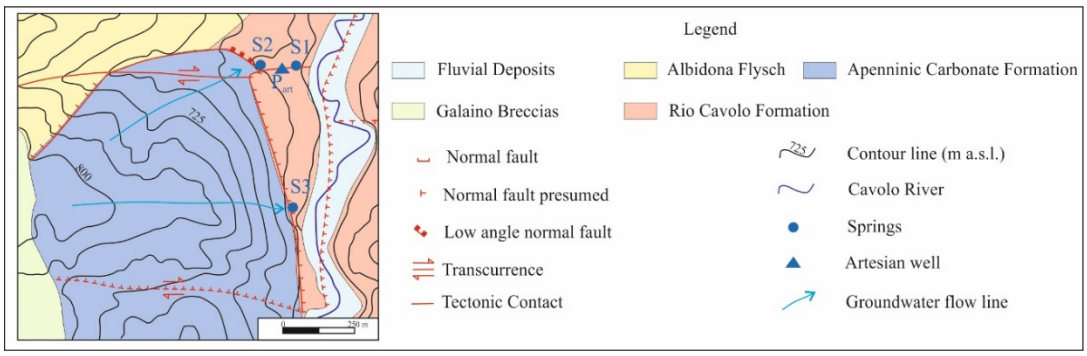

Figure 2. Hydrogeological map of the study area (from Rizzo et al. [19]); the blue points and the triangle show the location of the investigated springs S1 and S2 and the artesian well P_{art} (the geological sketch is taken from Olita [18], modified).

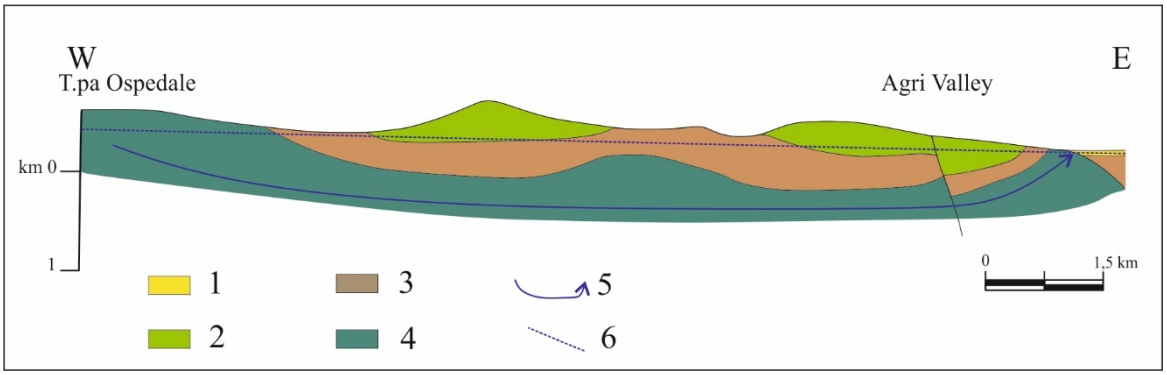

Figure 3. Hydrogeological schetch of the Scisti Silicei aquifer (Rizzo et al. [19]; based on the geological section of Menardi Noguera and Rea [24]): the (1) alluvial complex, (2) carbonate complex, (3) low-permeability complex, (4) Scisti Silicei complex, (5) groundwater flow line, (6) and hydraulic head.

2.2. Hydrogeological and Chemical-Physical Investigations

The discharge of the two springs S1 and S2 was measured in low flow (July 2018), in early recharge (October 2018) and in late recharge (March 2019). Moreover, it was measured hourly during a rainfall event to analyze the time lag between precipitation and springs' recharge increase. The flow rate of the P_{art} artesian well was not measurable but, based on some historical data, its order of magnitude is about 30 m^3/h [19]. In conjunction with discharge measurements and sample collection for chemical and microbiological analyses (March 2019), physico-chemical parameters such as temperature (°C), pH, electrical conductivity (EC µS/cm), redox potential (ORP millivolts) and total dissolved solids (TDS ppm), were measured with the multiparameter HANNA probe (mod. HI9828, HANNA Instruments, Villafranca Padovana, Italy).

Three water and two soil samples were collected to analyze PAHs and benzene, toluene, ethylbenzene, and xylene (BTEX) content. These data were compared with those acquired during sampling campaigns carried out in 2013 (unpublished data). One-liter (L) black glass bottles and 40-milliliter colorless glass vials (filled with water or soil) were used for PAHs and BTEX analyses, respectively. The analyses were performed at Biochemie Lab S.r.l. following the EPA 3510C 1996 + EPA 8270E 2018 protocol for PAHs and the EPA 5030C 2003 + EPA 8015D 2003 protocol for BTEX in water and EPA 3550C 2007 + EPA 8015D 2003 protocol for PAHs and the EPA 5021A 2014 + EPA 8015D 2003 protocol for BETX in soil [25–30].

2.3. Next-Generation Sequencing (NGS) for Bacterial Community Analyses

For bacterial community analyses, spring and groundwater samples (4 L) and two soil samples (0.5 g), collected close to the springs (0.5 m distance), were used. Water samples were filtered through sterile mixed esters of cellulose filters (S-Pak™ Membrane Filters, 47 mm diameter, 0.22 µm pore size,

Millipore Corporation, Billerica, MA, USA) within 24 h from the collection. Bacterial DNA extraction from filters and soils was performed using the commercial kit FastDNA SPIN Kit for soil (MP Biomedicals, LLC, Solon, OH, USA) and FastPrep®Instrument (MP Biomedicals, LLC, Solon, OH, USA). After the extraction, DNA integrity and quantity were evaluated by electrophoresis in 0.8% agarose gel containing 1μg/mL of Gel-RedTM (Biotium, Inc., Fremont, CA, USA). The bacterial community profiles in the samples were generated by next-generation sequencing (NGS) technologies at the Genprobio Srl Laboratory. Partial 16S rRNA gene sequences were obtained from the extracted DNA by polymerase chain reaction (PCR), using the primer pair Probio_Uni and Probio_Rev, targeting the V3 region of the bacterial 16S rRNA gene [31]. Amplifications were carried out using a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA), and PCR products were purified by the magnetic purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. Amplicon checks were carried out as previously described [31]. Sequencing was performed using an Illumina MiSeq sequencer (Illumina, Hayward, CA, USA) with MiSeq Reagent Kit v3 chemicals. The fastq files were processed using a custom script based on the QIIME software suite [32]. Paired-end read pairs were assembled to reconstruct the complete Probio_Uni/Probio_Rev amplicons. Quality control retained sequences with a length between 140 and 400 bp and mean sequence quality score > 20, while sequences with homopolymers > 7 bp and mismatched primers were omitted. To calculate downstream diversity measures, operational taxonomic units (OTUs) were defined at 100% sequence homology using DADA2 [33]; OTUs not encompassing at least two sequences of the same sample were removed. All reads were classified to the lowest possible taxonomic rank using QIIME2 [32,34] and a reference dataset from the SILVA database v132 [35]. The biodiversity of the samples (alpha-diversity) was calculated with the Shannon index. Similarities between samples (beta-diversity) were calculated by weighted uniFrac. The range of similarities is calculated between values 0 and 1. Principal Coordinate Analysis (PCoA) representations of beta-diversity were performed using QIIME2. In the PCoA, each dot represented a sample that is distributed in tridimensional space according to its own bacterial composition.

2.4. Enrichment and Isolation of Bacteria with Potential Hydrocarbon Degrading Ability

To isolate hydrocarbon-oxidizing bacteria, 1-mL aliquots of water from springs S1 and S2 and P_{art} well, and 5 g of soils collected close to the springs (0.5 m distance), were inoculated in sterile test tubes containing peptone water. Then, 1 mL of the suspensions was inoculated in 5 mL of liquid Bushnell-Haas (BH) medium ($MgSO_4$-0.2 g/L; $CaCl_2$-0.02 g/L; KH_2PO_4-1.0 g/L; K_2HPO_4-1.0 g/L; NH_4NO_3-1.0 g/L; $FeCl_3$-0.05 g/L) [36]. Diesel fuel was added at a concentration of 2% as the only carbon source, to select the hydrocarbon degrading bacteria. Cultures were, then, incubated at 28 °C for seven days with agitation. The enrichment step was repeated for seven cycles. From the fourth enrichment cycle, 100-μL aliquots of the cultures were spread on Bushnell-Haas (BH) agar medium supplemented with diesel fuel as a carbon source. Diesel fuel was supplied by diffusion through a soaked paper disk (9-mm diameter).

The colonies grown on the plates were repeatedly streaked over the entire surface of fresh BH agar medium supplemented with diesel fuel and, finally, on TSA (Tryptone Soy Agar) plates to be sure to obtain pure cultures.

2.5. Identification of the Bacterial Strains Isolated from Spring, Groundwater and Soil Samples

After the enrichment, the isolated bacterial strains were analyzed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) with the restriction endonuclease HaeIII, to group them on the basis of the restriction profiles. One representative strain for each ARDRA haplotype was selected for the 16S rDNA gene partial sequencing at BMR Genomics srl in Padua (Italy). The obtained sequences were then compared with those stored in the GenBank database at the NCBI (National Center for Biotechnology Information) by using the BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/blast). The partial 16S rDNA gene sequences were deposited in GenBank under the accession numbers MT703034 to MT703044.

2.6. Growth Response of Bacterial Isolates in the Presence of Different Hydrocarbons as the Sole Carbon Source

The potential degradation ability of some bacterial strains has been evaluated in BH agar medium containing different hydrocarbons (Naphthalene, Phenanthrene, Pristane and Hexadecane) as the sole carbon source. Naphthalene and Phenanthrene occur, at room temperature, in the solid state as granules; after spreading the individual bacterial strains onto the medium surface, the granules were added directly to the surface of the medium. The Pristane and the Hexadecane occur, at room temperature, in the liquid state and were delivered by diffusion from a soaked paper disk placed in the center of the plate. The plates were incubated at 30 °C for 4 weeks, monitoring the growth every week.

2.7. Determination of the Emulsifying Capacity and Emulsion Index

The assay used to determine the emulsifying capacity (EC%) and the emulsion index (E) is a re-adaptation of the method previously described by Mohebali et al. [37]; 2 mL of pure cultures of the isolated strains, grown in BH medium at 28 °C with agitation until the optical density (OD) at the wavelength of 600 nm reached 1.0 (OD measured with a Cary 50 UV-Vis spectrophotometer, VARIAN INC, Palo Alto, CA, USA), were transferred to test tubes and diesel fuel (0.2 mL) was added; the test tubes were vigorously shaken for 30 s and left standing for 10 min. The samples showing an emulsion over the liquid were selected and, once the emulsion was stabilized, more diesel fuel (0.2 mL) was added; the procedure was repeated until a distinct and clear fraction of non-emulsified diesel fuel was observed on the surface of the emulsion.

The emulsifying capacity (EC%) was calculated using the following formula:

$$\mathrm{EC} = \frac{\text{total diesel volume}}{\text{initial aqueous phase volume}} \times 100$$

The emulsion index (E) was calculated after the test for the emulsifying capacity; the variation of the emulsion thickness was measured after 24 (E_{24}) and 48 (E_{48}) h with the following formula:

$$\mathrm{E} = 100 \times \frac{\text{emulsion thickness at } t_x}{\text{emulsion thickness at } t_0}$$

where t_x is the time in which the measurement was taken (e.g., t_{24}, t_{48}) and t_0 is the test start time.

3. Results

3.1. Hydrogeological and Physico-Chemical Features

The flow rate of both hydrocarbon springs S1 and S2 slightly varied overtime during the observation period (1.3 to 1.7 m^3/h at spring S1; 1.0 to 1.2 m^3/h at spring S2), suggesting an active groundwater pathway. The spring regime, further analyzed through hourly measurements during a rainfall event (about 11 mm in a few tens of hours), demonstrated a very rapid and synchronous response of spring S1 (2.5 to 3.6 m^3/h) and spring S2 (2.0 to 2.5 m^3/h) to precipitation.

The main physico-chemical features of hydrocarbon springs and P_{art} well are synthesized in Table 1. The temperature ranged from 13.9 to 15.7 °C in spring waters, while it varied between 27.5 and 27.8 °C in P_{art} waters. The pH was constantly close to neutrality. Redox potential was slightly negative. The electrical conductivity was higher and more variable in P_{art}-water (1444 to 2433 μS/cm) than in springs waters (460 to 507 μS/cm).

Table 1. Physico-chemical features of spring- and groundwaters.

Sample	Date	Temperature	pH	Redox Potential	Electrical Conductivity
ID	dd/mm/yyyy	°C		mV	μS/cm
P_{art}	19/07/2018	27.8	6.8	−26.0	1444
P_{art}	29/10/2018	27.6	6.8	−30.0	2433
P_{art}	18/03/2019	27.5	6.8	−7.5	2432
S1	19/07/2018	15.6	6.9	−60.0	507
S1	29/10/2018	15.6	6.8	−51.8	494
S1	18/03/2019	15.7	6.9	−33.4	494
S2	19/07/2018	14.3	6.9	−20.0	460
S2	29/10/2018	15.6	6.9	−15.0	467
S2	18/03/2019	13.9	6.9	−4.0	493

3.2. Chemical Analyses of Spring, Groundwater and Soil Samples

Chemical analyses revealed detectable PAHs and no BTEX in both springs S1 and S2 (Table 2). Neither PAHs nor BTEX were detected in groundwater sampled from the P_{art} artesian well. These results agree with those obtained by ENI in 2013 (unpublished data).

Table 2. Results of chemical analyses of spring- and groundwater samples.

Parameter	Spring S1	Spring S2	P_{art} Well
Unit	μg/L	μg/L	μg/L
Benzene	<0.1	<0.1	<0.1
Ethylbenzene	<1.0	<1.0	<1.0
p-Xylene	<1.0	<1.0	<1.0
Styrene	<1.0	<1.0	<1.0
Toluene	<1.0	<1.0	<1.0
Benzo(a)anthracene	<0.002	<0.002	<0.002
Benzo(a)pyrene	<0.002	<0.002	<0.002
Benzo(b)fluoranthene	<0.002	0.354	<0.002
Benzo(k)fluoranthene	<0.002	0.0341	<0.002
Benzo(g,h,i)perylene	<0.002	0.199	<0.002
Chrysene	<0.002	<0.002	<0.002
Dibenzo(a,h)anthracene	<0.002	<0.002	<0.002
Indene(1,2,3-c,d)pyrene	<0.002	0.101	<0.002
Pyrene	<0.002	<0.002	<0.002
Acenaphthene	<0.002	<0.002	<0.002
Acenaphthylene	<0.002	<0.002	<0.002
Anthracene	<0.005	0.726	<0.005
Naphthalene	0.00231	<0.002	<0.002
Phenanthrene	<0.002	0.465	<0.002
Fluoranthene	<0.005	0.166	<0.005
Fluorene	<0.005	<0.005	<0.005
Benzo(J)fluoranthene	<0.001	0.0140	<0.001
Dibenzo(a,e)pyrene	<0.001	<0.001	<0.001
Dibenzo(a,l)pyrene	<0.001	<0.001	<0.001
Dibenzo(a,i)pyrene	<0.001	<0.001	<0.001
Dibenzo(a,h)pyrene	<0.001	<0.001	<0.001
Σ PAHs	<0.002	0.689	<0.002

Consistent with the results obtained in spring water samples, several PAHs were detected in soils collected close to both the investigated springs. Higher concentrations were found close to spring S2 (Table 3).

Table 3. Results of chemical analyses of soil samples.

Parameter	Soil S1	Soil S2
Unit	mg/kg	mg/kg
Benzene	<0.01	<0.01
Etilbenzene	<0.05	<0.05
Toluene	<0.05	<0.05
Xylenes	<0.05	<0.05
o-Xylene	<0.05	<0.05
p,m-Xylenes	<0.05	<0.05
Benzo(a)anthracene	0.16	0.95
Benzo(a)pyrene	<0.01	<0.01
Benzo(b)fluoranthene	<0.05	3.93
Benzo(g,h,i)perylene	0.28	1.45
Benzo(k)fluoranthene	<0.05	0.58
Chrysene	0.21	1.23
Dibenzo(a,e)pyrene	<0.01	<0.01
Dibenzo(a,h)anthracene	<0.01	0.34
Dibenzo(a,h)pyrene	<0.01	<0.01
Dibenzo(a,i)pyrene	<0.01	<0.01
Dibenzo(a,l)pyrene	<0.01	0.10
Indene(1,2,3-c,d)pyrene	0.05	0.19
Pyrene	<0.05	0.61
Σ PAHs	<1.0	8.25

3.3. 16S Ribosomal RNA Gene Next-Generation Sequencing (NGS)

MiSeq runs produced 294,432 final reads (Table 4). The 16S rRNA gene sequences generated in this study have been deposited in the NCBI Sequence Read Archive under the accession numbers PRJNA629324 and PRJNA636951. The rarefaction analysis, a measure used to estimate the alpha diversity in samples and gauge whether or not sequencing efforts captured the microbial diversity, showed a relatively higher biodiversity in soil samples and spring S2-water (Shannon index values ranging from 7.59 to 8.88) compared to spring S1-water and P_{art}-groundwater (index values of 3.40 and 4.32, respectively; Figure S3). Principal Coordinate Analysis (PCoA) based on weighted uniFrac index revealed a clear separation of soil, spring, and groundwater samples and highlighted marked differences between the S1 spring water microbial community and all the others (Figure 4).

The NGS results allowed us to obtain detailed information about the composition of microbial communities in spring water, groundwater and soil samples.

Table 4. Number of 16S rDNA sequences obtained after next-generation sequencing (NGS) analysis for spring water, groundwater and soil samples.

Sample	Final Read Number
S1 Water	49,512
S1 Soil	61,621
S2 Water	61,065
S2 Soil	68,893
P_{art} Water	53,341

The phylum *Proteobacteria* was dominant in most of the analyzed samples, reaching relative abundance values of up to 93.60% (sample P_{art}) (Figure 5). Other phyla abundantly represented were *Epsilonbacteraeota* in the spring water sample S1 (52.48%), and *Actinobacteria*, found in the two soil samples at percentages of 16.38% and 22.20%.

In line with the PCoA results, significant differences between the two spring water microbial communities were detectable already at this level. In fact, *Epsilonbacteraeota*, *Bacteroidetes*, and *Proteobacteria* were the three major phyla in water from the spring S1 with relative abundance values of 52.48%, 23.63%, and 20.67%, respectively, whereas *Proteobacteria* dominated in S2 spring water (78.63%) followed by *Patescibacteria* (4.51%) and *Actinobacteria* (3.17%).

At a lower taxonomic level (Figure 6), the genus *Hydrogenophaga*, affiliated to the phylum *Proteobacteria*, was found at higher percentages in P_{art} (34.09%) and S2 water samples (15.17%). *Hydrogenophaga* and some other bacterial genera retrieved in the samples, such as *Flavobacterium*, *Gordonia*, *Sulfuritalea*, and *Rhodoferax*, are known to include microorganisms able to oxidize hydrocarbons [38–43].

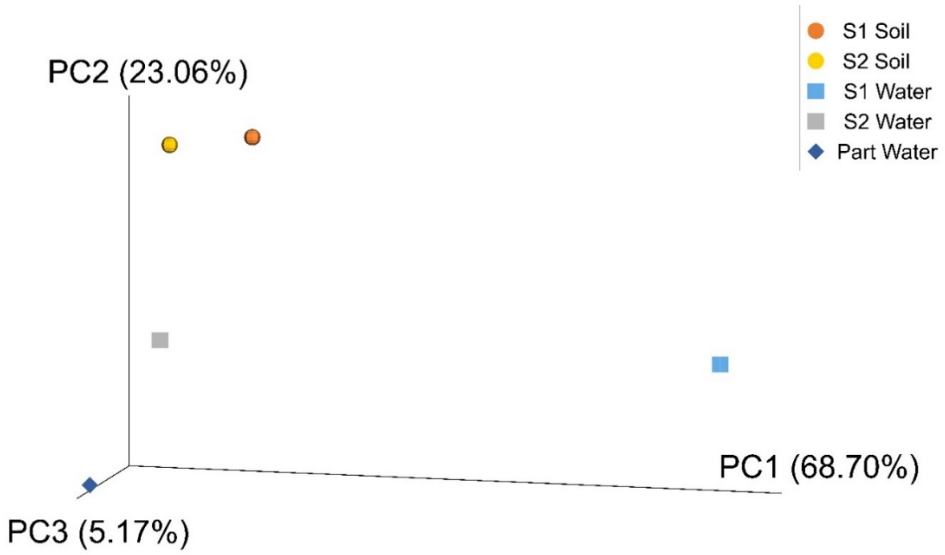

Figure 4. Principal Coordinate Analysis (PCoA). The plot was generated using a weighted uniFrac distance matrix. Soil, spring water, and groundwater samples are shown.

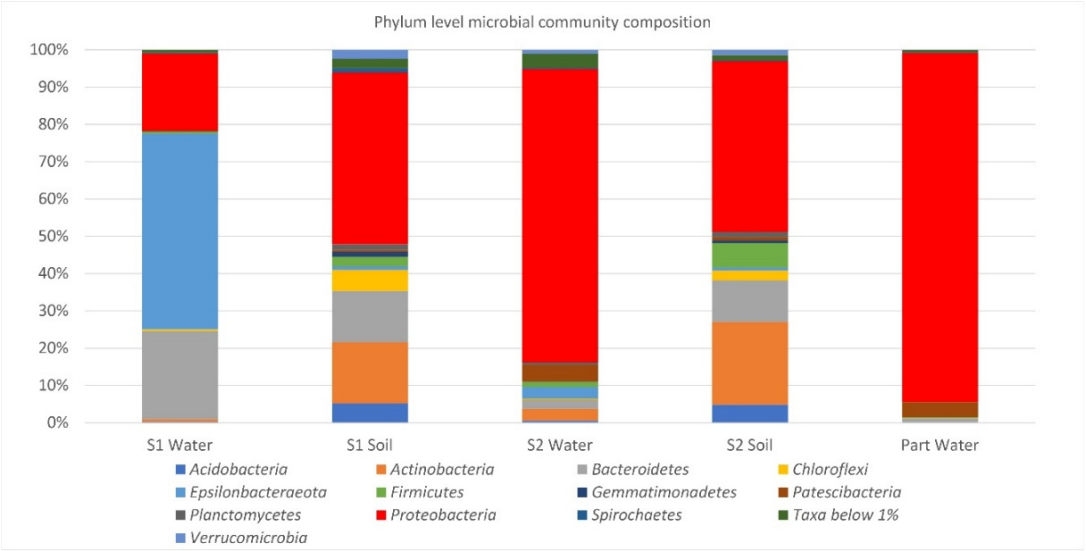

Figure 5. Phylum level microbial community composition in spring water, Part well, and soil samples.

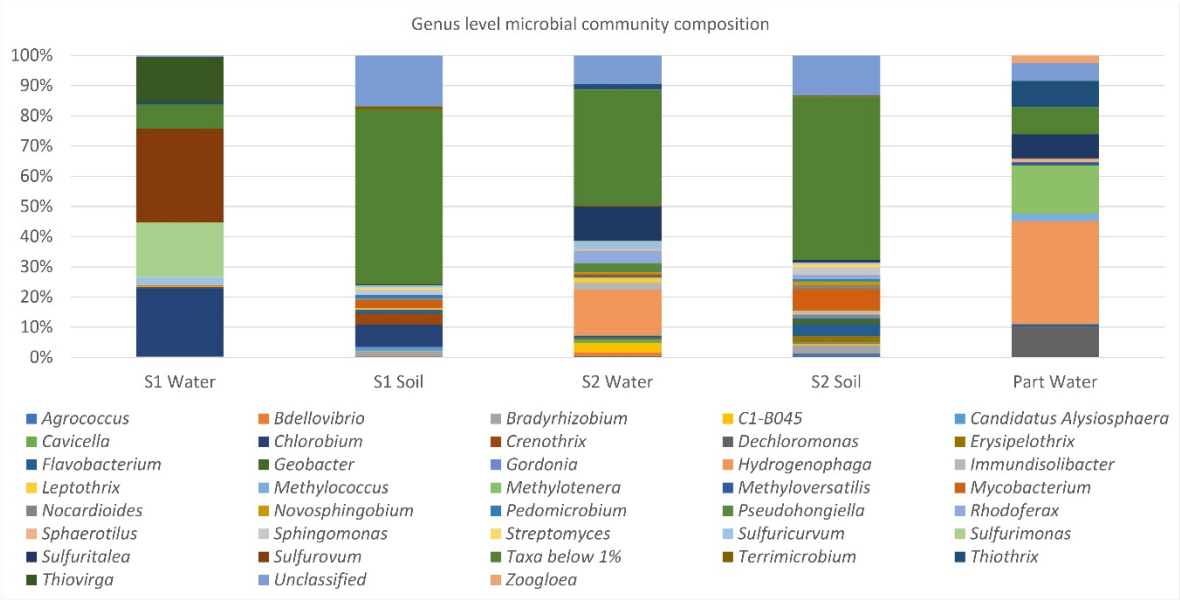

Figure 6. Genus level microbial community composition in spring water, P_{art} well, and soil samples.

3.4. Isolation and Identification of Bacterial Strains

Following the seven enrichment cycles on BH medium with diesel fuel as the only carbon source, 26 bacterial strains with different colony morphology were isolated from springs S1 and S2, P_{art} artesian well and soils.

An identification number from 1 to 26 was assigned to each strain. The 26 bacterial isolates were subjected to ARDRA analysis that allowed us to group them based on electrophoretic profiles. ARDRA analysis was also performed on five strains (1C–5C) obtained from S1 and S2 spring waters in previous experiments carried out at the study site (unpublished data). Seventeen bacterial isolates, representing all the ARDRA haplotypes, were chosen for 16S rRNA gene partial sequencing. Eleven bacterial strains were identified (Table 5), while the remaining isolates could not be rendered perfectly axenic in isolation procedures and, therefore, the sequencing failed. Some of the 16S rRNA gene sequences showed an identity < 97% with those stored in the GenBank database, suggesting that the bacterial isolates could be representatives of novel genera or species (Table 5).

Table 5. Results of 16S rRNA gene partial sequencing of bacterial strains isolated from spring water, groundwater and soil samples.

Strain	Sequence Accession Number	Most Closely Related Organism (Accession Number)	Identity (%)
3C	MT703034	*Achromobacter spanius* strain LMG 5911 (NR_025686)	97.80
1C	MT703035	*Pseudomonas protegens* strain CHA0 (NR_114749)	99.78
3	MT703036	*Dyella japonica* strain NBRC 102414 (NR_114075)	90.69
4	MT703037	*Pseudomonas protegens* strain CHA0 (NR_114749)	98.42
7	MT703038	*Cupriavidus metallidurans* CH34 (NR_074704)	98.16
8	MT703039	*Pseudomonas protegens* strain CHA0 (NR_114749)	98.19
10	MT703040	*Stenotrophomonas maltophilia* strain ATCC 13637 (NR_112030)	99.19
15	MT703041	*Stenotrophomonas tumulicola* strain T5916-2-1b (NR_148818)	90.86
16	MT703042	*Stenotrophomonas maltophilia* strain ATCC 13637 (NR_112030)	86.38
18	MT703043	*Dyella terrae strain* JS14-6 (NR_044540)	92.50
20	MT703044	*Dyella terrae strain* JS14-6 (NR_044540)	91.00

3.5. Growth Test

Five bacterial strains (1C, 2C, 3C, 4C and 5C), isolated in a previous experiment from S1 and S2 spring waters, and identified by sequencing of partial 16S rDNA together with the new isolates, were subjected to growth tests with four different recalcitrant hydrocarbons, naphthalene, phenanthrene, pristane and hexadecane (Table 6). These isolates were identified as *Achromobacter* sp. (3C) and *Pseudomonas* sp. (1C, 2C, 4C and 5C).

Table 6. Growth test results after 4 weeks (bacterial growth: +slight; ++moderate; +++high).

Isolate	Naphthalene	Phenanthrene	Pristane	Hexadecane
1C	+	+	+	++
2C	+	+++	+++	++
3C	+	++	++	++
4C	+	+++	++	++
5C	+	+	+	++

As shown in Table 6, some of the *Pseudomonas* sp. isolates (2C and 4C) have returned the best results when supplementing phenanthrene and pristane as the only carbon sources. All the isolates gave equivalent results for the growth on medium containing naphthalene and hexadecane.

3.6. Determination of the Emulsifying Capacity and Emulsion Index

The 11 identified strains were tested for emulsion capacity. Moreover, one strain of *Escherichia coli* and one of *Bacillus subtilis* were used as controls (Table 7).

Table 7. Emulsifying capacity results (EC%) (- absence; + presence).

Strain	EC	EC%
Proteobacteria bacterium strain 3	-	0
Pseudomonas sp. strain 4	+	60
Cupriavidus sp. strain 7	-	0
Pseudomonas sp. strain 8	+	60
Stenotrophomonas sp. strain 10	-	0
Proteobacteria bacterium strain 15	-	0
Proteobacteria bacterium strain 16	+	30
Proteobacteria bacterium strain 18	+	60
Proteobacteria bacterium strain 20	+	60
Pseudomonas sp. strain 1C	+	40
Achromobacter sp. strain 3C	+	50
Escherichia coli (negative control)	-	0
Bacillus subtilis (positive control)	+	50

Seven out of the 11 bacterial strains produced emulsion with diesel fuel, most of which with an EC% greater than 50%. As shown in Figure 7, the stability of the emulsions (E%) after 24 (t24) and 48 h (t48) was higher for *Pseudomonas* sp. strain 8 and *Proteobacteria* bacterium strain 18. After 24 and 48 h, a decrease of 17% and 20%, and 15% and 23%, was observed for the strains 8 and 18, respectively. The emulsion of the *Proteobacteria* bacterium strain 16 was much less stable with a difference from t_0 to t_{24} of 70% and exhausted after 48 h.

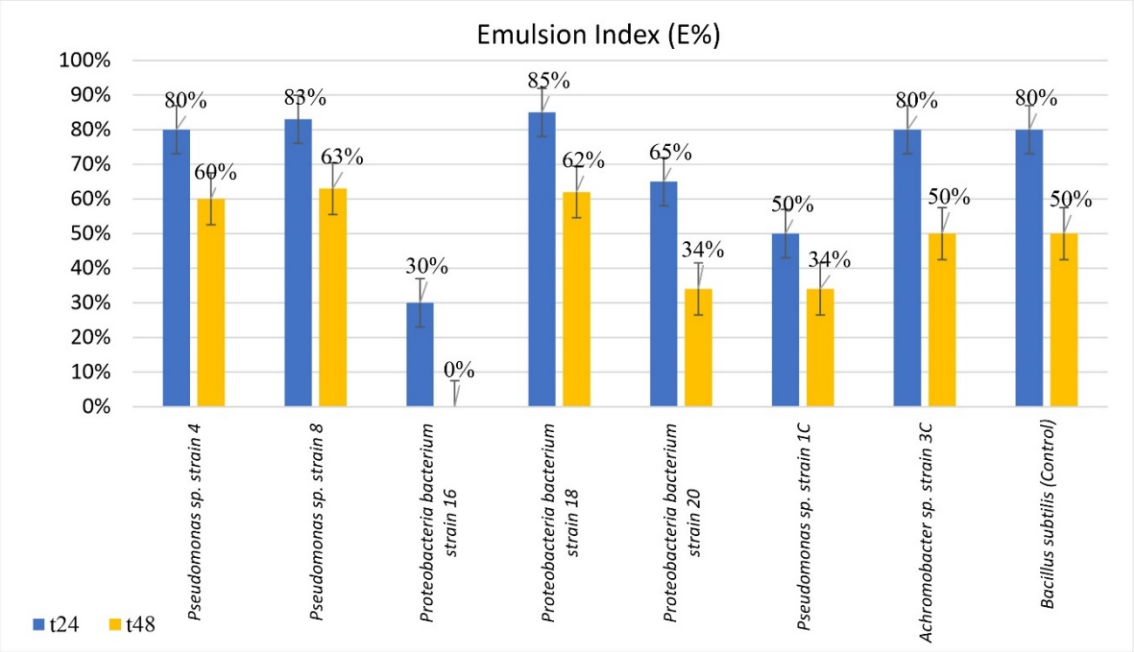

Figure 7. Histogram with emulsion index results (E%) of the strains that showed emulsifying capacity, respectively, at time t_{24} and t_{48}. The error used in the histogram is "the standard error".

4. Discussion and Conclusions

Petroleum hydrocarbon contamination is an issue of major concern worldwide. These compounds represent the most common environmental pollutants and their presence is destructive to the ecosystem and economic and human health [44]. The clean-up of hydrocarbon-contaminated sites is expensive and time consuming; however, bioremediation is a cost-effective and environmentally safe approach for petroleum hydrocarbon contamination (PHC) removal [44]. This technology involves the use of living organisms such as microbes and/or plants to reduce/degrade, eliminate and transform contaminants present in soils, sediments and water and has gained wider acceptance in recent years for all its potential [45]. In fact, although oil pollution is difficult to treat, indigenous bacteria can ultimately degrade or metabolize most petroleum hydrocarbons encountered in the environment because of their energetic and carbon needs for growth and reproduction, as well as the requirement to relieve physiological stress caused by their presence [46–48]. Accordingly, petroleum hydrocarbon-degrading bacteria, which have evolved as a result of existing in close proximity to naturally occurring petroleum hydrocarbons in the environment, represent suitable candidates for the treatment of oil polluted sites and to achieve the best purification effect [49–51].

An understanding of the temporal and spatial structures, functions, interactions, and population dynamics of microbial communities is critical for biotechnological development, environmental protection, and human health [52] so much that several methods have been employed to reveal microbial community composition, function and responses to environmental changes, in various environments and different contexts [53–60]. The main goal of this research was to analyze microbial communities in a site in southern Italy characterized by the presence of hydrocarbons of natural origin, by using a multidisciplinary approach based on microbiological, geological and hydrogeological investigations.

In detail, bacterial communities of two springs (S1 and S2), the surrounding soils, and groundwater (P_{art} well) were studied through a combination of molecular and culture-dependent methodologies to explore the biodiversity at the study site, to isolate microorganisms with degradative abilities and to assess their potential to develop effective strategies to restore the environmental quality.

The two hydrocarbon springs S1 and S2 are linked to the recharge in a local aquifer system and distributed along a fault zone that favors the rise of fluids and hydrocarbons within the shallow low-

permeability media. This hydrogeological behavior of the fault zone is not surprising when analyzing the possible roles (conduit, barrier, combined conduit-barrier systems) that these zones can play from the hydraulic point of view, depending on the relative percentage of fault core and damage zone [61–67]. In many aquifer systems worldwide, comparable with the studied one, both structural and hydrogeological approaches demonstrated the possible migration of fluids within fault zones acting as high- or low-flow discontinuities [68–74]. This evidence was further confirmed by the presence of H_2S at spring S1.

Chemical analyses of hydrocarbons highlighted the existence of several and different PAHs, such as Naphthalene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(g,h,i)perylene, Indene(1,2,3-c,d)pyrene, Chrysene, Anthracene, Phenanthrene, Fluoranthene, Benzo(j)fluoranthene, Dibenzo(a,h)anthracene, Pyrene and Dibenzo(a,l)pyrene in spring waters and soils. Generally, polycyclic aromatic hydrocarbons are chemicals with various structures and varied toxicity, stable, persistent in the environment, and resistant to degradation [75,76]. Chemically, they are comprised of two or more benzene rings bonded in linear, cluster, or angular arrangements [75]. The increase in the number of fused rings leads to higher hydrophobicity and recalcitrance to microbial degradation [77]. The continuous interaction with hydrocarbons could have influenced the bacterial communities of the two springs and the soils, naturally selecting some strains capable to biodegrade these pollutants.

The NGS results allowed us to obtain detailed information about the composition of microbial communities and revealed the dominance of species belonging to the phylum *Proteobacteria*. In this very large phylum of Gram-negative organisms, the majority of the formally described genera of hydrocarbon-degrading bacteria is included [78]; thus, it is likely that the continuous outflow of oil over time led to a stable and highly specialized microbiota, particularly adapted to grow and thrive in those environmental conditions. However, in addition to *Proteobacteria*, it was possible to also detect other autochthonous hydrocarbon-oxidizing bacteria affiliated to other phyla (e.g., species of the genera *Flavobacterium* and *Gordonia*), strengthening the assumption that natural exposure to pollutants can have impacted the structure and function of microbial populations. As expected, a higher biodiversity was found in soils compared to the aquatic ecosystems, generally lacking inputs of fresh, easily available organic carbon. In addition, beta-diversity analysis revealed a clear separation of soil, spring water, and groundwater samples and highlighted marked differences between S1 spring water microbial community and all the others. Although it is not surprising to find diverse microbial communities in different habitats due to their peculiar physico-chemical properties, it is likely that the observed results for the two springs reflect the influence of specific hydrogeological and other environmental factors that, on a local scale, shape bacterial community composition. As a matter of fact, P_{art}-groundwater is fed by a deep confined aquifer. Differently, the springs S1 and S2 are fed by a common groundwater coming from the nearby carbonate aquifer, mixed with different fluids (rising along a fault zone) in different proportions. This is clearly demonstrated by differences in some physico-chemical features (Table 1) and hydrocarbon concentrations (Table 2), and are further supported when taking into consideration the different mean residence time of spring waters, determined through tritium analyses (S1 = 5.6 to 6.9 tritium unit (TU), S2 = 6.8 to 7.5 TU; the spring fed by the only carbonate aquifer = 8.4 to 9.1 TU in Rizzo et al. [19]). These results are also in agreement with findings in other hydrogeological settings, where differences in microbial communities were clearly explained through the differences in (i) the aquifer type, (ii) the mixing between shallow groundwater and ascending fluids, (iii) the mixing between waters characterized by different salinity [53].

The traditional cultivation-based approach, performed by applying a selective pressure with diesel fuel supplementations, led to the isolation and identification of 11 aerobic hydrocarbon-oxidizing proteobacteria. Most of the bacteria isolated and identified belong, or are related, to genera or species known for their degradation abilities of polycyclic aromatic hydrocarbons [79–85]. The results are in line with those obtained by chemical analyses, which showed higher concentrations of these compounds at the investigated site. It is interesting to note that microorganisms of the genera *Hydrogenophaga*, *Sulfuritalea* and *Sulfurovum*, whose abundant presence was revealed by DNA-based

analyses, have not been isolated after the enrichment procedures. This "paradox" can be explained by the theory of the "Great Plate Count Anomaly" [86], which refers to the observation that only a small fraction (0.01–1%) of the microorganisms present in the environment can be cultivated in the laboratory. Despite this, the cultivation procedures led to the isolation of bacterial strains showing low values of 16S rRNA gene sequence identity with known species, contributing to get insights into the hidden microbial diversity of the analyzed ecosystem.

Some of the isolates were found to be able to grow better when phenanthrene was supplied as the sole carbon source compared to other simpler aliphatic and aromatic hydrocarbons. Subsequently, their emulsifying capacity was assessed and the emulsion index was calculated. Seven out of the 11 isolated bacterial strains produced emulsion with diesel fuel (most of them showing EC values greater than 50%) with high stability after 24 h and, in some cases, after 48 h. The tests have clearly demonstrated the capability of some strains to increase hydrocarbon bioavailability, most likely through the production of biosurfactants. These compounds are naturally derived surfactants produced from biological entities (especially microorganisms), which can be utilized as a cost-effective and eco-friendly mean to enhance bioremediation of oil components, including PAHs, in the natural environment. Fungi, bacteria, and yeasts belonging to different species and strains are known for producing biosurfactants of a diverse variety of molecular structures. Amongst the bacteria domain, the genera of *Pseudomonas*, *Bacillus*, and *Acinetobacter* dominate the literature space as excellent producers of biosurfactants [87]. For example, *Bacillus subtilis* is known for its ability to enhance diesel solubility [88] and to improve its degradation by producing surfactin, a lipoprotein-type biosurfactant [89].

In the late 1960s, biosurfactants attracted attention as hydrocarbon-dissolving agents as potential replacements for synthetic surfactants (usually toxic, hardly degraded by microorganism, and causing damage to the environment) especially in the food, pharmaceutical, and oil industries [90]. Bioemulsifiers get accumulated at the interphase between the two immiscible phases by which they can reduce the surface tension, thereby increasing the solubility and emulsification of the immiscible phases. Accordingly, these compounds may convert insoluble substrate into soluble substrates, increasing their bioavailability and making them usable by the microorganisms [91].

In conclusion, the data collected in this study pave the way for further investigations finalized at exploiting both (i) the degradation ability of the bacterial isolates and/or microbial consortia to remediate hydrocarbon-contaminated sites through strategies like biostimulation or bioaugmentation, and (ii) the capability to produce molecules with a good promoting effect for the restoration of oil polluted matrices. In addition, the isolation of strains showing a 16S rRNA gene sequence identity < 97% with those available in the genetic sequence database, constitutes a significant result and represents a further exciting challenge to fully unravel the existing biodiversity at the study site and expand the current knowledge on biodegradation processes, also through the description and characterization of novel bacterial species.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4441/12/8/2090/s1, Figure S1: Geological section W-E crossing the Agri Valley, Figure S2: Hydrocarbon springs S1 and S2, Figure S3: Rarefaction curves of spring water, groundwater, and soil samples collected at the study site.

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