processes

MDPI

Article

Bioremediation of Polycyclic Aromatic Hydrocarbons from Industry Contaminated Soil Using Indigenous *Bacillus* spp.

Prisha Mandree [1,*], Wendy Masika [1], Justin Naicker [1], Ghaneshree Moonsamy [1], Santosh Ramchuran [1,2] and Rajesh Lalloo [1]

[1] Chemicals Cluster, Council for Scientific and Industrial Research, Meiring Naude Road, Pretoria 0001, South Africa; wmasika@csir.co.za (W.M.); jemmanuel777@gmail.com (J.N.); gmoonsamy@csir.co.za (G.M.); sramchuran@csir.co.za (S.R.); rlalloo@csir.co.za (R.L.)
[2] School of Life Sciences, University of KwaZulu-Natal, Durban 4041, South Africa
* Correspondence: pnaicker@csir.co.za

Abstract: Polycyclic aromatic hydrocarbons (PAHs) are reportedly toxic, ubiquitous and organic compounds that can persist in the environment and are released largely due to the incomplete combustion of fossil fuel. There is a range of microorganisms that are capable of degrading low molecular weight PAHs, such as naphthalene; however, fewer were reported to degrade higher molecular weight PAHs. *Bacillus* spp. has shown to be effective in neutralizing polluted streams containing hydrocarbons. Following the growing regulatory requirement to meet the PAH specification upon disposal of contaminated soil, the following study aimed to identify potential *Bacillus* strains that could effectively remediate low and high molecular weight PAHs from the soil. Six potential hydrocarbon-degrading strains were formulated into two prototypes and tested for the ability to remove PAHs from industry-contaminated soil. Following the dosing of each respective soil system with prototypes 1 and 2, the samples were analyzed for PAH concentration over 11 weeks against an un-augmented control system. After 11 weeks, the control system indicated the presence of naphthalene (3.11 $\mu g \cdot kg^{-1}$), phenanthrene (24.47 $\mu g \cdot kg^{-1}$), fluoranthene (17.80 $\mu g \cdot kg^{-1}$) and pyrene (28.92 $\mu g \cdot kg^{-1}$), which illustrated the recalcitrant nature of aromatic hydrocarbons. The soil system dosed with prototype 2 was capable of completely degrading (100%) naphthalene, phenanthrene and pyrene over the experimental period. However, the accumulation of PAHs, namely phenanthrene, fluoranthene and pyrene, were observed using prototype 1. The results showed that prototype 2, consisting of a combination of *Bacillus cereus* and *Bacillus subtilis* strains, was more effective in the biodegradation of PAHs and intermediate products. Furthermore, the bio-augmented system dosed with prototype 2 showed an improvement in the overall degradation (10–50%) of PAHs, naphthalene, phenanthrene and pyrene, over the un-augmented control system. The following study demonstrates the potential of using *Bacillus* spp. in a bioremediation solution for sites contaminated with PAHs and informs the use of biological additives for large-scale environmental remediation.

Keywords: *Bacillus*; bioremediation; hydrocarbon pollution; polycyclic aromatic hydrocarbons; PAH biological removal

Citation: Mandree, P.; Masika, W.; Naicker, J.; Moonsamy, G.; Ramchuran, S.; Lalloo, R. Bioremediation of Polycyclic Aromatic Hydrocarbons from Industry Contaminated Soil Using Indigenous *Bacillus* spp. *Processes* **2021**, *9*, 1606. https://doi.org/10.3390/pr9091606

Academic Editors: Raluca Maria Hlihor, Petronela Cozma and Avelino Núñez-Delgado

Received: 24 May 2021
Accepted: 21 August 2021
Published: 8 September 2021

1. Introduction

Environmental damage due to oil spills has resulted in the need for sustainable strategies to rehabilitate contaminated sites. Contamination of the environment with oil results in pollution, which affects terrestrial and marine life, commercial fisheries, recreational resources and public health. The oil can also contaminate run-off water to the sewer, which results in an increase in total petroleum hydrocarbon (TPH) concentration. Globally, the concentration of petroleum hydrocarbons contaminating soil ranges between 1.2 and 237 $g \cdot kg^{-1}$ [1]. Industry operations are therefore threatened by the growing regulatory requirements for safe and responsible disposal of contaminated soil and water and overall

preservation of the environment. Currently, the presence of volatile organic compounds (VOCs) and semi-volatile organic compounds (sVOCs) are prohibited in discharged trade effluent in South Africa [2], and a range of lower limits are also stipulated for TPH contaminating trade effluent and polycyclic aromatic hydrocarbons (PAHs) contaminating soil [3]. PAHs, specifically, are reportedly toxic, ubiquitous and organic compounds that can persist in the environment and are released into the environment largely due to the incomplete combustion of fossil fuel. Petroleum hydrocarbons, such as PAHs, also occur in high concentrations in petroleum sludge. The improper disposal of petroleum sludge has a negative effect on the environment, such as modification in the chemical and physical properties of soil, resulting in nutrient deficiency and stunted growth of vegetation. High viscous petroleum sludge is also easily entrained in the soil due to its porosity [4].

There is a range of microorganisms that are capable of degrading low molecular weight PAHs, such as naphthalene; however, fewer microorganisms have been observed to degrade higher molecular weight PAHs [5]. Toxic compounds, such as fluoranthene, are a major component of petroleum sludge and is a non-alternant high molecular weight PAH that has a five-member ring. Fluoranthene, including other high molecular weight PAHs, such as pyrene, benzo(a)pyrene and benzo(b)fluoranthene, are generally recalcitrant and resistant to microbial attack and are detrimental to the environment and human health [6].

Microbial populations from various genera have been detected in petroleum-contaminated soil and water, which suggests the ability of the organisms to use hydrocarbon compounds as a substrate for survival [7,8]. Isolated bacteria from brackish water capable of hydrocarbon degradation included *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Enterobacter*, *Escherichia*, *Flavobacterium*, *Norcadia*, *Pseudomonas*, *Staphylococcus* and *Vibrio* [8]. Bacteria were also found to degrade crude oil from a polluted water stream and included *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus subtilis*, *Bacillus* spp., *Alcaligenes* sp., *Acinetobacter lwoffi*, *Flavobacterium* sp., *Micrococcus roseus* and *Corynebacterium* sp. [9]. The most important hydrocarbon-degrading genera in soil environments include *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Collimonas*, *Corynebacterium*, *Dietzia*, *Flavobacterium*, *Gordonia*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas* and *Variovorax* [10–12]. Bioremediation is therefore suggested as an approach for remediation of contaminated soil sites due to the conversion of contaminants to harmless end products. Biodegradation of the hydrocarbons by natural populations of microorganisms is considered the primary mechanism for the removal of hydrocarbon pollutants from the environment [9]. The identification of PAH-degrading microorganisms specifically is significant in the development of effective remediation technologies [13]. *Bacillus* spp., in particular, were previously identified from polluted streams containing hydrocarbons and are known for their bioremediating properties [9,14]. In the study by Masika et al., *Bacillus* spp. were shown to effectively remediate TPH from contaminated effluent from industry [15]. The biodegradation within soil systems, however, is more complex due to interactions between the soil matrix and hydrocarbons [10,16]. A few studies reported the use of *Bacillus* spp. to degrade toxic PAHs specifically. In the study by Habib et al., *Bacillus megaterium* could degrade 72.44% of pyrene within 7 days [17]. In another study by Tarafdar et al., *Bacillus thuringiensis* was shown to degrade anthracene, one of the 16 United States Environmental Protection Agency's (EPA) priority chemicals [18].

While the bio-augmentation of contaminated soil using indigenous *Bacillus* spp. is suggested as a feasible solution to enhance existing microbial populations and improve the degradation of PAHs, it is necessary to demonstrate bioremediation effectiveness in controlled bio-augmented small-scale systems. This is required to inform the use of biological products for large-scale applications, in comparison to traditional methods.

The main goal of this research was to compare the removal of low and high molecular weight PAHs, using known *Bacillus* spp. as mixed consortia in bio-augmented systems. Two consortia of indigenous *Bacillus* spp. containing nutrient additives were developed and tested in the treatment of soil contaminated with greases, oils and fuel, obtained from a

selected industry site. It was hypothesized that the application of microbial populations of *Bacillus* strains previously used to treat TPH in contaminated water [15] would effectively remediate PAHs from contaminated soil.

2. Research Design

Following the aim of the research and hypothesis, the proposed research approach to evaluate PAHs was aligned to previous research, according to Masika et al. [15]. The core studies and focus of the research presented in this paper included the formulation of two product prototypes (*Bacillus* spp.) and the evaluation of PAH degradation potential in small-scale soil systems (Figure 1).

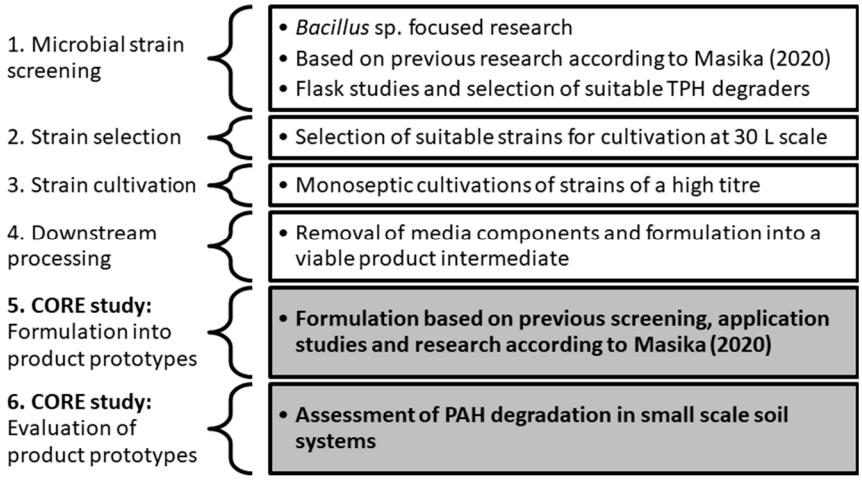


Figure 1. Proposed research design for evaluation of PAH degradation.

3. Materials and Methods

3.1. Microbial Strain Selection

3.1.1. Sampling and Isolation

Soil and water samples were collected from various hydrocarbon-contaminated environments across Gauteng (South Africa). A total of 38 sites were visited. Selective screening and isolation were performed using a modified protocol developed to obtain spore-forming *Bacillus* species (spp.) from environmental samples [19], as described in the study by Masika et al. [15]. Liquid samples (1 mL) were processed by mixing and transferring to sterile tryptone soy broth (TSB), soil samples (1 g) were weighed and transferred, whereas swab samples were added directly to the sterile media. The inoculated media (200 mL TSB each contained in Erlenmeyer flasks) were incubated on a rotary platform shaker at 32 °C and 180 rpm for a period of 5 days. For selective isolation of *Bacillus* spp. a sample (4 mL) of the culture broth containing spores was added to 9 mL of sterile nutrient broth (Unilab; prepared at a concentration of 16 $g{\cdot}L^{-1}$ sterilized for 15 min at 121 °C) contained in a McCartney bottle. The suspension was vortexed, and a sample (4 mL) was transferred to a sterile empty Falcon tube and incubated in an oven (Series 2000, Scientific, Johannesburg, South Africa) for 30 min at 45 °C. Following incubation, the sample was removed from the oven, and a 50% $v{\cdot}v^{-1}$ ethanol solution was added to make up to a final volume of 20 mL. The mixture was then incubated at room temperature for 60 min to allow for the dehydration of cells. Following dehydration, the tubes were centrifuged, and the supernatant was decanted and discarded. The remaining pellet was dried to evaporate any residual ethanol. The pellet was reconstituted in sterile water, serial dilutions were performed, and the dilutions were plated onto nutrient agar supplemented with polymyxin B, which aided the growth of the bacteria. Plates were incubated for 48 h. Colonies that germinated after the isolation were purified by passaging on plate count agar (PCA). Isolates were screened and selected based on colony morphology, gram stain, qualitative catalase enzyme activity and sporulation efficiency. The catalase test was conducted on all purified

microorganisms according to the methods outlined by Lalloo et al. [20]. This technique was used to potentially differentiate *Bacillus* spp. (catalase positive) from non-*Bacillus* spp. (catalase-negative).

3.1.2. Molecular Identification

Following the preliminary assessment of hydrocarbon degradation, molecular identification of selected isolates was performed (Inqaba Biotec, Pretoria, South Africa). DNA was extracted, and the 16S target region was amplified using DreamTaq™ DNA Polymerase (Thermo Fisher Scientific, Massachusetts, USA), and the primers used were Forward 27F: AGAGTTTGATCCTGGCTCAG and Reverse 1492R: GGTTACCTTGTTACGACT. PCR products were gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, California, Irvine, CA, USA) and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyzer. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Irvine, CA, USA) were analyzed using CLC Main Workbench 7 followed by Basic Local Alignment Search Tool (BLAST) search using the database produced by The National Center for Biotechnology Information (NCBI). The BLAST results were used to draw a phylogenetic tree to compare the sequence homologies to *B. cereus*, *B. thuringiensis* and *B. anthracis*. The *Bacillus* strains were stored as master cell banks in cryovials (2 mL) at −80 °C as part of the Council for Scientific and Industrial Research (CSIR, Pretoria, South Africa) culture collection.

3.1.3. Strain Selection Based on Preliminary Hydrocarbon Degradation

According to Figure 1, the following microbial strain screening and selection approach was used. A total of 115 presumed *Bacillus* species were isolated and purified from the environmental samples. Of the 115 isolates tested, approximately 97% tested Gram-positive, 100% tested positive for catalase activity and 94% were spore forming. These *Bacillus* strains were stored in the culture collection above and further assessed for their hydrocarbon degradation efficacy. The colorimetric screening was used to rapidly screen for potential hydrocarbon-degrading bacteria using the redox indicator, 2,6 dichlorophenol indophenol (2,6 DCPIP) in Bushnell–Haas (BH) media together with a hydrocarbon substrate [17]. Following this method, 20 potential strains were identified. These strains were subjected to further testing by measuring the performance using diesel as the hydrocarbon substrate. For each strain, sterile tryptone soy broth (TSB) (30 $g \cdot L^{-1}$) was supplemented with hydrocarbon substrate (1 $v \cdot v^{-1}$%) and inoculated. The cultures were incubated, and at the desired cell concentration, 1 mL of each culture was extracted and added into a 6-well plate containing BH media. The plates were further incubated, and samples were extracted at 0 and 16 h in order to determine the degradation of total petroleum hydrocarbons (TPH). For the purpose of screening and selection, gas chromatography (GC) analysis was performed using the Agilent 7890A GC system plus 7693 Autosampler, equipped with flame ionization detector (FID), using the fused silica capillary column Rxi-1ms with dimensions of 30 m × 0.25 mm × ID 1.0 µm (Restek, Bellefonte, PA, USA). The standard used was the total recoverable petroleum hydrocarbons (TRPH) standard (Restek, PA, USA). Hydrocarbon degrading strains were selected based on the methodology. A total of six strains, based on their performance, were finally selected for further evaluation.

3.2. High Cell Density Batch Cultivation

3.2.1. Fermentation Process for the Production of Hydrocarbon-Degrading Strains at 30 L Scale

High cell density batch fermentations were performed for each strain selected above using Biostat C-DCU 30 L (working volume) bioreactors (Sartorius Stedim Biotech, Goettingen, Germany). For each strain, sterile growth media was prepared in a 2 L Fernbach flask. For the preparation of the inoculum, each cryopreserved strain, stored at −80 °C in the CSIR culture collection, was used to inoculate a flask above, which was subsequently incubated at 32 °C and 180 rpm on an Innova 2300 rotary platform shaker (New Brunswick Scientific,

St Albans, UK). The initial volume of each bioreactor consisted of sterile liquid media composed of salts, a nitrogen source and antifoam. A separately sterilized carbon solution was added to the media following in situ sterilization. At the desired transfer age, inoculum for each strain was transferred into the respective bioreactor. The pH was maintained at 6.8 using 10% $v{\cdot}v^{-1}$ H_2SO_4 or 25% $v{\cdot}v^{-1}$ NH_4OH. The air-flow rate was set at approximately 1.6 $v{\cdot}v^{-1}{\cdot}min^{-1}$. The stirrer speed was gradually ramped up to 800 rpm, and dissolved oxygen concentration above 30% saturation was maintained. The cultivation temperature was maintained at 32 °C, and each strain was cultivated until complete sporulation was achieved. Each culture was then stabilized and the fermentation broth was harvested from the respective bioreactor.

3.2.2. Strain Recovery

Solid-liquid separation of the fermentation broth was performed using centrifugation. For each strain, the fermentation broth was centrifuged using a BRPX-207 disc stack centrifuge (Alfa Laval, Lund, Sweden) operated at a bowl speed of 6158 rpm. The fermentation broth was fed from a holding tank into the centrifuge at a flow rate of 120 $L{\cdot}h^{-1}$. The pellet from within the centrifuge bowl was automatically purged every 20 min. The optical density (OD) of the supernatant was measured to limit losses to $\leq$10%. The final recovered pellet was used for the production of the product prototypes.

3.3. Preparation of Mixed Culture Prototypes for Efficacy Testing

The solid pellet fraction obtained after centrifugation, as described in Section 3.2.2, was used to prepare the mixed culture prototypes. The pellet containing spores of each organism were reconstituted in a nutrient activation mix containing key proteins, carbohydrates and minerals, and formulated to a viable cell concentration of 1×10^8 $CFU{\cdot}mL^{-1}$. Two microbial prototypes were formulated by mixing equal proportions of each purified bacterial culture as follows:

Prototype 1 consisted of *Bacillus velezensis* strains GPA7.1 and GPA6.2, as well as, *Bacillus subtilis* strain GPA11.2.

Prototype 2 consisted of *Bacillus subtilis* strains P402 and D014, as well as, *Bacillus cereus* strain P401.

The two product prototypes were used to treat hydrocarbon contaminated soil and tested against the untreated (or un-augmented) negative control system (referred to as control in the following results).

3.4. In Vitro Hydrocarbon Degradation Assessment

Following the preparation of two product prototypes consisting of *Bacillus* strains, the study was performed to test the hypothesis that application of microbial populations of *Bacillus* strains previously used to treat TPH in contaminated water would effectively remediate PAHs from contaminated soil.

3.4.1. Assessment and Source of Contaminated Soil

The following study was conducted on hydrocarbon contaminated soil obtained from a selected industry site in Johannesburg, South Africa (GPS coordinates, S 26°8′33.918″, E 28°12′25.369″). The hydrocarbon contamination is attributable to the maintenance and cleaning of heavy industrial equipment. The unsterilized soil was excavated, and all visible surface litter removed.

3.4.2. Configuration and Setup of Bench-Scale Evaluation System

Approximately 250 g of a representative sample of the soil was dispensed into a 250 mL Schott bottle and analyzed for PAHs according to USEPA Method 8270E [21]. Once the soil was confirmed for the presence of petroleum hydrocarbons (>1%), the study was conducted over 11 weeks. Equal quantities (8.5 kg) of the contaminated soil samples were dispensed into three plastic containers to maintain a depth of approximately 4 cm. Two

soil test systems were inoculated with 0.1% ($m \cdot m^{-1}$) of prototypes 1 and 2, respectively, at the start of the trial. The remaining control test system was left un-augmented for the duration of the study. Water (20 mL) was added to each test system to maintain a soil moisture content of 10–15% ($m \cdot m^{-1}$), according to [7,22]. For each test system, the biological prototypes and water were thoroughly mixed into the soil. Each test system was covered with a perforated plastic lid and placed in an incubation room at 30 °C with a relative humidity of 60% for the duration of the trial (Figure 2).

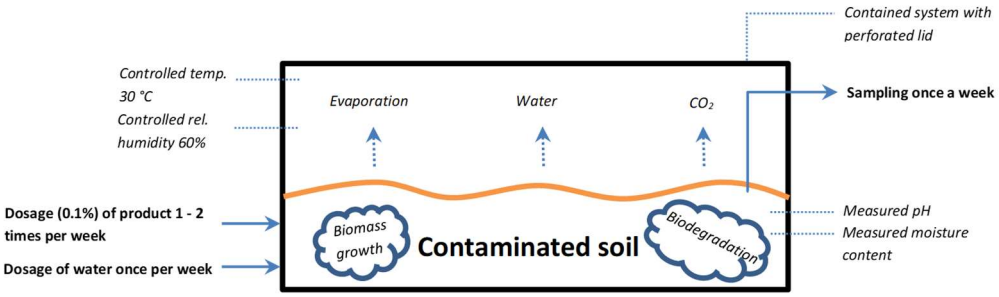


Figure 2. Diagrammatic representation of test system.

3.4.3. Monitoring and Evaluation of the Test Systems

For the inoculated test systems, the respective prototypes were dosed (0.1% on a dry basis) into the soil once a week for the first five weeks and twice a week thereafter. Water was dosed once a week according to Equation (2) (Section 3.4.4 (b)) to maintain moisture content between 10–15% ($m \cdot m^{-1}$) for the duration of the study. Each system was toiled on a weekly basis, and samples (250 g) were taken weekly using 250 mL Schott bottles, sealed and analyzed thereafter. One replicate was performed for each treatment due to the large volume of soil sample required for extraction and analysis of hydrocarbons and to prevent disturbances within the system. The samples were measured externally at a SANAS (South African National Accreditation System) accredited laboratory that is used frequently by the industry to determine whether environmental samples are within specification.

3.4.4. Analyses

(a) pH

The dissolution pH and moisture content were monitored for the duration of the study. The procedure for pH analysis was adapted using the USEPA Method 9045D [23], whereby 20 g of soil was added to 20 mL of distilled water contained in a Schott bottle with a stirrer. The soil suspension was continuously stirred for 5 min and then left to stand for 15 min. The aqueous phase (~20 mL) was removed by decanting into a sterile McCartney bottle.

(b) Moisture content

The moisture content was determined using a Mettler Toledo Excellence HS153 Moisture analyzer (Mettler Toledo, Columbus, OH, USA). Two grams of the soil sample from the bulk soil was used to perform the analyses. The instrument was set at the following conditions; drying temperature: 105 °C, switch-off criterion: 1 mg/50 s, moisture content: moisture content (%) on a dry weight basis. The drying process was initiated, and once the drying process was complete (approximately 8–10 min), the moisture content (%) was recorded. The mass of the soil on a dry basis was calculated according to Equation (1). Water was added to each test system on a dry basis according to Equation (2).

$$Mass\ of\ the\ sample\ (dry\ basis) = \left(1 - \frac{measured\ moisture\ content}{100}\right) \times mass\ of\ the\ sample\ (wet\ basis) \quad (1)$$

$$Mass\ of\ water\ to\ be\ added = \left(0.15 - \frac{measured\ moisture\ content}{100}\right) \times mass\ of\ bulk\ soil\ (dry\ basis) \quad (2)$$

(c) PAH concentration

Polycyclic aromatic hydrocarbons were extracted from the soil samples (250 g) and measured according to the modified USEPA Method 8270E [21]. Analyses were performed using the Bruker 450-C GC and Bruker 300 MS systems (Bruker Corporation, MA, USA), respectively, and the fused silica capillary column Rxi-5Sil MS with dimensions of 30 m × 0.25 mm × ID 0.25 µm (Restek, PA, USA). The injection volume was between 0.5 and 2 µL, and the inlet flow rate was 100 mL·min^{-1} (split flow). A full scan MS mode was used (*m/z* 35–500). The method of analysis used was specifically for the assessment of PAH degradation in small-scale soil systems (Figure 1).

(d) Statistical methods

Regression analysis was used to compare the slopes and standard error of the regressed hydrocarbon concentrations over 11 weeks across the control and test systems, according to the methods outlined by Andrade and Estévez-Pérez [24].

4. Results and Discussion

4.1. Microbial Strain Identification and Preliminary Assessment of Hydrocarbon Degradation

The BLAST search of 6 *Bacillus* strains selected for evaluation showed ~100% sequence similarity (16S rRNA) to known deposited sequences in the NCBI, and their identification was confirmed at a species level to *cereus*, *subtilis* and *velezensis* (Table 1). Strains GPA7.1 and GPA6.2 demonstrated TRPH degradation >90%; this was followed by GPA11.2 with degradation >80% and finally D014 and P402 with degradations >70%. The results obtained are aligned to the literature findings on *Bacillus* spp. hydrocarbon degradation. According to the literature, the most important hydrocarbon-degrading bacterial genera in soil environments include *Bacillus* spp. [10,11,25]. A typical substrate for *Bacillus* spp. includes toluene and crude oil [10].

Table 1. Molecular identification of strains and TRPH degradation (%) after 16 h.

Strain	Molecular Identification	TRPH (%)
GPA7.1	*Bacillus velezensis*	92.94 ± 1.23
GPA11.2	*Bacillus subtilis*	84.86 ± 1.84
GPA6.2	*Bacillus velezensis*	95.99 ± 0.48
P401	*Bacillus cereus*	65.65 ± 6.30
D014	*Bacillus subtilis*	78.83 ± 4.50
P402	*Bacillus subtilis*	78.83 ± 4.50

4.2. High Cell Density Cultivations of Selected Strains

All six strains were successfully cultivated to a high cell density of >10 g·L^{-1} dry cell weight in 30 L bioreactors, compared to accepted biomass production of >1 g·L^{-1} as per the literature findings [26]. The fermentation time taken to reach the target cell density for each strain was between 38 and 46 h post-inoculation. High cell density, also indicated by cell concentration (cell·mL^{-1}), and high sporulation efficiency is required to cost-effectively formulate products to a market-accepted viable cell concentration (>1 × 10^{8} CFU·mL^{-1}) in order for the product to be efficacious and meet end-user requirements. The process leading from a vegetative bacterial cell to a spore is triggered by the depletion of the cell's nutrient source or changes in aspects of the environment. In the spore form, microorganisms are virtually metabolically inactive. Spores are preferred in product formulation as they exhibit a higher degree of resistance to inactivation from physical sources, such as heat, UV or extreme desiccation, therefore exhibiting great longevity, which is useful for product shelf-life [27]. All strains cultivated exceeded the target cell concentration of 1.0 × 10^{10} cell·mL^{-1}. Strains GPA7.1, GPA11.2, GPA6.2 and P401 obtained a high sporulation efficiency (>90%) (Table 2); according to Escobar [28], high sporulation efficiency is classified as being >85%. All cultivations were deemed acceptable for the purpose of testing hydrocarbon-degrading potential in soil.

Table 2. Summary of cell concentration (cell·mL^{-1}) and sporulation efficiency (%) obtained post-fermentation for the selected strains.

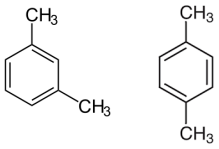
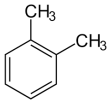
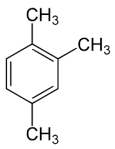
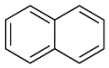
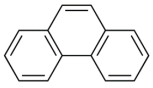
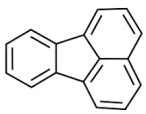
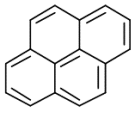
Strain	Final Cell Concentration (Cell·mL^{-1})	Sporulation Efficiency (%)
GPA7.1	2.29×10^{10}	90
GPA11.2	1.62×10^{10}	91
GPA6.2	1.51×10^{10}	76
P401	2.10×10^{10}	95
D014	3.3×10^{10}	92
P402	1.84×10^{10}	81

4.3. In Vitro Hydrocarbon Degradation Assessment of Prototypes Containing Mixed Cultures

4.3.1. Assessment of PAH Contaminants in Soil

PAHs occur in high concentrations near coal gasification sites and tar oil distillation plants. PAHs emanating from fuel combustion, automobiles, spillage of petroleum products and waste incineration are also significant sources of pollution into the environment [29]. Construction equipment and activities contribute to the contamination of soil with greases, oils and fuels, which contain PAHs [30]. Furthermore, the combustion of fuels, particularly when using construction equipment, results in the accumulation of PAHs in the surface layer of soil [31]. The research findings are consistent with the initial analyses of the contaminated soil obtained from an industry site that rents out construction equipment. The initial analyses indicated a significant concentration of PAH contaminants in the soil (Table 3).

Table 3. Concentrations of aromatic and PAH contaminants in the soil.

Hydrocarbon	Concentration (µg·kg^{-1})	Chemical Description	Molecular Weight (g·mol^{-1})
M+P Xylene	19		106.16
O-Xylene	18		106.16
1,2,4 Trimethyl benzene	19		120.19
Naphthalene	29		128.17
Phenanthrene	46		178.23
Fluoranthene	34		202.25
Pyrene	70		202.26

According to specifications stipulated and reported for the protection of water, for industry and for protection of ecosystems in South Africa [3], the conclusion drawn was that the initial concentration of contaminants in the soil exceeded certain limits specified for the protection of ecosystems. The study was conducted to determine whether the hydrocarbons could be remediated for the protection of natural ecosystems and to determine the efficacies of the respective treatment systems.

4.3.2. Removal of Polycyclic Aromatic Hydrocarbons in Test Systems

After 74 days, test systems utilizing both prototypes 1 and 2 showed degradation at varying degrees versus the untreated negative control system. Results from the test system showed that the compounds, namely M+P Xylene, O-Xylene and 1,2,4-trimethyl benzene, were completely removed from all three systems after 14 days. The complete removal of M+P Xylene, O-Xylene and 1,2,4-trimethyl benzene from all three systems is attributed to their low molecular weight (Table 3) and volatility versus the high molecular weight aromatics; generally, an increase in a PAH molecular weight results in a corresponding increase in hydrophobicity and electrochemical stability [6,32]. After 74 days, the control system indicated the presence of naphthalene (3.11 $\mu g \cdot kg^{-1}$), phenanthrene (24.47 $\mu g \cdot kg^{-1}$), fluoranthene (17.80 $\mu g \cdot kg^{-1}$) and pyrene (28.92 $\mu g \cdot kg^{-1}$); PAH degradation of naphthalene was 89.30%, and degradation of the remaining three compounds was between 45% and 60% (Figure 3). This illustrates the recalcitrant nature of aromatic hydrocarbons and justifies the requirement for biological treatment. Any removal of hydrocarbons in the control system could be attributed to bioremediation by natural populations of microorganisms occurring in the soil; these removal efficiencies of the natural population were further enhanced by maintaining optimum moisture, temperature and humidity conditions [9]. Regression analysis showed no significant difference in the rate of degradation ($mg \cdot L^{-1} \cdot h^{-1}$), determined by inferring slope and standard error values, between the untreated negative control system and test systems utilizing prototypes 1 and 2 (p-value > 0.05). Hydrocarbon degradation generally follows non-linear regression due to the formation of intermediates, and therefore drawing conclusions based solely on the rate of degradation was insufficient [18]. The differences in control and test systems were therefore attributed to the absence or presence of PAHs after 74 days, according to Figure 3. The intermediates formed during the test period and the specific degradation pathways of the strains used in the product prototypes were outside the scope of this study.

The accumulation of PAHs, namely phenanthrene, fluoranthene and pyrene, were observed after 74 days of treatment using prototype 1 (Figure 4), and there was no improvement in degradation versus the control. In comparison, prototype 2 was capable of completely degrading (100%) naphthalene, phenanthrene and pyrene (Figure 5). The accumulation of one compound, namely fluoranthene, after 74 days of treatment, was observed in the control system (47.81% degradation) and while using prototype 1 (25.58% degradation) and prototype 2 (28.05% degradation). However, the results are consistent with findings from the literature, where the reported efficiency of degradation for soil bacteria is between 0.13% and 50% [9]. In the study performed by Rabodonirina et al., bacterial strains identified in soil, including *Bacillus simplex* and *Bacillus pumilus*, achieved degradation (%) of low molecular weight PAHs fluorene (65–86%) and phenanthrene (86–95%) after a 72 day incubation period; however, no significant degradation was observed for higher molecular weight PAHs [33]. According to Gupta et al., *Bacillus* spp. were reported to degrade the high molecular weight PAH, pyrene, from soil up to 48% [34]. The performance of prototype 2 was therefore deemed suitable when comparing the degradation (%) of PAHs using *Bacillus* spp. reported in the literature.

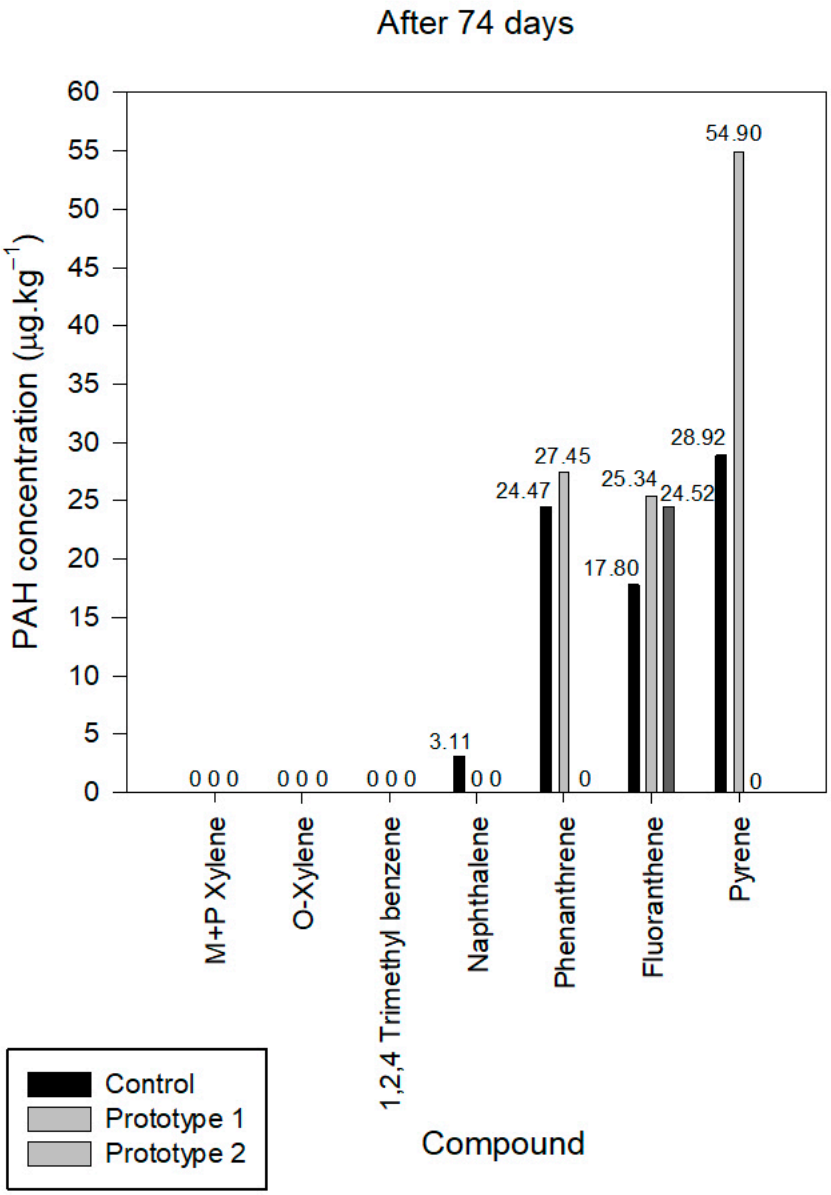


Figure 3. Polycyclic aromatic hydrocarbon (PAH) concentrations (µg·kg^{-1}) for the control and test systems after 74 days. n = 1.

Interestingly, PAH degradation (%) using prototype 1 decreased with an increase in the molecular weight of the compound, which demonstrates the difficulty in degrading higher molecular weight compounds using prototype 1. Hydrocarbons differ in their susceptibility to microbial attack and can generally be ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes > high molecular weight aromatics [10]. Therefore, high molecular weight aromatics are the most difficult to break down. The microbial degradation of low molecular weight PAHs, such as naphthalene and phenanthrene, has been well documented and the biochemical pathways elucidated [14,29]. However, the environmental persistence of PAHs increases as their molecular size increases to four or five benzene rings, indicating that chemical and biological degradation is slow due to only a limited number of microbes or microbial populations being able to degrade these compounds. It has also been shown that low molecular weight hydrocarbons can be metabolized by pure strains, whereas higher molecular weight organisms may depend on the combination of different populations [5].

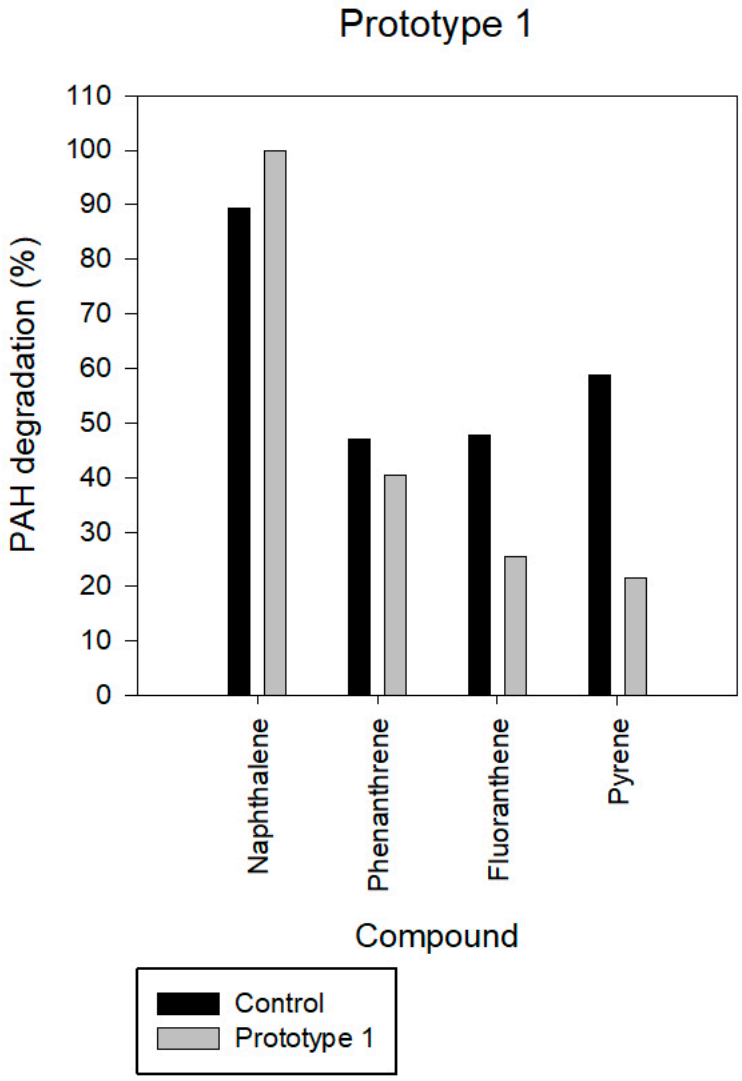


Figure 4. Polycyclic aromatic hydrocarbon (PAH) degradation (%) using prototype 1 versus the control system after 74 days. n = 1.

Prototypes 1 and 2, were both formulated using only *Bacillus* spp. however, only prototype 2 was successful in the complete degradation (100%) of the three compounds, namely naphthalene, phenanthrene and pyrene, under the subjected environmental conditions and performed better than the untreated negative control system. The degradation of hydrocarbons from the control system can be attributed to the natural population of microorganisms in the soil, as well as the evaporation of hydrocarbons from the system. Therefore, the net improvement in the degradation of hydrocarbons using prototype 2 can be calculated by considering the efficiency of the control system. Improvements in degradation when using prototype 2 over the control system were 10.71%, 53.04% and 41.19% for naphthalene, phenanthrene and pyrene, respectively. Prototype 2 was unsuccessful in the removal of fluoranthene. Prototype 1 was selected for testing based on the ability to degrade hydrocarbons between 84% and 96% (Table 1) in a water system; however, the accumulation of PAHs was observed in a soil system treated with prototype 1. It is therefore postulated that the hydrocarbon-degrading efficacy of microorganisms differs in water and soil systems. A contributing factor to the difference in performance between the prototypes includes the contaminant (hydrocarbon) bioavailability. Microbial surfactants can promote the bacterial growth of petroleum hydrocarbons by increasing surface area between oil and water through emulsification, which results in an increase of the contaminant bioavailability to the degrading microorganisms [16]. If this mechanism

of degradation is adopted, then the removal of hydrocarbons needs to be promoted by (1) availability of the contaminant and (2) accessibility of the contaminant. The composition of the product added must not limit the accessibility of the contaminant; the composition of the soil (containing clay particles, for example) may also affect the accessibility of the contaminant. It is possible the soil constituents in particular, could be detrimental to the growth of the microorganisms. With fine soil particles, the pores between the grains are smaller, restricting the flow of water and gas; this leads to a lack of oxygen in the soil [7]. Furthermore, it was also found that the presence of certain hydrocarbons may have an inhibitory effect on overall degradation [14].

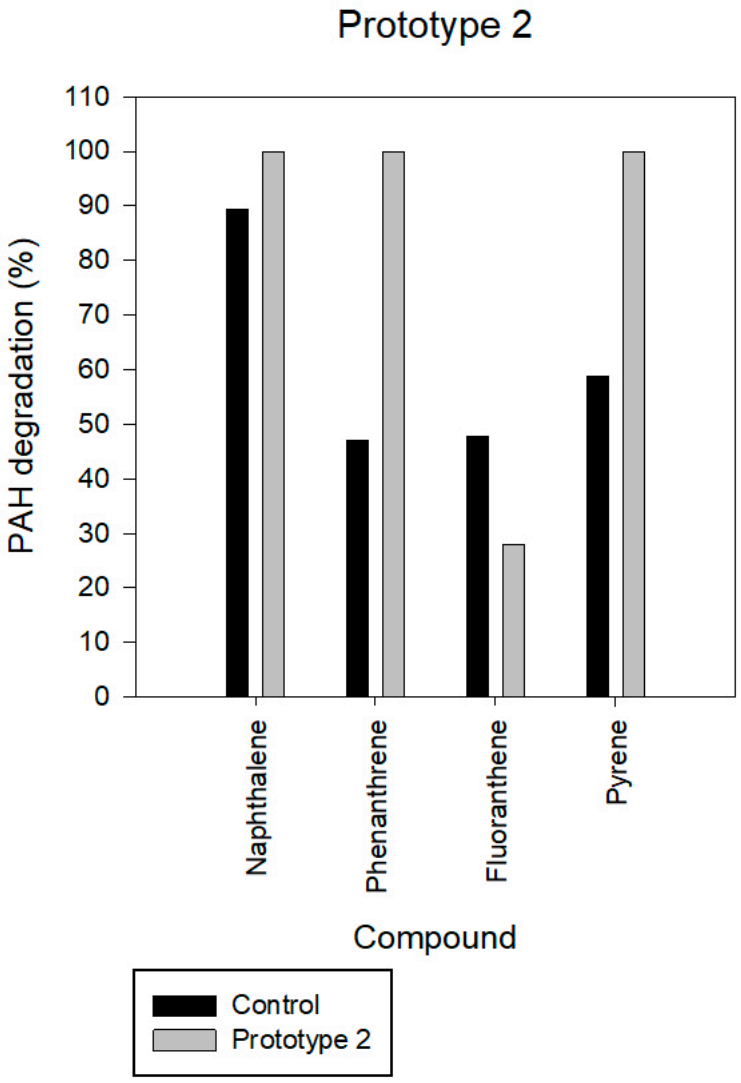


Figure 5. Polycyclic aromatic hydrocarbon (PAH) degradation (%) using prototype 2 versus the control system after 74 days. n = 1.

The performance of prototype 2 consisting of *Bacillus* spp. was favorable when considering the degradation (%) achieved using other microorganisms reported in the literature. Numerous fungal species, such as *Mycobacterium* sp., were reported to degrade PAHs [35]. The transformation of PAHs using fungal species may involve several enzymatic pathways [35]. The degradation (%) of PAHs from media containing pyrene and phenanthrene by *Mycobacterium* sp. was demonstrated using two-phase partitioning bioreactors under optimum conditions [36]; pyrene and phenanthrene were completely degraded (100%) between a 70–80 h test period. In the study by Kim and Lee, initial experiments indicated a 58% degradation of phenanthrene by *Penicillium* sp. and 35% degradation of phenanthrene

by *Aspergillus terreus* [37]. In PAH contaminated soil, *Penicillium* sp. alone resulted in the degradation (%) of four PAHs, namely anthracene (45%), fluoranthene (15%), phenanthrene (64%) and pyrene (46.5%), after a five-week period [37]. Whereas, *Penicillium* sp. co-cultured with *Rhodococcus* sp. resulted in the more efficient degradation (%) of anthracene (66.7%), fluoranthene (16%), phenanthrene (72.8%) and pyrene (68.4%) [37]. Both *Mycobacterium* and *Rhodococcus* species have glycolipids in their cell walls, which may contribute to the interaction with hydrophobic PAHs and subsequent uptake [36]. In the study by Ma et al., *Pseudomonas* sp. was cultivated in enriched media containing PAHs and was shown to degrade more than 90% of fluorene and phenanthrene, and approximately 40% of anthracene at 18, 28 and 37 °C during the 96 h test period [38]. Notably, the dosage regime and performance of prototype 2 consisting of *Bacillus* spp. can be simulated for real-world applications compared to other microorganisms reported in the literature.

The biotransformation pathways of *Bacillus* spp. contributing to the differences in performance of the two product prototypes is also not fully understood. Typically, hydrocarbon-utilizing bacteria will accumulate hydrocarbons within the cell and assimilate the compounds for normal cell functioning [9,32] however, the biotransformation pathway of aromatic compounds using a diverse microbial population is complex [14]. Furthermore, intermediate hydrocarbon product formation was observed between 28 and 74 days of treatment due to the synthesis of hydrocarbons using various elucidated pathways [14,29,32]. In general, PAHs are recalcitrant due to complex mixtures of compounds that co-exist [29], and therefore, it is postulated that while prototype 2 was capable of completely degrading three higher molecular weight compounds, it was unable to completely degrade fluoranthene due to the dynamics within the system that were outside the scope of this study. Deoxygenases are responsible for the aerobic oxidation of lower molecular weight aromatic hydrocarbons compounds; genes encoding these deoxygenases have been cloned and sequenced. Comparatively, less information is available on bacterial genes encoding proteins for the degradation of higher molecular weight PAHs, such as phenanthrene, anthracene, pyrene and fluoranthene.

5. Conclusions

Prototype 2 was more effective in degrading polycyclic aromatic hydrocarbons (PAHs), including intermediate compounds, compared to prototype 1 and the un-augmented control system. Bio-augmentation using prototype 2 resulted in the degradation of naphthalene (100%), phenanthrene (100%) and pyrene (100%). This confirms the improved performance of biologically augmented soil systems. However, this is strain and consortia dependent, and therefore the research hypothesis that top-performing TPH degrading strains can effectively degrade PAHs holds true for prototype 2 only. In a dynamic system consisting of naturally occurring microbial populations and varying environmental conditions, prototype 2 is more suited to degrading high molecular weight contaminants and recalcitrant intermediates from the soil. A bio-augmented soil system using prototype 2 offers an improvement (%) in naphthalene, phenanthrene and pyrene removal, over the un-augmented natural system, of 10.71%, 53.04% and 41.19%, respectively. Further work is required in determining the optimum consortium requirements for fluoranthene degradation. The following study contributes to the evidence that *Bacillus* spp. could be effective in removing hydrocarbons from contaminated sites. Development work underway includes assessing the possibility of using these strains as commercially formulated products for the bioremediation of polycyclic aromatic hydrocarbons.

Author Contributions: P.M. was responsible for conceptualisation of the study, testing of the methodology, analysis and interpretation of the data, deriving an application of the prototypes and writing the original draft. W.M. was responsible for investigating the top performing strains that could be used for hydrocarbon remediation. G.M. provided supervision for activities related to investigating the top performing strains. J.N. was responsible for setup of the experiment, optimisation, conducting the experiment, and collecting data. R.L. contributed to conceptualisation and methodology development of the study, provided supervision and contributed to reviewing and editing of the

document. S.R. provided supervision and contributed to reviewing and editing of the document. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. Parliamentary Grant funding was used to conduct the research. The funding body did not participate in the design and execution of the study, or writing of the original draft.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare that they have no competing interest.

References

1. Andrade, J.M.; Estévez-Pérez, M.G. Statistical comparison of the slopes of two regression lines: A tutorial. *Anal. Chim. Acta* **2014**, *838*, 1–12. [CrossRef]
2. Atlas, R.M. Microbial Degradation of Petroleum Hydrocarbons: An Environmental Perspective. *Microbiol. Rev.* **1981**, *45*, 180–209. [CrossRef]
3. Chikere, C.B.; Okpokwasili, G.C.; Chikere, B.O. Monitoring of microbial hydrocarbon remediation in the soil. *3 Biotech* **2011**, *1*, 117–138. [CrossRef]
4. Das, N.; Chandran, P. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. *Biotechnol. Res. Int.* **2011**, *2011*, 941810. [CrossRef]
5. Department of Environmental Affairs National Norms and Standards for the Remediation of Contaminated Land and Soil Quality. *Gov. Gaz.* **2012**, *561*, 1–16.
6. Escobar, V.V.; López, S.M.; Uribe, L.F.P.; Correa, E.M.R.; Gaviria, T.Z.C.; Castillo, J.J.M.; Roldán, L.E.A. Process for Increasing Biomass and Spores Production of Plant Growth Promoting Bacteria of the Bacillus Genus. U.S. Patent No. 9,839,222, 12 December 2017.
7. Gałazka, A.; Gałazka, R. Phytoremediation of polycyclic aromatic hydrocarbons in soils artificially polluted using plant-associated-endophytic bacteria and Dactylis glomerata as the bioremediation plant. *Pol. J. Microbiol.* **2015**, *64*, 241–252. [CrossRef]
8. Ghazali, F.M.; Rahman, R.N.Z.A.; Salleh, A.B.; Basri, M. Biodegradation of hydrocarbons in soil by microbial consortium. *Int. Biodeterior. Biodegrad.* **2004**, *54*, 61–67. [CrossRef]
9. Ghosal, D.; Ghosh, S.; Dutta, T.K.; Ahn, Y. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): A review. *Front. Microbiol.* **2016**, *7*, 1837. [CrossRef] [PubMed]
10. Giunta, M.; Lo Bosco, D.; Leonardi, G.; Scopelliti, F. Estimation of gas and dust emissions in construction sites of a motorway project. *Sustainability* **2019**, *11*, 7218. [CrossRef]
11. Gkorezis, P.; Daghio, M.; Franzetti, A.; Van Hamme, J.D.; Sillen, W.; Vangronsveld, J. The interaction between plants and bacteria in the remediation of petroleum hydrocarbons: An environmental perspective. *Front. Microbiol.* **2016**, *7*, 1836. [CrossRef] [PubMed]
12. Gupta, G.; Kumar, V.; Pal, A.K. Microbial Degradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons with Emphasis on Pyrene. Polycyclic Aromatic Compounds. *Taylor Fr.* **2019**, *39*, 124–138. [CrossRef]
13. Habib, S.; Johari, W.L.W.; Shukor, M.Y.; Yasid, N.A. Screening of Hydrocarbon-degrading Bacterial Isolates Using the Redox Application of 2, 6-DCPIP. *Bioremediat. Sci. Technol.* **2017**, *5*, 13–16.
14. Hamamura, N.; Olson, S.H.; Ward, D.M.; Inskeep, W.P. Microbial population dynamics associated with crude-oil biodegradation in diverse soils. *Appl. Environ. Microbiol.* **2006**, *72*, 6316–6324. [CrossRef] [PubMed]
15. Haritash, A.K.; Kaushik, C.P. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J. Hazard. Mater.* **2009**, *169*, 1–15. [CrossRef] [PubMed]
16. Ichor, T.; Okerentugba, P.; Okpokwasili, G. Biodegradation of Total Petroleum Hydrocarbon by Aerobic Heterotrophic Bacteria Isolated from Crude Oil Contaminated Brackish Waters of Bodo Creek. *J. Bioremediat. Biodegrad.* **2014**, *5*, 1–6. [CrossRef]
17. Johnson, O.A.; Affam, A.C. Petroleum sludge treatment and disposal: A review. *Environ. Eng. Res.* **2019**, *24*, 191–201. [CrossRef]
18. Kelly, D.P.; Ardley, J.K.; Wood, A.P. Cultivation of Methylotrophs. In *Hydrocarbon and Lipid Microbiology Protocols—Springer Protocols Handbooks*; Springer: Berlin/Heidelberg, Germany, 2014; pp. 249–268. [CrossRef]
19. Kim, J.D.; Lee, C.G. Microbial degradation of polycyclic aromatic hydrocarbons in soils by bacterium-fungus co-cultures. *Biotechnol. Bioprocess Eng.* **2007**, *12*, 410–416. [CrossRef]
20. Lalloo, R.; Ramchuran, S.; Ramduth, D.; Görgens, J.; Gardiner, N. Isolation and selection of *Bacillus* sas potential biological agents for enhancement of water quality in culture of ornamental fish. *J. Appl. Microbiol.* **2007**, *103*, 1471–1479. [CrossRef]
21. Lawal, A.T. Polycyclic aromatic hydrocarbons: A review. Cogent Environmental Science. *Cogent* **2017**, *3*, 537–567. [CrossRef]
22. Leahy, J.G.; Colwell, R.R. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.* **1990**, *54*, 305–315. [CrossRef]

23. Lee, K.C.; Darah, I.; Ibrahim, C.O. A laboratory scale bioremediation of Tapis crude oil contaminated soil bybioaugmentation of Acinetobacter baumannii T30C. *Afr. J. Microbiol. Res.* **2016**, *5*, 2609–2615. [CrossRef]
24. Lei, Y.J.; Zhang, J.; Tian, Y.; Yao, J.; Duan, Q.S.; Zuo, W. Enhanced degradation of total petroleum hydrocarbons in real soil by dual-frequency ultrasound-activated persulfate. *Sci. Total. Environ.* **2020**, *748*, 141414. [CrossRef] [PubMed]
25. Ma, J.; Xu, L.; Jia, L. Degradation of polycyclic aromatic hydrocarbons by *Pseudomonas* sp. JM2 isolated from active sewage sludge of chemical plant. *J. Environ. Sci.* **2012**, *24*, 2141–2148. [CrossRef]
26. MacLeod, C.T.; Daugulis, A.J. Interfacial effects in a two-phase partitioning bioreactor: Degradation of polycyclic aromatic hydrocarbons (PAHs) by a hydrophobic Mycobacterium. *Process. Biochem.* **2005**, *40*, 1799–1805. [CrossRef]
27. Masika, W.S.; Moonsamy, G.; Mandree, P.; Ramchuran, S.; Lalloo, R.; Kudanga, T. Biodegradation of petroleum hydrocarbon waste using consortia of *Bacillus* sp. *Bioremediat. J. Taylor Fr.* **2020**, *25*, 72–79. [CrossRef]
28. Mohandass, R.; Rout, P.; Jiwal, S.; Sasikala, C. Biodegradation of Benzo[a]pyrene by the mixed culture of *Bacillus cereus* and *Bacillus vlretl* isolated from the petrochemical industry. *J. Environ. Biol.* **2012**, *33*, 985–989.
29. Nicholson, W.L.; Munakata, N.; Horneck, G.; Melosh, H.J.; Setlow, P. Resistance of Bacillus Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 548–572. [CrossRef]
30. Obayori, O.S.; Salam, L.B. Degradation of polycyclic aromatic hydrocarbons: Role of plasmids. *Sci. Res. Essays* **2010**, *5*, 4096–4109.
31. Pogodda, S. Requirements for Discharge of Trade Effluent into the Public Sewers. 2013; p. 18571442. Available online: https://www.pub.gov.sg/Documents/requirements_UW.pdf (accessed on 4 July 2018).
32. Rabodonirina, S.; Rasolomampianina, R.; Krier, F.; Drider, D.; Merhaby, D.; Net, S.; Ouddane, B. Degradation of fluorene and phenanthrene in PAHs-contaminated soil using Pseudomonas and Bacillus strains isolated from oil spill sites. *J. Environ. Manag.* **2019**, *232*, 1–7. [CrossRef]
33. Seo, J.-S.; Keom, Y.-S.; Li, Q.X. Bacterial degradation of aromatic compounds. *Int. J. Environ. Res. Public Health* **2009**, *6*, 278–309. [CrossRef]
34. Sooch, B.S.; Kauldhar, B.S.; Puri, M. Types, Structure, Applications and Future Outlook. *Microb. Enzym. Technol. Food Appl.* **2020**, 241–254. [CrossRef]
35. Tarafdar, A.; Sinha, A.; Masto, R.E. Biodegradation of anthracene by a newly isolated bacterial strain, *Bacillus thuringiensis* AT.ISM.1, isolated from a fly ash deposition site. *Lett. Appl. Microbiol.* **2017**, *65*, 327–334. [CrossRef] [PubMed]
36. USEPA Method 9045D: Soil and Waste pH. United States Environmental Protection Agency, (November). Available online: http://biblioteca.usac.edu.gt/tesis/08/08_2469_C.pdf (accessed on 6 July 2018).
37. USEPA Method 8270E: Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS). United States Environmental Protection Agency. 2018. Available online: https://www.epa.gov/sites/default/files/2020-10/documents/method_8270e_update_vi_06-2018_0.pdf (accessed on 8 July 2018).
38. Wang, W.; Wang, L.; Shao, Z. Polycyclic aromatic hydrocarbon (PAH) degradation pathways of the obligate marine PAH degrader *Cycloclasticus* sp. strain P1. *Appl. Environ. Microbiol.* **2018**, *84*, e01261-18. [CrossRef] [PubMed]