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Biodegradation of High Concentrations of Aliphatic Hydrocarbons in Soil from a Petroleum Refinery: Implications for Applicability of New Actinobacterial Strains

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Featured Application: Microbial strains *Gordonia rubripertincta* CWB2 and *Rhodococcus erythropolis* S43 revealed good potential for bioremediation of soils highly contaminated by petroleum hydrocarbons.

Abstract: At present, there is great demand for new resistant and metabolically active strains of biodegrading bacteria capable of degrading high concentrations of petroleum pollutants. In this study, we undertook a series of pot-based biodegradation experiments on soil from a petroleum refinery lagoon heavily polluted with aliphatic hydrocarbons (81.6 ± 2.5 g·kg^{-1} dry weight) and metals. Periodical bioaugmentation with either a mixture of isolated degraders identified as *Bacillus* sp. and *Ochrobactrum* sp. or biostimulation with nutrient medium, singly or in combination, did not produce any significant decrease in hydrocarbons, even after 455 days. Inoculation with *Gordonia rubripertincta* CWB2 and *Rhodococcus erythropolis* S43 in iron-limited media, however, resulted in a significant decrease in hydrocarbons 45 days after bioaugmentation. These actinobacterial strains, therefore, show significant potential for bioremediation of such highly polluted soils.

Keywords: petroleum hydrocarbons decontamination; *Bacillus*; *Ochrobactrum*; *Gordonia*; *Rhodococcus*; siderophores

1. Introduction

Petroleum hydrocarbons represent one of the most abundant and most frequent pollutants on Earth. Oil mining, transporting and processing are major sources of this pollution. Oil production tends to follow the rise in human population numbers so the future outlook of petroleum contamination is

rather negative [1,2]. Modern bioremediation techniques represent both an environmentally friendly and economically feasible means of eliminating petroleum pollutants from soils and sludges [3–5]. Petroleum hydrocarbons are fairly biodegradable, however their biodegradation can be slow or even fail when hydrocarbon concentrations are high or when they occur in combination with other pollutants (e.g., heavy metals) [6]. A range of bioremediation techniques has been developed in recent years, including biopiling, biocomposting and rhizoremediation; however, these methods are generally based either on biostimulation (i.e., improvement of conditions for indigenous strains of biodegrading microorganisms), bioaugmentation (i.e., introduction of allochthonous biodegrading microbial strains) or a combination of both [3,7–9]. Seeking new resistant and metabolically active biodegrading strains capable of degrading of high concentrations of pollutants is important for successful application of bioremediation techniques. Techniques for improvement of biodegrading strains [10] (adaptation for higher toxicities, genetic engineering etc.) have been developed, however the major source of new strains remains their isolation from contaminated soils [9,11–13].

Actinobacteria are one of the microbial groups of special interest for bioremediation purposes due to their large genomes, rich metabolism, secondary metabolites, high resilience to toxic pollutants and ability to biodegrade many organics from small volatile to long chain hydrocarbons [14,15]. Recently new actinobacterial strains applicable for bioremediation have been described or characterized, e.g., *Gordonia* sp. capable of biodegradation of phthalic esters [16], *Gordonia polyisoprenivorans* capable of cleavage of poly(*cis*-1,4-isoprene) [17], *Gordonia rubripertincta* CWB2 capable of biodegradation of styrene [10], or *Gordonia phthalatica* sp. capable of biodegradation of various phthalic esters [18].

Despite significant progress in the prediction of bioremediation processes, a preliminary laboratory experiment is a standard tool before the bulk treatment is commenced. The aim of this study was to test, in a laboratory pot experiments emulating potential bulk treatments, different variants of bioremediation of soil from oil refinery contaminated by high concentrations of aliphatic petroleum hydrocarbons ($C_{10}-C_{40}$ ~80 $g \cdot kg^{-1}$ dry weight). In the initial long-term experiment (Experiment A), we subjected a mixture of two bacterial isolates routinely used in bioremediation applications (*Ochrobactrum* sp. and *Bacillus* sp.) to variants of periodic bioaugmentation and biostimulation. In a second experiment (Experiment B), we treated the soil with two recently isolated strains of actinobacteria, *Gordonia rubripertincta* CWB2 [10] and *Rhodococcus erythropolis* S43 [19]. As these strains are known producers of siderophores, we tested the hypothesis that iron bioavailability could influence the overall biodegradation performance of the strains used.

2. Materials and Methods

2.1. Soil Used and Its Characteristics

The loamy soil was obtained from the refinery lagoon. It was sampled and characterized within our previous study [20]. Briefly, the soil was transported to the laboratory and air-dried. Stones were removed from the soil by sieving through a 1 cm sieve and the soil was mixed to obtain a homogeneous test material.

Within this study we have analysed the aliphatic hydrocarbon content, total organic carbon (TOC), pH and water-extractable (presumably bioavailable) metal fractions (see Section 2.4. for details of analyses). The soil characteristics are summarized in Table 1.

Table 1. Soil characteristics (average ± standard deviation, n = 4).

Parameter	Concentration
Hydrocarbon content	
Aliphatics C_{10}-C_{40} [g·kg^{-1} dwt]	80.7 ± 2.2
Sum of PAHs [mg·kg^{-1} dwt] [1]	95 ± 29
Water extractable elements [2]	[mg/kg dwt]
Al	68.2 ± 1.2
As	<0.1
Ba	46.3 ± 1.1
Ca	67478 ± 713
Cd	<0.02
Co	<0.02
Cr	<0.02
Cu	11.4 ± 0.4
Fe	161.1 ± 5.1
K	3253 ± 40
Li	<0.1
Mg	2444 ± 46
Mn	242 ± 1.6
Mo	5.8 ± 0.4
Na	1652 ± 81
Ni	9.4 ± 0.0
P	2.6 ± 0.3
Pb	<0.07
S	2480 ± 51
Sb	<0.1
Se	<0.1
Sn	<0.3
Sr	244.5 ± 5.0
Ti	<0.02
Zn	159.4 ± 4.8
Zr	<0.02
Chemical properties	
TOC [g/kg dwt]	14.3 ± 0.4
pH	7.76 ± 0.10

dwt = dry weight; TOC = Total organic carbon; PAH = polyaromatic hydrocarbons; [1] Kakosová et al. 2017 [20]; [2] Presummably bioavailable = first fraction of sequential BCR extraction (see Section 2.5.)

2.2. Cultivation of Biodegrading Microorganisms

Bacterial Salt Medium (BSM; [21]) comprising of KH_2PO_4 0.17 g·L^{-1}, K_2HPO_4 0.13 g·L^{-1}, $(NH_4)_2SO_4$ 0.71 g·L^{-1}, $MgCl_2 \cdot 6H_2O$ 0.034 g·L^{-1} and a trace metal solution ($MnCl_2 \cdot 4H_2O$ 1 g·L^{-1}, $CaCl_2 \cdot 2H_2O$ 26 g·L^{-1}, $FeSO_4 \cdot 7H_2O$ 0.6 g·L^{-1} and $Na_2MoO_4 \cdot 2H_2O$ 2 g·L^{-1}) dosed at 1 mL·L^{-1} was used for cultivation of biodegrading bacteria. The BSM was supplemented with 1% diesel oil (summer diesel for moderate climate compliant with EN590 [22] purchased from an ÖMV petrol station, sterilised by filtering through a 0.45 µm filter) as a sole source of carbon and energy. Cultivations were carried out with aeration in batch mode at 25 ± 1 °C. For experiment A the intensively aerated fermenter (20 L) was used, for experiment B Erlenmeyer flasks (V = 250 mL, 6 × 60 mL of media) on rotary shaker were used. Cultivation of the culture for experiment A was carried out perpetually throughout the experiment. Each week, after withdrawal of the biodegrading culture for bioaugmentation (see Section 2.4 for details), the bioreactor was refilled by a fresh BSM and sterile diesel in order to have a permanently high amount of biomass for bioaugmentation available (see Section 2.5 for details).

2.3. Microbial Cultures

The biodegradation culture used in Experiment A was obtained previously from another soil polluted over the long term by petroleum hydrocarbon received from a collaborating remediation company. A portion of 1 g of polluted soil was cultivated in Erlemeyer flasks (60 mL) as described in Section 2.2. and repetitively transferred to fresh media (overall ~3 months and 6 transfers). Before use for experiment A the bacteria were stored frozen (−40 °C) in sterile glycerol. The culture was identified as a mixture of *Ochrobactrum* sp. and *Bacillus* sp. using Sanger sequencing on an ABI3500 Genetic Analyser (ThermoFisher Scientific, Waltham, MA, USA). Total DNA from the bacterial cultures was isolated using a Fast DNA Spin Kit for Soil (MP Biomedicals, Santa Anna, CA, USA) according to the manufacturer's protocol. Polymerase chain reactions (PCR) were performed to amplify the whole gene using the primers pAf (3 π-GAGTTTGATCCTGGCTCAG-5′) in position 8–18 within the *16S rDNA* gene and pHr (3′-AAGGAGGTGATCCAGCCGCA-5′) in position 1542-1522 within the *16S rDNA* gene [23]. The PCR products were then purified by using the Sure Clean kit (Bioline, London, UK). A second PCR run was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit with pAf and pHr primers. The products were cleaned on NucleoSeq columns (Machery Nagel, Düren, Germany) then sequenced using capillary electrophoresis on an ABI3500 Genetic Analyser. The Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI, Bethesda, MB, USA) was used for sequence classification.

The actinobacteria *Gordonia rubripertincta* CWB2 and *Rhodococcus erythropolis* S43 used in Experiment B were isolated in Freiberg, Germany and partly characterized [10,19,24–26]. Strain S43 was obtained from a former mining area heavily contaminated with heavy metals and arsenic, while strain CWB2 was isolated from plant potting soil using styrene as sole carbon source.

2.4. Experimental Setup

Experimental variants are summarized in Table 2. Experiment A (455 days) involved several variants of periodic bioaugmentation using a mixture of *Ochrobactrum* sp. and *Bacillus* sp. and biostimulation using BSM as a source of mineral nutrients. Significantly shorter experiment B (45 days) involved single initial bioaugmentation with *G. rubripertincta* CWB2 and *R. erythropolis* S43 and biostimulation with siderophore desferrioxamine B (DFOB).

Table 2. Description of experimental variants.

	Set-Up, Maintenance
Experiment A	2.0 kg soil, periodic addition of . . .
AC (control)	Distilled water only
AA (bioaugmentation)	*Bacillus* sp. + *Ochrobactrum* sp. suspended in water
AS (biostimulation)	Mineral nutrients BSM
AD (combination)	*Bacillus* sp. + *Ochrobactrum* sp. suspended in mineral nutrients medium (BSM)
Experiment B	0.5 kg soil, initial addition of . . .
BC (control)	water (60 mL)
BD (DFOB)	2 mM Desferrioxamine (60 mL)
BG1 (Fe^{2+})	*G. rubripertincta* CWB2 + 60 mL Fe^{2+}-containing BSM
BG2 (no Fe)	*G. rubripertincta* CWB2 + 60 mL Fe-free BSM
BG3 (Fe^{3+})	*G. rubripertincta* CWB2 + 60 mL Fe-free BSM + $FeCl_3$ (5 g·kg^{-1} soil)
BR1 (Fe^{2+})	*R. erythropolis* S43 + 60 mL Fe^{2+}-containing BSM
BR2 (no Fe^{2+})	*R. erythropolis* S43 + 60 mL Fe-free BSM
BR3 (Fe^{3+})	*R. erythropolis* S43 + 60 mL Fe-free BSM + $FeCl_3$ (5 g·kg^{-1} soil)

Contaminated soil (2 kg per pot for Experiment A and 0.5 kg per pot for Experiment B (less soil in experiment B was used because we did not have enough soil from the same sampling) was placed in a series of 2-L plastic pots. Four replicates were prepared for each variant. The soil was mixed manually each week in order to provide aeration. Experiments were carried out in an air-conditioned laboratory with constant temperature (25 °C ± 1.0 °C).

Soil humidity of experiment A was maintained at 10–15% (m/m) through a weekly addition of either distilled water, mineral nutrients solution (i.e., BSM), suspension of microorganisms or their combinations. Details are summarized in Table 2. Concentrated suspension was prepared from the culture, which was perpetually grown in fermenter as described in Section 2.2. An aliquot of 300 mL was withdrawn from a fermenter (average OD_{600} 0.60 ± 0.17), centrifuged at 4000 rpm for 10 min and split into equal parts. This inoculum was then resuspended in 37.5 mL of sterile BSM or sterile water (see Table 2 for details), inoculated into the soil and the soil was mixed. For non-inoculated variants AC and AS the same volume (37.5 mL) of sterile distilled water or sterile BSM was used respectively (Table 2).

Experiment B was set-up in the beginning only. *G. rubripertincta* CWB2 was grown to OD_{600} = 0.23, *R. erythropolis* S43 to OD_{600} = 0.14, the suspensions from all Erlenmeyer flasks (6 × 60 mL) were put together, separated into three centrifugation tubes, centrifuged (4000 rpm, 10 min), resuspended in appropriate media (Table 2) and inoculated. The humidity was maintained at 10–15% (m/m) by weekly addition of distilled water.

2.5. Sampling and Analysis

Samples in Experiment A were taken approximately every 90 days. Experiment B was sampled on the beginning and in the end only. The soil was homogenized and ~100 g was withdrawn using spatula. Approximately half of the sample was stored in the freezer for further analyses, aliquot of ~10 g was used fresh for determination of respiration and activity of dehydrogenases. Aliquot of ~10 g was dried overnight (105 °C) for determination of the humidity.

Aliphatic petroleum hydrocarbons were determined by GC-FID (Master GC, DANI Instruments, Milan, Italy) in hexane extract according to Standard EN 14039 [27], as previously described [28].

Soil respiration was determined through absorption of CO_2 in NaOH and reverse titration, using the methods described by [29].

Dehydrogenase activity (DHA) was determined via reduction of triphenyltetrazolium to triphenylformazan during direct incubation of soil sample with the substrate solution according to Standard ISO 23753-1 [30].

Sequential BCR four-step-extraction [31,32] estimating metal fraction of different accessibility was carried out using the methods described by [33]. Briefly the soil sample was sequentially digested using water, acetic acid (0.11 mol/L), hydroxylamine hydrochloride (0.1 mol/L, pH 2), and warm hydrogen peroxide (8.8 mol/L, pH 2.5). The residuum was digested using aqua regia. Metal concentrations in extracts were determined by ICP-OES (Optima 8000, Perkin Elmer, Waltham, MA, USA).

TOC was determined from dry samples according to ISO Standard 14235 [34].

2.6. Statistics

Statistical evaluation was calculated from four replicates of each variant. Data were evaluated using the Statistica 12 and Past 3.0 statistical software packages. The Shapiro–Wilks test indicated that the hydrocarbon and TOC data were normally distributed, while Levene's test showed group variances to be homogenous. Hence, we applied one-way analysis of variance (ANOVA) for the overall comparison. As the respiration data and DHA homogeneity of variance were non-normally distributed, we used the non-parametric Kruskal–Wallis test in these cases. In addition, we performed Fisher's Least Significant Difference (LSD) post-hoc tests for better understanding the differences in mean values computed for the soil parameters. Tukey's pairwise post-hoc tests were used for identification of significant changes compared to initial values (A, B) and to final values of the controls (AC, BC).

3. Results

Initial and final values of both experiments are summarized in Table 3. Hydrocarbon concentrations are depicted in Figure 1.

Table 3. Initial and final values for soil parameters (average ± std. deviation from four replications of each variant). Bold face indicates a significant change compared to the initial value, asterisks denote significance compared to control at the end of experiment. Lower-case letters denote homogenous groups according to the Fisher's Least Significant Difference (LSD) test ($\alpha = 0.05$).

Variant	$C_{10}-C_{40}$ [g·kg^{-1} dwt]	DHA [mU·kg^{-1} dwt]	Respiration [U·kg^{-1} dwt]	TOC [%]	Fe [1] [kg^{-1} dwt]
	Experiment A–*Mixed culture* (455 days)				
Day 0	81.6 ± 2.5	9.9 ± 3.2	9.4 ± 2.2	14.9 ± 0.5	NA
Day 455					
AC (control)	78.4 ± 0.9	0.9 ± 0.3	1.7 ± 0.3	14.7 ± 0.6	NA
AA (bioaugmentation)	86.2 ± 3.4	6.4 ± 0.8 ***	2.5 ± 0.8 *	15.1 ± 0.4	NA
AS (biostimulation)	87.8 ± 3.3	2.4 ± 1.1 **	2.9 ± 0.7 ***	14.3 ± 0.7	NA
AD (biostim. + bioaug.)	78.1 ± 3.1	10.1 ± 1.8 ***	4.3 ± 0.4 ***	15.1 ± 2.9	NA
	Experiment B-*G. rubripertincta* CWB2, *R. erythropolis* S43 (45 days)				
Day 0	†† 79.7 ± 2.0c	3.4 ± 1.6a *	11.7 ± 0.9c **	13.5 ± 0.4a	197 ± 6a
Day 45					
BC (control)	78.2 ± 2.2bc	0.8 ± 0.9a	†† 0.6 ± 0.9a	†† 13.3 ± 0.5a	198 ± 7a
BD (DFOB)	60.3 ± 0.7de **	†† 4.3 ± 1.3a **	[A] 0 ± 0a	13.0 ± 0.6a	223 ± 32a
BG1 (Fe^{2+})	54.2 ± 4.6e **	6.7 ± 4.0ab *	2.6 ± 1.6a	12.8 ± 0.6a	224 ± 26a
BG2 (no Fe)	66.4 ± 7.5ad **	3.7 ± 3.5a	[A] 0 ± 0a	13.4 ± 0.4a	213 ± 24a
BG3 (Fe^{3+})	75.1 ± 5.8bc	†† 7.2 ± 9.5ab	††† 0.4 ± 0.2a	13.1 ± 0.9a	221 ± 15a *
BR1 (Fe^{2+})	70.2 ± 0.9ab **	† 40.8 ± 7.8c **	6.5 ± 1.7b **	† 13.0 ± 0.6a	220 ± 41ab
BR2 (no Fe^{2+})	†† 67.0 ± 3.6ad **	17.0 ± 9.8b **	12.0 ± 4.0c **	13.5 ± 0.4a	253 ± 18b *
BR3 (Fe^{3+})	73.2 ± 8.5abc	52.4 ± 13.5c **	7.9 ± 1.4b **	13.1 ± 0.7a	208 ± 41a
	Comparison of microorganisms considered as single factors				
Day 45					
G. rubripertincta CWB2 (BG1 to BG3)	65.2 ± 10.5 **	5.9 ± 6.5 **	1.0 ± 1.4	13.1 ± 0.7	219 ± 20 *
R. erythropolis S43 (BR1 to BR3)	70.2 ± 5.9 **	36.7 ± 18.1 ***	8.8 ± 3.5 ***	13.2 ± 0.6	227 ± 36

Normality: Shapiro-Wilks test († $p < 0.1$; †† $p < 0.05$; ††† $p < 0.01$); Overall comparison: ANOVA or Kruskal–Wallis test (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.01$); [1] Water extractable iron (presumably bioavailable, first step of sequential BCR extraction). DHA = Dehydrogenase activity. [A] no varibility in data (all values equal to zero)—variable omitted in all tests.

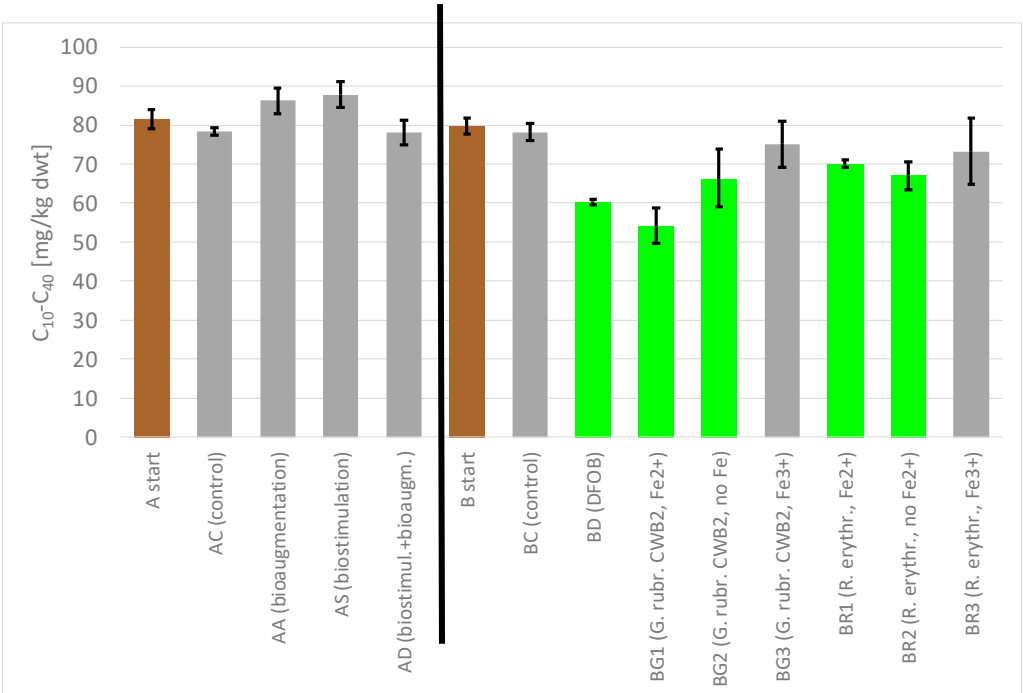


Figure 1. Aliphatic hydrocarbon concentrations in both experiments. Brown colour indicates initial values, light-green colour indicates significant decrease compared to control (Tukey's pairwise post-hoc tests, $p < 0.05$).

3.1. Experiment A

Despite long-term (455 days) periodic bioaugmentation and biostimulation of the polluted soil, very little variation was observed in the test parameters in Experiment A (see Table 3 and Supplementary Table S1). In particular, significant decrease was observed of neither hydrocarbon concentrations nor TOC values in any of the variants. By contrast, microbial activity (soil respiration and DHA) showed an overall decrease over time. While DHA in the control variant AC exhibited peaks after 121 days (with all values higher than the control), no such peaks were observed in soil respiration. Final (day 455) values of DHA and respirations were in all cases significantly higher than control (AC). The highest DHA value was determined for variant AD, i.e., a combination of biostimulation and bioaugmentation.

3.2. Experiment B

While the control showed no significant change in aliphatic hydrocarbon concentrations compared to the initial values, a significant decrease in aliphatic hydrocarbon concentrations was observed in five test variants in just 45 days, a period one order of magnitude shorter than that in Experiment A (Table 3 and Supplementary Table S1). The highest reduction of aliphatic hydrocarbon was achieved by *G. rubripertincta* CWB2 in Fe^{2+}-containing BSM (variant BG1), the results being significantly lower (LSD test, $p < 0.1$) than the other four variants (Table 3). Significant values (LSD test, $p < 0.1$ to <0.001) were also obtained for *G. rubripertincta* CWB2 in Fe-free BSM (variant BG2), *R. erythropolis* S43 in Fe^{2+}-containing BSM (variant BR1), *R. erythropolis* S43 in Fe-free BSM (variant BR2) and through biostimulation with DFOB (variant BD). Variants BG3 and BR3, both with Fe^{3+} addition, showed no significant biodegradation.

Initial hydrocarbon concentrations in the test soil corresponded to ~7% TOC and despite the significant decrease of aliphatic hydrocarbons, no significant change in TOC was observed in any variant. Supposing complete mineralization of aliphatic hydrocarbons, the decrease to 64.9 ± 8.8 (i.e., average of successful variants BD, BG1, BG2, BR1 and BR2) should have corresponded to a decrease in TOC of 1.3% (i.e., the final TOC value of 12.2%). In fact, the final TOC value from the successful variants (13.0 ± 0.6%) corresponded to only around 40% of theoretical mineralization, roughly confirmed by the amount of respired CO_2 throughout the experiment. Hence, approximately 60% of the hydrocarbons removed were either transformed into biomass or only partially transformed to other soil components; a combination of both most likely.

While both actinobacterial strains proved successful at biodegrading of the soil hydrocarbons, some differences were observed. *G. rubripertincta* CWB2 proved slightly better at biodegradation than *R. erythropolis* S43 (final values of 65.2 ± 10.5 and 70.2 ± 5.9 mg.kg^{-1} dwt, respectively; significant at $p < 0.095$). On the other hand, the use of *R. erythropolis* S43 led to significantly higher microbial activity, i.e., DHA (*t*-test, $p < 1.2 \times 10^{-5}$) and respiration (*t*-test, $p < 3.9 \times 10^{-7}$), suggesting improved regeneration of soil functioning following bioaugmentation by *R. erythropolis* S43.

4. Discussion

4.1. Applicability for Bulk Bioremediation

While petroleum hydrocarbons can be generally biodegraded reasonably well, many factors can reduce the effectiveness of the biodegradation process [9,35–39]. Prior to bulk bioremediation measures, therefore, it is common to undertake laboratory tests similar to those undertaken in this study in order to assess the effectiveness of the proposed bioremediation procedures [4,28,35,39–41]. As such, our two-tier experimental process reflects a process of trial and error in the search for a successful combination of bacterial medium and process.

The experiments described were initiated by the need of bioremediation of soil highly polluted by petroleum hydrocarbons in the order of thousands of tons. Before such bulk bioremediation, trial small-scale experiments are necessary. In our case we started with massive repetitive bioaugmentation

using *Ochrobactrum* sp. and *Bacillus* sp., microbial consortium routinely used in previous bioremediation applications (commercial activities, not published), and compared them to biostimulation using BSM and combination (Experiment A). This experiment, despite its long time period, proved this approach inapplicable for a bulk remediation method as no decrease of hydrocarbons was detected in any of the variants. The reasons for the unsuccessful application of an otherwise successful consortium can be only speculated upon, and are likely to be too high a concentration and age of the petroleum contamination along with the inhibiting presence of heavy metals. Nevertheless, it confirms that growth of microbes on the petroleum hydrocarbons as a sole source of carbon and energy in liquid media does not implicate biodegradation in real soils. At least microbial activities throughout the test suggested a positive effect on soil function regeneration. Absolute values of microbial activities were however generally low and corresponded more with developing, damaged or poor soils than healthy soils [29,42].

Consequently we had to try different approaches, especially testing new strains of highly resistant actinobacteria (Experiment B). In this case, a significant decrease of aliphatic hydrocarbons was achieved in several variants despite an order of magnitude shorter experimental period. The positive result cannot be explained by different amount of bioaugmented bacteria, as the experiment A was bioaugmented periodically with high amounts of degrading bacteria, while experiment B was inoculated only once in the beginning roughly by minimally two orders of magnitude lower amount of bacteria. The results of Experiment B suggest that both the *G. rubripertincta* CWB2 and *R. erythropolis* S43 strains show clear promise for applications in bioremediation of petroleum contaminated soil; although the actual mechanism by which they achieve biodegradation remains somewhat unclear.

4.2. Possible Role of Siderophores

Actinobacteria have long been considered suitable candidates for biodegradation studies due to their high metabolic capability [43–46]. Two properties in particular are of relevance in this case, (a) the number and diversity of degradative pathways for hydrocarbons, and (b) the production of secondary metabolites such as biosurfactants [47–49] and siderophores [26,50,51]. These properties allow actinobacteria to survive under a wide range of environmental conditions and to adapt to, or even change, local conditions by secreting metabolites. As such, the presence of actinobacteria and its secretion of metabolites will generally affect the microbial community. This is difficult to investigate in soil samples, however, as the distribution of bacteria and metabolites will depend on the soil's properties and mobilisation potential. In this study, siderophores clearly appeared to act as a promoting factor on the hydrocarbon degradation potential. Recently, it has also been demonstrated that citrate and DFOB, two typical microbial metabolites relevant for metal mobilisation and transport, were able to mobilise a number of metals and metalloids, depending on the conditions [52]. As such, the availability of trace elements of importance to microbial life may be affected by such processes.

Both actinobacterial strains investigated in this study are proven producers of siderophores, which bind Fe^{3+} and other trivalent metals, thereby affecting their bioavailability [26,53,54]. As such, we included iron treatment and DFOB siderophore biostimulation within our test variants of experiment B. Theoretically, the presence of Fe^{3+} should suppress siderophore production, allowing microorganisms to have a non-iron limiting metabolism [55]; however, the effects of Fe^{2+}, DFOB and siderophores may differ. While Fe^{2+} can support reductive processes, thus making other metals or metalloids bioavailable, siderophores, including DFOB, predominantly mobilise trivalent metals or metalloids, although the results obtained have been somewhat contradictory [26,53,55]. In our own study, a significant decrease in hydrocarbon concentration was observed following biostimulation by DFOB, even without bioaugmentation (variant BD), thereby suggesting a clear positive effect by siderophores on biostimulation of autochthonous microflora and their biodegradation activity. Similar observations have also been reported for siderophores of a *Rhodococcus* strain, which can stimulate the growth of other bacterial organisms [50]. Test variants where Fe^{3+} was added (BG3 and BR3), on the other hand, produced no significant hydrocarbon decrease when compared with those variants where only Fe^{2+} was added (BG1 and BR1) or where no iron was added (variants BG2 and BR2).

This suggests a positive effect on hydrocarbon degradation following production of siderophores (likely to have been repressed in variants BG3 and BR3 by high levels of Fe^{3+}). One test of this would be to assess the concentration of water-extractable (presumably bioavailable) iron obtained as fraction 1 of the sequential BCR extraction. Despite a slight increase in water-extractable iron in all variants, concentration variability was relatively high and only variants BG3 and BR2 exhibited significant results ($p < 0.1$). Grouping the variants together to promote the differences revealed a significant increase in water-extractable iron after treatment with *G. rubripertincta* CWB2 (joint variants BG1 and BG2; *t*-test, $p = 0.064$) but no significant change following addition of *R. erythropolis* S43 (joint variants BR1 and BR2; *t*-test, $p = 0.118$). Consideration of each strain as a factor (Table 3) indicated significance for *G. rubripertincta* CWB2 only.

A second possible mechanism includes either mobilisation or detoxification of other metals [26,53] (see Table 1 for a list of water-extractable metals). Possible toxicity could be expected from aluminium (Al), copper (Cu), manganese (Mn), nickel (Ni), strontium (Sr), and zinc (Zn). Of the trivalent metals possibly mobilised by siderophores (see above), only the water-extractable fraction of Al exceeded the detection limit (80.1 ± 9.5 mg·kg^{-1} dwt), although there was no significant difference in Al concentration between variants (data not shown). For non-trivalent metals, only Sr and Zn showed a significant decrease compared to the control (data not shown). Sequential BCR extraction with ICP-OES, however, was unable to detect metal speciation; hence, further research is needed to confirm this as a possible action mechanism.

Overall, our analysis indicates that not only did both actinobacterial strains tested biodegrade hydrocarbons but that may also have contributed to the biostimulation of biodegradation by indigenous microflora. Siderophore production, iron mobilisation and metal detoxification may all have contributed to the biostimulation. While both actinobacterial strains tested resulted in a significant decrease in hydrocarbons after 45 days of bioaugmentation, *G. rubripertincta* CWB2 appears to be slightly better at this than *R. erythropolis* S43. As such, these actinobacterial strains show significant potential for bioremediation of highly polluted soils, although further research is needed to fully identify the action mechanism involved and the nature of the siderophores produced.

5. Conclusions

Two attempts to biodegrade high concentration of petroleum hydrocarbons (81.6 ± 2.5 g·kg^{-1} $C_{10}-C_{40}$ dwt) in soil from petroleum refinery were carried out in long-term pot experiments. While bioaugmentation of a mixture of *Bacillus* sp. and *Ochrobactrum* sp., capable of growth on diesel as a sole source of carbon and energy, did not result in significant decrease of hydrocabons even after 455 days, the use of *Gordonia rubripertincta* CWB2 as well as *Rhodococcus erythropolis* S43 resulted in significant hydrocarbon decrease even after 45 days. The results show the applicability of these actinobacterial strains for the bioremediation of soils contaminated with high concentrations of petroleum hydrocarbons.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/8/10/1855/s1, Table S1: Results from intermediate samplings not presented in Table 3, raw data of both experiments.

Author Contributions: J.T. contributed to the design of both experiments, and undertook data evaluation and preparation of the manuscript. C.O.E. carried out Experiment B and contributed to the analysis and data evaluation. S.K., P.K., L.S. and P.V.-D. undertook the chemical analysis. J.P. performed the statistical evaluation of the data. O.O.B. contributed to the data evaluation. P.H. and A.Š. contributed to the design and completion of Experiment A and undertook data evaluation. M.C. carried out the genetic analysis of the microorganisms used in Experiment A, while E.K. contributed to the sampling and analysis of Experiment A. D.T. isolated strain CWB2 and maintained both CBW2 and S43 novel isolates. All authors drafted the manuscript in its final version.

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References

1. Hook, M. Depletion rate analysis of fields and regions: A methodological foundation. *Fuel* **2014**, *121*, 95–108. [CrossRef]
2. Hook, M.; Hirsch, R.; Aleklett, K. Giant oil field decline rates and their influence on world oil production. *Energy Policy* **2009**, *37*, 2262–2272. [CrossRef]
3. Varjani, S.J. Microbial degradation of petroleum hydrocarbons. *Bioresour. Technol.* **2017**, *223*, 277–286. [CrossRef] [PubMed]
4. Lim, M.W.; Von Lau, E.; Poh, P.E. A comprehensive guide of remediation technologies for oil contaminated soil—Present works and future directions. *Mar. Pollut. Bull.* **2016**, *109*, 14–45. [CrossRef] [PubMed]
5. Singh, P.; Jain, R.; Srivastava, N.; Borthakur, A.; Pal, D.B.; Singh, R.; Madhav, S.; Srivastava, P.; Tiwary, D.; Mishra, P.K. Current and emerging trends in bioremediation of petrochemical waste: A review. *Crit. Rev. Environ. Sci. Technol.* **2017**, *47*, 155–201. [CrossRef]
6. Varjani, S.J.; Gnansounou, E.; Pandey, A. Comprehensive review on toxicity of persistent organic pollutants from petroleum refinery waste and their degradation by microorganisms. *Chemosphere* **2017**, *188*, 280–291. [CrossRef] [PubMed]
7. Azubuike, C.C.; Chikere, C.B.; Okpokwasili, G.C. Bioremediation techniques-classification based on site of application: Principles, advantages, limitations and prospects. *World J. Microbiol. Biotechnol.* **2016**, *32*. [CrossRef] [PubMed]
8. dos Santos, J.J.; Maranho, L.T. Rhizospheric microorganisms as a solution for the recovery of soils contaminated by petroleum: A review. *J. Environ. Manag.* **2018**, *210*, 104–113. [CrossRef] [PubMed]
9. Varjani, S.J.; Upasani, V.N. A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. *Int. Biodeterior. Biodegrad.* **2017**, *120*, 71–83. [CrossRef]
10. Heine, T.; Zimmerling, J.; Ballmann, A.; Kleeberg, S.B.; Ruckert, C.; Busche, T.; Winkler, A.; Kalinowski, J.; Poetsch, A.; Scholtissek, A.; et al. On the Enigma of Glutathione-Dependent Styrene Degradation in Gordonia rubripertincta CWB2. *Appl. Environ. Microbiol.* **2018**, *84*. [CrossRef] [PubMed]
11. Korotkevych, O.; Josefiova, J.; Praveckova, M.; Cajthaml, T.; Stavelova, M.; Brennerova, M.V. Functional adaptation of microbial communities from jet fuel-contaminated soil under bioremediation treatment: Simulation of pollutant rebound. *FEMS Microbiol. Ecol.* **2011**, *78*, 137–149. [CrossRef] [PubMed]
12. Kolvenbach, B.A.; Helbling, D.E.; Kohler, H.-P.E.; Corvini, P.F.X. Emerging chemicals and the evolution of biodegradation capacities and pathways in bacteria. *Curr. Opin. Biotechnol.* **2014**, *27*, 8–14. [CrossRef] [PubMed]
13. Troegl, J.; Chauhan, A.; Ripp, S.; Layton, A.C.; Kuncova, G.; Sayler, G.S. Pseudomonas fluorescens HK44: Lessons Learned from a Model Whole-Cell Bioreporter with a Broad Application History. *Sensors* **2012**, *12*, 1544–1571. [CrossRef] [PubMed]
14. Singh, B.P.; Gupta, V.K.; Passari, A.K. *Actinobacteria: Diversity and Biotechnological Applications: New and Future Developments in Microbial Biotechnology and Bioengineering*; Elsevier Science: Amsterdam, The Netherlands, 2018.
15. Amoroso, M.J.; Benimeli, C.S.; Cuozzo, S.A. *Actinobacteria: Application in Bioremediation and Production of Industrial Enzymes*; CRC Press: Boca Raton, FL, USA, 2013.
16. Fan, S.H.; Wang, J.H.; Li, K.; Yang, T.; Jia, Y.; Zhao, B.S.; Yan, Y.C. Complete genome sequence of Gordonia sp YC-JH1, a bacterium efficiently degrading a wide range of phthalic acid esters. *J. Biotechnol.* **2018**, *279*, 55–60. [CrossRef] [PubMed]
17. Andler, R.; Hiessl, S.; Yucel, O.; Tesch, M.; Steinbuchel, A. Cleavage of poly(cis-1,4-isoprene) rubber as solid substrate by cultures of Gordonia polyisoprenivorans. *New Biotechnol.* **2018**, *44*, 6–12. [CrossRef] [PubMed]

18. Jin, D.C.; Kong, X.; Jia, M.H.; Yu, X.Y.; Wang, X.X.; Zhuang, X.L.; Deng, Y.; Bai, Z.H. Gordonia phthalatica sp. nov., a di-n-butyl phthalate-degrading bacterium isolated from activated sludge. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 5128–5133. [CrossRef] [PubMed]
19. Sultana, M.; Vogler, S.; Zargar, K.; Schmidt, A.C.; Saltikov, C.; Seifert, J.; Schlomann, M. New clusters of arsenite oxidase and unusual bacterial groups in enrichments from arsenic-contaminated soil. *Arch. Microbiol.* **2012**, *194*, 623–635. [CrossRef] [PubMed]
20. Kakosova, E.; Hrabak, P.; Cernik, M.; Novotny, V.; Czinnerova, M.; Trogl, J.; Popelka, J.; Kuran, P.; Zoubkova, L.; Vrtoch, L. Effect of various chemical oxidation agents on soil microbial communities. *Chem. Eng. J.* **2017**, *314*, 257–265. [CrossRef]
21. Cejkova, A.; Masak, J.; Jirku, V. Use of mineral nutrients and surface-active substances in a biodegradation process modulation. *Folia Microbiol.* **1997**, *42*, 513–516. [CrossRef]
22. Standardization, BS EN 590 Automotive Fuels—Diesel—Requirements and Test Methods. 2004. Available online: http://www.envirochem.hu/www.envirochem.hu/documents/EN_590_2009_hhV05.pdf (accessed on 27 August 2018).
23. Hastings, R.C.; Ceccherini, M.T.; Miclaus, N.; Saunders, J.R.; Bazzicalupo, M.; McCarthy, A.J. Direct molecular biological analysis of ammonia oxidising bacteria populations in cultivated soil plots treated with swine manure. *FEMS Microbiol. Ecol.* **1997**, *23*, 45–54. [CrossRef]
24. Esuola, C.O.; Babalola, O.O.; Heine, T.; Schwabe, R.; Schlomann, M.; Tischler, D. Identification and characterization of a FAD-dependent putrescine N-hydroxylase (GorA) from Gordonia rubripertincta CWB2. *J. Mol. Catal. B Enzym.* **2016**, *134*, 378–389. [CrossRef]
25. Oelschlägel, M.; Kaschabek, S.R.; Zimmerling, J.; Schlömann, M.; Tischler, D. Co-metabolic formation of substituted phenylacetic acids by styrene-degrading bacteria. *Biotechnol. Rep. (AMST)* **2015**, *6*, 20–26. [CrossRef] [PubMed]
26. Schwabe, R.; Obst, B.; Mehnert, M.; Tischler, D.; Wiche, O. Gallium Mobilization in Soil by Bacterial Metallophores. *Solid State Phenom.* **2017**, *262*, 513–516. [CrossRef]
27. Standardization, E.c.f. EN 14039 Characterization of Waste—Determination of Hydrocarbon Content in the Range of C10 to C40 by Gas Chromatography. 2005. Available online: https://www.sis.se/api/document/preview/39058/ (accessed on 27 August 2018).
28. Kuran, P.; Troegl, J.; Novakova, J.; Pilarova, V.; Danova, P.; Pavlorkova, J.; Kozler, J.; Novak, F.; Popelka, J. Biodegradation of Spilled Diesel Fuel in Agricultural Soil: Effect of Humates, Zeolite, and Bioaugmentation. *Sci. World J.* **2014**. [CrossRef]
29. Frouz, J.; Cajthaml, T.; Kribek, B.; Schaeffer, P.; Bartuska, M.; Galertova, R.; Rojik, P.; Kristufek, V. Deep, subsurface microflora after excavation respiration and biomass and its potential role in degradation of fossil organic matter. *Folia Microbiol.* **2011**, *56*, 389–396. [CrossRef] [PubMed]
30. Organization, ISO 23753–1 Soil Quality—Determination of Dehydrogenase Activity in Soils—Part 1: Method Using Triphenyltetrazolium Chloride (TTC). 2005. Available online: https://www.evs.ee/preview/iso-23753-1-2005-en.pdf (accessed on 27 August 2018).
31. Quevauviller, P.; Ure, A.; Muntau, H.; Griepink, B. Improvement of Analytical Measurements within the BCR-Programme: Single and Sequential Extraction Procedures Applied to Soil and Sediment Analysis. *Int. J. Environ. Anal. Chem.* **1993**, *51*, 129–134. [CrossRef]
32. Ure, A.M.; Quevauviller, P.; Muntau, H.; Griepink, B. Speciation of Heavy Metals in Soils and Sediments. An Account of the Improvement and Harmonization of Extraction Techniques Undertaken Under the Auspices of the BCR of the Commission of the European Communities. *Int. J. Environ. Anal. Chem.* **1993**, *51*, 135–151. [CrossRef]
33. Janos, P.; Vavrova, J.; Herzogova, L.; Pilarova, V. Effects of inorganic and organic amendments on the mobility (leachability) of heavy metals in contaminated soil: A sequential extraction study. *Geoderma* **2010**, *159*, 335–341. [CrossRef]
34. Organization, ISO 14235 Soil Quality—Determination of Organic Carbon in Soil by Sulfochromic Oxidation. 1998. Available online: https://www.evs.ee/preview/iso-14235-1998-en.pdf (accessed on 27 August 2018).
35. Moliterni, E.; Rodriguez, L.; Fernandez, F.J.; Villasenor, J. Feasibility of Different Bioremediation Strategies for Treatment of Clayey and Silty Soils Recently Polluted with Diesel Hydrocarbons. *Water Air Soil Pollut.* **2012**, *223*, 2473–2482. [CrossRef]

36. Moliterni, E.; Jimenez-Tusset, R.G.; Rayo, M.V.; Rodriguez, L.; Fernandez, F.J.; Villasenor, J. Kinetics of biodegradation of diesel fuel by enriched microbial consortia from polluted soils. *Int. J. Environ. Sci. Technol.* **2012**, *9*, 749–758. [CrossRef]
37. Tyagi, M.; da Fonseca, M.M.R.; de Carvalho, C. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation* **2011**, *22*, 231–241. [CrossRef] [PubMed]
38. Dandie, C.E.; Weber, J.; Aleer, S.; Adetutu, E.M.; Ball, A.S.; Juhasz, A.L. Assessment of five bioaccessibility assays for predicting the efficacy of petroleum hydrocarbon biodegradation in aged contaminated soils. *Chemosphere* **2010**, *81*, 1061–1068. [CrossRef] [PubMed]
39. Benyahia, F.; Abdulkarim, M.; Zekri, A.; Chaalal, O.; Hasanain, H. Bioremediation of Crude Oil Contaminated Soils: A Black Art or an Engineering Challenge? *Process Saf. Environ. Prot.* **2005**, *83*, 364–370. [CrossRef]
40. Taccari, M.; Milanovic, V.; Comitini, F.; Casucci, C.; Ciani, M. Effects of biostimulation and bioaugmentation on diesel removal and bacterial community. *Int. Biodeterior. Biodegrad.* **2012**, *66*, 39–46. [CrossRef]
41. Lin, T.C.; Pan, P.T.; Young, C.C.; Chang, J.S.; Chang, T.C.; Cheng, S.S. Evaluation of the optimal strategy for ex situ bioremediation of diesel oil-contaminated soil. *Environ. Sci. Pollut. Res.* **2011**, *18*, 1487–1496. [CrossRef] [PubMed]
42. Helingerova, M.; Frouz, J.; Santruckova, H. Microbial activity in reclaimed and unreclaimed post-mining sites near Sokolov (Czech Republic). *Ecol. Eng.* **2010**, *36*, 768–776. [CrossRef]
43. Drzyzga, O. The strengths and weaknesses of Gordonia: A review of an emerging genus with increasing biotechnological potential. *Crit. Rev. Microbiol.* **2012**, *38*, 300–316. [CrossRef] [PubMed]
44. Arenskotter, M.; Broker, D.; Steinbuchel, A. Biology of the metabolically diverse genus Gordonia. *Appl. Environ. Microbiol.* **2004**, *70*, 3195–3204. [CrossRef] [PubMed]
45. Elsayed, Y.; Refaat, J.; Abdelmohsen, U.R.; Fouad, M.A. The Genus Rhodococcus as a source of novel bioactive substances: A review. *J. Pharmacogn. Phytochem.* **2017**, *6*, 83–92.
46. Bell, K.; Philp, J.; Aw, D.; Christofi, N. The genus Rhodococcus. *J. Appl. Microbiol.* **1998**, *85*, 195–210. [CrossRef] [PubMed]
47. Franzetti, A.; Gandolfi, I.; Bestetti, G.; Smyth, T.J.P.; Banat, I.M. Production and applications of trehalose lipid biosurfactants. *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 617–627. [CrossRef]
48. Pi, Y.R.; Chen, B.; Bao, M.T.; Fan, F.Q.; Cai, Q.H.; Ze, L.; Zhang, B.Y. Microbial degradation of four crude oil by biosurfactant producing strain *Rhodococcus* sp. *Bioresour. Technol.* **2017**, *232*, 263–269. [CrossRef] [PubMed]
49. Pagilla, K.R.; Sood, A.; Kim, H. Gordonia (nocardia) amarae foaming due to biosurfactant production. *Water Sci. Technol.* **2002**, *46*, 519–524. [CrossRef] [PubMed]
50. Carrano, C.J.; Jordan, M.; Drechsel, H.; Schmid, D.G.; Winkelmann, G. Heterobactins: A new class of siderophores from Rhodococcus erythropolis IGTS8 containing both hydroxamate and catecholate donor groups. *Biometals* **2001**, *14*, 119–125. [CrossRef]
51. Bosello, M.; Zeyadi, M.; Kraas, F.I.; Linne, U.; Xie, X.L.; Marahiel, M.A. Structural Characterization of the Heterobactin Siderophores from Rhodococcus erythropolis PR4 and Elucidation of Their Biosynthetic Machinery. *J. Nat. Prod.* **2013**, *76*, 2282–2290. [CrossRef] [PubMed]
52. Wiche, O.; Tischler, D.; Fauser, C.; Lodemann, J.; Heilmeier, H. Effects of citric acid and the siderophore desferrioxamine B (DFO-B) on the mobility of germanium and rare earth elements in soil and uptake in Phalaris arundinacea. *Int. J. Phytoremediat.* **2017**, *19*, 746–754. [CrossRef] [PubMed]
53. Anke, M.K.; Szymańska, K.; Schwabe, R.; Wiche, O.; Tischler, D. On the Immobilization of Desferrioxamine-Like Siderophores for Selective Metal Binding. *Solid State Phenom.* **2017**, *262*, 517–520. [CrossRef]
54. Retamal-Morales, G.; Mehnert, M.; Schwabe, R.; Tischler, D.; Schlömann, M.; Levicán, G.J. Genomic Characterization of the Arsenic-Tolerant Actinobacterium, Rhodococcus erythropolis S43. *Solid State Phenom.* **2017**, *262*, 660–663. [CrossRef]
55. Hider, R.C.; Kong, X.L. Chemistry and biology of siderophores. *Nat. Prod. Rep.* **2010**, *27*, 637–657. [CrossRef] [PubMed]



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