# Effect of the Application of *Ochrobactrum* sp.-Immobilised Biochar on the Remediation of Diesel-Contaminated Soil

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*Article*

# Effect of the Application of *Ochrobactrum* sp.-Immobilised Biochar on the Remediation of Diesel-Contaminated Soil

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**Abstract:** The immobilisation of bacteria on biochar has shown potential for enhanced remediation of petroleum hydrocarbon-contaminated soil. However, there is a lack of knowledge on the effect of bacterial immobilisation on biosolids-derived biochar for the remediation of diesel-contaminated soil. The aim of this current study was to assess the impact of the immobilisation on biosolids-derived biochar of an autochthonous hydrocarbonoclastic bacteria, *Ochrobacterium* sp. (BIB) on the remediation of diesel-contaminated soil using a laboratory based mesocosm study. Additionally, the effect of fertiliser application on the efficacy of the BIB treatment was investigated. Biochar (BC) application alone led to significantly higher hydrocarbon removal compared to the control treatment at all sampling times (4,887 – 11,589 mg/kg higher). When *Ochrobacterium* sp. was immobilised on biochar (BIB), the hydrocarbon removal was higher than BC by 5,533 mg/kg and 1,607 mg/kg at weeks 10 and 22, respectively. However, when BIB was co-applied with fertiliser (BIBF), the hydrocarbon removal was lower than BIB by 6,987 – 11,767 mg/kg. Quantitative PCR analysis revealed that the gene related to *Ochrobacterium* sp. was higher in BIB than in the BC treatment, which likely contributed to higher hydrocarbon removal in the BIB treatment. The findings of this study demonstrate that bacteria immobilisation on biosolids-derived biochar is a promising technique for the remediation of diesel-contaminated soil. Future studies should focus on optimising the immobilisation process for enhanced hydrocarbon removal.

**Keywords:** bioaugmentation; biochar; bioremediation; biodegradation; hydrocarbon; petroleum; pyrochar; sewage sludge

## 1. Introduction

Crude oil is an important natural resource for energy generation and for producing raw materials for industry. The 2% average per annual increase in global oil consumption (million tonnes) between 1965 and 2020 attests to how relevant oil remains to the world [1]. Nonetheless, petroleum/crude oil's leakage and accidental release into the environment frequently occurs in the course of the exploration, refining, transportation, and storage of petroleum/crude oil and its derived products [2]. Soil pollution by petroleum hydrocarbon is a common problem in many countries [3, 4]. For example, between 2006 and June 2022 in Nigeria, 4,102 crude and refined oil spills on land were reported in the country's oil spill monitor [4]. Diesel is a product of crude oil, which is a frequent progenitor for petroleum hydrocarbon pollution in the environment [5]. Acute and chronic effects on humans and plants occur after exposure to diesel oil [6]. For example, Bona, Rezende, Santos and Souza [7] reported that the seedling growth of *Schinus terebinthifolius* was significantly affected by exposure to diesel.

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Remediation of crude oil-contaminated soils is necessary due to the detrimental effects of exposure to this contaminant [8]. Although several remediation techniques are available for oil/petroleum hydrocarbon-impacted environments [9], remediation of contaminated soil remains a concern. This is owing to the shortcomings associated with existing remediation techniques. For example, thermal desorption is expensive and prone to secondary pollution [10]. Recently, biochar has gained relevance in the remediation of hydrocarbon-contaminated soil. Biochar is a carbon-based product obtained from biomass's thermochemical decomposition, including waste materials in an oxygen-limited environment [11]. The application of biochar to contaminated soil has been shown to enhance hydrocarbon removal by up to 2.1-fold [12-14]. For example, Aziz, Ali, Farooq, Jamal, Liu, He, Guo, Urynowicz and Huang [12] showed that adding sludge-derived biochar led to a minimum 101% higher removal compared to the control treatment. Biodegradation is one of the ways through which biochar is thought to accelerate the remediation of hydrocarbon-contaminated soil [12]. Biodegradation of organic contaminants is facilitated by microbial communities. It is possible that the contaminated soil may not have the right autochthonous hydrocarbon-degrading microbial community [15]. The above scenario could impede the biodegradation effect of biochar on hydrocarbon removal. The co-application of biochar with bioaugmentation, including microbes, has been advocated in the remediation of petroleum hydrocarbon-contaminated soil [16]. Bioaugmentation can involve the use of autochthonous, allochthonous, or genetically engineered microbes [17], with the autochthonous form being the cheapest and least stressful to use from a regulatory and commercial perspective. Evidence in published literature confirms that biochar's co-application with bioaugmentation (microbe) leads to increased hydrocarbon removal relative to biochar treatment on its own [18-21].

The co-application of biochar with microbes has been conducted using immobilised, free-living, or other associations [18, 19, 22]. Compared to free-living co-application, the immobilised form is more effective in remediating hydrocarbon-contaminated soil [23, 24]. This is likely because the advantage derived from the immobilisation of bacteria to biochar may not be realised with free-living bacterial addition [16]. Despite attempts to study the effect of bacteria-immobilised biochar (BIB) on the remediation of petroleum hydrocarbon-contaminated soil [18, 20, 24-27], there is a lack of knowledge regarding the role of BIB in the remediation of diesel-contaminated soil when biochar is produced from biosolids. Using biosolids-derived biochar in bacterial immobilisation offers an alternative approach to managing problematic waste. The rapid rise in the amount of biosolids generated makes biosolids management in a sustainable way a major problem for our contemporary world [28].

Considering that the effect of bacteria immobilised biochar (BIB) on hydrocarbon removal has been examined in the absence [18, 20, 21, 25] and presence [23, 24] of supplementary nitrogen and phosphate, it is important to see if fertiliser addition would be beneficial for BIB in the remediation of diesel-contaminated soil when biosolids-derived biochar is used for bacteria immobilisation. Since the goal of adding nutrients to hydrocarbon-contaminated soils is to compensate for the alteration in the carbon-to-nitrogen (C/N) ratio induced by the hydrocarbon, a soil with a high C/N ratio (27) was used for this study.

The functional group or chemical structure can be used to group petroleum hydrocarbons [29]. The RemScan technology, an alternative to gas chromatography / mass spectrometry (GC/MS) analysis with benefits in terms of cost and speed, provides an idea of the concentration of the total $C_{10}$–$C_{40}$ present in the soil [30, 31]. However, it does not give an idea of the changes in the functional groups or chemical structure of the hydrocarbon with time or treatments. With the integration of Fourier transform infrared (FTIR) spectroscopy in remediation, it is possible to decipher changes in the functional groups or chemical structure. Fourier transform infrared spectroscopy has been widely applied to study changes in functional groups of contaminants and can be rapidly used to characterise different functional groups, including the aliphatic and aromatic [29]. This understanding can offer insight into the influence of time and treatment on hydrocarbon fraction. Therefore, the RemScan was used to study the TPH in this study, while FTIR was deployed for the assessment of functional groups.

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This study examines the effect of bacteria immobilised biochar (BIB) on the remediation of diesel-contaminated soil. The specific objectives were to: (i) isolate bacteria from hydrocarbon-contaminated soil and compare their hydrocarbon degradation efficiency; (ii) evaluate the effect of bacteria immobilised biochar on the remediation of diesel-contaminated soil; (iii) assess the role of fertiliser on the efficacy of bacteria immobilised biochar on hydrocarbon removal; (iv) assess the abundance of gene related to *Ochrobactrum* sp. (OCB) and encoding for the total bacterial population (16S rRNA); and (v) assess the degradation of the diesel using FTIR spectroscopy.

## 2. Materials and Methods

### *2.1. Soil and Biochar*

Pristine soil was obtained from Whittlesea, Melbourne, Victoria, and had a pH of 7.6, total carbon of 2.23%, total nitrogen of 0.22%, and total phosphorus content of 313 mg/kg [32]. The diesel-contaminated soil used for bacteria isolation was from a previous study using the same soil as the current study, only amended with biochar and sodium azide (unpublished) (BN).

The biochar used was produced from biosolids obtained from the Mount Martha Wastewater treatment plant operated by South East Water Corporation, Melbourne, Australia. Before pyrolysis, the biosolids were dried in the incubator for over 18 h and transferred into a crucible and pyrolysed in a muffle furnace at 900 °C for 3 h at a heating rate of 10 °C/min. The produced biochar was passed through a 1 mm mesh. The biochar had a volatile matter of 3.15 ± 0.21
%, fixed carbon of 20.18 ± 5.26%, and ash content of 76.26 ± 4.79%.

### *2.2. Isolation of bacteria from diesel-contaminated soil*

Bacteria were isolated from soil BN as mentioned in Section 2.1. The methods previously described in the literature with modifications were used for bacterial immobilisation [18, 33, 34]. Briefly, soil (2.5 g) was added to minimal salt media (MSM, $KH_2PO_4$ 15 g/l, NaCl 2.5 g/l, $Na_2HPO_4$ 33.9 g/l, and $NH_4Cl$ 5 g/l, 25 ml) with diesel at a concentration of 10 ml/l and incubated for 7 days at 30 °C at 150 rpm for the first cycle. Following incubation, an aliquot (2.5 ml) from the previous cycle of incubation was transferred to fresh MSM (22.5 ml) with a higher diesel concentration and incubated at 30 °C at 150 rpm for 5 d. This was repeated three times, with an increment of the diesel concentration at each cycle. The range of concentration of diesel in the fresh MSM culture after the first cycle was around 200 – 800 ml/l, with the concentration of the diesel increased at each cycle.

From the final MSM culture, serial dilution of the culture was performed, and an aliquot (100 µl) spread on Lueri Bertani (LB) agar. Plates were incubated at 30 °C for 7 d. Colonies were streaked onto LB agar plates and isolates purified. Three distinct colonies were isolated after a series of isolation, namely, isolates A, B, and C.

### *2.3. Identification of bacteria isolates*

The three (3) bacteria isolates were identified using Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS). This method of bacterial identification involves mass spectrometry and was carried out in a MALDI-TOF MS device (Bruker Microflex LT, Germany) and Flex Control software [35]. Briefly, a toothpick was used to pick bacterial samples from a culture plate to a spot on the target plate. The bacterium was smeared on the spot on the target plate. This was followed by adding 70% formic acid (1 µl), mixing thoroughly with a toothpick, and letting it dry completely. An aliquot (1 µl) of the matrix was later added to the dry sample and left to dry. The target plate containing the dried sample was placed on the dock of the equipment and was read.

Further identification was carried out for Isolate C using 16S rRNA Sanger sequencing. Colonies of the bacteria isolates were suspended in PrepMan buffer (100 µl) and was sent to the Australian Genome Research Facility Ltd (AGRF) in Melbourne, VIC, Australia, for Sanger sequencing.

### *2.4. Assessment of the efficacy of bacterial isolates to remediate diesel-contaminated soil*

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The three isolated bacteria (A, B, and C) were examined for their hydrocarbon-degrading efficiency by introducing them individually to a diesel-contaminated soil, with a total petroleum hydrocarbon (TPH) concentration of 32,400 ± 937 mg/kg. For each of the bacterial isolates, colonies from the streak plate were cultured in LB broth for 19 h at 150 rpm and 30 °C to attain an optical density at 600 nm of 0.8 - 2. To ensure that an equal optical density at 600 nm of 1.09 was used for the three bacteria, the volume was normalised. The cells were washed in 0.9% NaCl and resuspended in 0.9% NaCl after washing. An equal volume of bacterium suspension A, B, and C was added to separate pots containing diesel-contaminated soil, in triplicate. A control treatment was included, and all pots were incubated at room temperature in the laboratory, with sampling on days 7, 14, and 37 to determine the hydrocarbon concentration.

*2.5. Bacterial immobilisation on biochar*

The bacterium exhibiting the greatest efficacy from the preliminary experiment in Section 2.4 was chosen for immobilisation on biochar (isolate C). The bacteria were streaked on an LB agar and incubated at 30 °C for at least 24 h. Distinct colonies were transferred to LB broth and cultured at 30 °C with shaking (150 rpm) for 19 h. The bacteria were centrifuged at 5,000 rpm for 10 min, then rinsed with 0.9% NaCl three to four times. The bacterial pellets were resuspended in sterile 0.9% NaCl. A heterotrophic bacteria count was assessed by dilution on LB agar incubated for more than 24 h at 30 °C. The heterotrophic bacterial count after culturing was $6.5*10^9 \pm 1.4*10^8$ CFU/ml.

A 1:5 (w/v) ratio was used to immobilise the bacterium to the biochar [20]. Biochar (6 g) and bacterial suspension (30 ml) were transferred to a 50 ml centrifuge tube incubated for 24 h at 30 °C and 150 rpm. The mixture was centrifuged at 1,000 rpm for 20 min, followed by another 5 min centrifugation at 1,000 rpm. The resultant pellet was washed with 0.9% NaCl three times and centrifuged at 1,000 rpm for 20 min. The immobilised biochar was dried in a biosafety cabinet for 6 d at room temperature, followed by drying at 30 °C for 4 d in the same cabinet.

*2.6. Bioremediation mesocosm experiment*

Pristine soil (sieved using a 4 mm sieve) was contaminated with diesel at 6.4% (v/w). The soil was mixed and left in the fume hood for 24 h. The soil was mixed, and 180 g dispensed into 30 glass containers, then placed inside a plastic mesocosm of equal diameter. The appropriate treatments were added to different mesocosms in triplicate, as described in Table 1. For the bacteria only treatment, bacteria (45 ml) were centrifuged and resuspended in 5 ml of 0.9% NaCl. For all other treatments, NaCl (5 ml, 0.9 %) was added to the mesocosms. Water (1.5 – 4.2 ml) was added at least once every two weeks for the first 5 weeks. From week 6, the moisture content was regulated to 11 – 18% once every week by adding water when necessary. The soil was mixed at least once every week and sampled at weeks 3, 6, 10, 14, 18 and 22.

**Table 1.** Description of treatments used for the remediation of diesel-contaminated soil.

| **Treatments** | **Description** | **Identification Code** |
|---|---|---|
| Control | No amendment | C |
| Bacteria | 5 ml Bacteria suspension ($6.5*10^9$ CFU/ml) | B |
| Fertiliser* | 2% w/w of fertiliser | F |
| Biochar | 5% w/w of biochar | BC |
| Biochar with fertiliser* | 5% w/w of biochar + 2% w/w of fertiliser | BCF |

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| | | |
|---|---|---|
| Bacteria immobilised biochar | 5% w/w of biochar immobilised bacteria | BIB |
| Bacteria immobilised biochar co-application with fertiliser* | 5% w/w of biochar immobilised bacteria + 2% w/w of fertiliser | BIBF |

*Fertiliser used: Yates thrive all-purpose soluble fertiliser - NPK 25: 5: 8.8.

*2.7. Total petroleum hydrocarbon (TPH) analysis*

The TPH concentration was assessed using RemScan Technology (Ziltek, South Australia, Australia) [31]. The device utilises a diffuse reflectance (mid)-infrared Fourier transform (DRIFT) spectrometer for assessment of TPH [31]. The amount of soil sampled for RemScan analysis was >20n g. Before the measurement of TPH, the soil samples were air-dried for 24 h in the fume hood, ground in most cases (except week 0 and 3) and passed through a 2 mm sieve.

*2.8. Molecular microbiological analysis*

2.8.1. Isolation of DNA from soil and bacteria samples

DNA was isolated from the soil, bacteria, biochar, and bacteria immobilised biochar samples using the Power Soil Kit (Qiagen, Hilden, Germany). For the bacteria isolate, a sterile loop was used to take some of the bacterial isolates and transfer them to the power beads tube. A sample weight of 0.25 g was used for the soil, biochar, and bacterial immobilised biochar. The DNA extraction was conducted using the manufacturer's instructions.

2.8.2. Quantitative PCR (qPCR) analysis

Real-time PCR was carried out to quantity the 16S rRNA encoding for the total bacteria population (16S rRNA) and that related to Ochrobactrum (OCB) using a Qiagen Rotor Gene machine (Qiagen, Maryland, USA). A 20 µl reaction was used for amplification of the two genes assessed, which comprised of 0.4 µl forward primer (10 pmol/µl), 0.4 µl reverse primer (10 pmol/µl), 8.2 µl nuclease free water, 10 µl Kapa SYBR Fast qPCR master mix, and 1 µl DNA sample [36]. The primers used for the 16S rRNA were 341-F (5'CCT ACGGGAGGCAGCAG3') and 518-R (5'ATTACCGCGGCTGCTGG3') [37], while 5'CTACCAAGGCGACGATCCAT3' and 5' GGGGCTTCTTCTCCGGTTAC3' were used for the OCB gene as forward and reverse primers, respectively. The primer for the OCB gene was obtained from the National Centre for Biotechnology Information website, with ascension number (DM110786.1). Before use of this primer, PCR, and gel electrophoresis was carried out using the bacteria DNA. Details are provided in Text S1.

Both genes were amplified using the same cycling conditions. The conditions: (i) initial denaturation step at 95 °C for 5 min; (ii) 40 cycles at 95 °C denaturation for 10 s; (iii) annealing at 55 °C for 30 s; (iv) 72 °C extension for 30 s; and (v) 80 °C primer dimer removal and signal acquisition (10 s) [38]. A standard curve for each gene was created using serial dilutions of the cleaned PCR products of the gene [38]. A plot of the cycle threshold (CT) values from the serial dilutions versus the log of their original copy number was prepared, and then a standard curve was produced using linear regression [36]. To calculate the copies of each gene, the CT value was correlated with the standard curve of the gene of interest and reported as $\log_{10}$ gene copy number/g dry soil [39].

*2.10. Fourier transform infrared (FTIR) analysis of the soil*

For FTIR analysis, soil samples were dried for at least 12 h in the fume hood. FTIR (OMNI spectra) identified functional groups of soils by scanning 400-4000 $cm^{-1}$ with 32 scanning times at 4 resolutions. The spectra were reported in an absorbance mode. The sample used for FTIR analysis were both ground and sieved (2 mm sieve) soil.

*2.11. Statistical and kinetic analysis*

All soil analyses were carried out in replicates. Results were expressed as the mean of the replicates and the standard deviation. One-way analysis of variance (ANOVA), was used to assess the statistical difference at $p<0.05$ using Minitab software (Minitab, Pennsylvania, USA). Microsoft Excel (Microsoft, Washington, USA) was used to plot the kinetic curves of the different treatments.

First-order kinetics was used for the kinetics of bioremediation in this study [40]. The equation of the first-order kinetics is as follows:

$$C_t = C_0 . \exp(-kt), \quad (1)$$

where $C_t$ is the concentration of the contaminant at the time t (mg/kg), k is the first-order kinetic constant ($day^{-1}$), $C_0$ is the concentration at the beginning (mg/kg), and t is the time (day) [40]. The half-life ($DT_{50}$) of biodegradation was calculated using (2):

$$DT_{50} = \ln 2/k \quad (2)$$

where $k$ represents the rate constant ($day^{-1}$) [41].

## 3. Results and Discussion

*3.1. Isolation, identification, and screening of autochthonous hydrocarbonoclastic bacteria*

Three distinct bacteria were isolated from the diesel-contaminated soil (isolates A, B, and C). MALDI-TOF MS examination identified isolates A, B, and C as *Achromobacter denitrificans*, *Stenotrophomonas* sp., and *Ochrobactrum grignonense*, respectively. The confidence scores were 1.85, 2.14, and 1.89 for identification of isolates A, B, and C, respectively. Isolate C was further identified using Sanger Sequencing with a 100% identity with *Ochrobactrum* sp. Bacteria belonging to the Achromobacter, Stenotrophomonas, and Ochrobactrum genus have previously been isolated from soils contaminated with petroleum hydrocarbon [42-45].

The isolates were examined for their ability to remediate diesel-contaminated soil through bioaugmentation (Figure 1). Initially at day 7, all bacteria amended treatments control showed similar TPH concentration profiles with the control ($p<0.05$). However, by day 14, TPH concentration was significantly lower in soils with isolates A and C than the control. At the end of incubation, only treatments amended with isolates B and C had a significantly lower hydrocarbon concentration ($p<0.05$) compared to the control (Figure 1). Wu, Dick, Li, Wang, Yang, Wang, Xu, Zhang and Chen [46] observed that bioaugmentation only showed a significant difference from the control from week 2. Comparing treatment with isolates B and C, soils amended with isolate C showed both the lower average and standard deviation and on this basis was selected for biochar immobilisation and subsequent incubation study.

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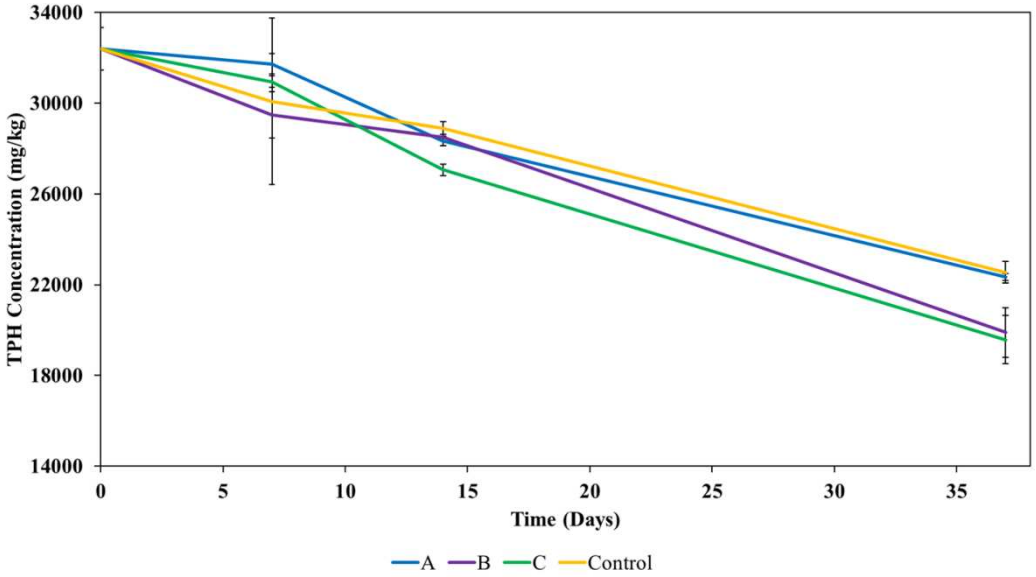


**Figure 1.** Total Petroleum Hydrocarbon (TPH) removal over 37 days in contaminated soils bioaugmented with hydrocarbon isolates and the control (unamended soil). Values are the mean of triplicate measurements, while the error bar represents the standard deviation of the mean. A: *Achromobacter denitrificans*; B: *Stenotrophomonas* sp.; C: *Ochrobactrum* sp.; Control: No bacterial amendment.

### *3.2. Immobilisation of bacteria on biochar*

To assess whether the immobilisation of the bacteria on the biochar was successful, quantitative PCR, and proximate analysis were carried out on the biochar before and after immobilisation. The number of the 16S rRNA gene copies increased by 1.1 $Log_{10}$ gene copies/g following immobilisation on the biochar, confirming the adsorption of bacteria on the biochar. Further, proximate analysis of the biochar showed an increase in both the volatile matter and fixed carbon after bacterial immobilisation on the biochar, while the ash content decreased after immobilisation (Table S1).

### *3.3. Remediation of contaminated soil*

#### 3.3.1. Impact of biochar on remediation of contaminated soil

To assess the efficacy of the addition of biochar to hydrocarbon contaminated soils (6.2% total petroleum hydrocarbon concentration), TPH concentration was monitored over 22 weeks across different treatments (Figure 2). The results showed that the hydrocarbon concentration decreased in all the biochar treatments and the control at the end of incubation (Figure 2 a, and d), which is consistent with the literature [12, 13, 47]. At week 3, negligible removal occurred in the control treatment; however, in the biochar treatment, a significant reduction of 11,993 mg/kg occurred. The hydrocarbon removal in the biochar treatment remained higher ($p < 0.05$) than the control throughout the incubation, with 17% greater removal at week 22 (Figure 2 a and d). The greater difference in hydrocarbon removal between both treatments in the early stages of remediation in the biochar treatment was consistent with our previous work involving biosolids biochar [13]. The fate of the components of crude oil in terms of degradation varies, with n-alkanes easier to degrade compared to the branched alkanes [48]. It is possible that the readily degradable fraction of the hydrocarbon was consumed faster by the microbes in the biochar treatment [49] resulting in a reduction in the rate of remediation in later stages. It is also possible that some metabolites produced during biodegradation inhibit microbial metabolism and, subsequently, hydrocarbon removal [50]. Biochar can serve as a biostimulating agent and thus enhance hydrocarbon removal because of its ability to support soil microbial communities by providing habitat or improving soil properties [51]. Our

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results on the effectiveness of biochar in enhancing hydrocarbon removal agree with other reports in the literature [12, 13, 47, 52].

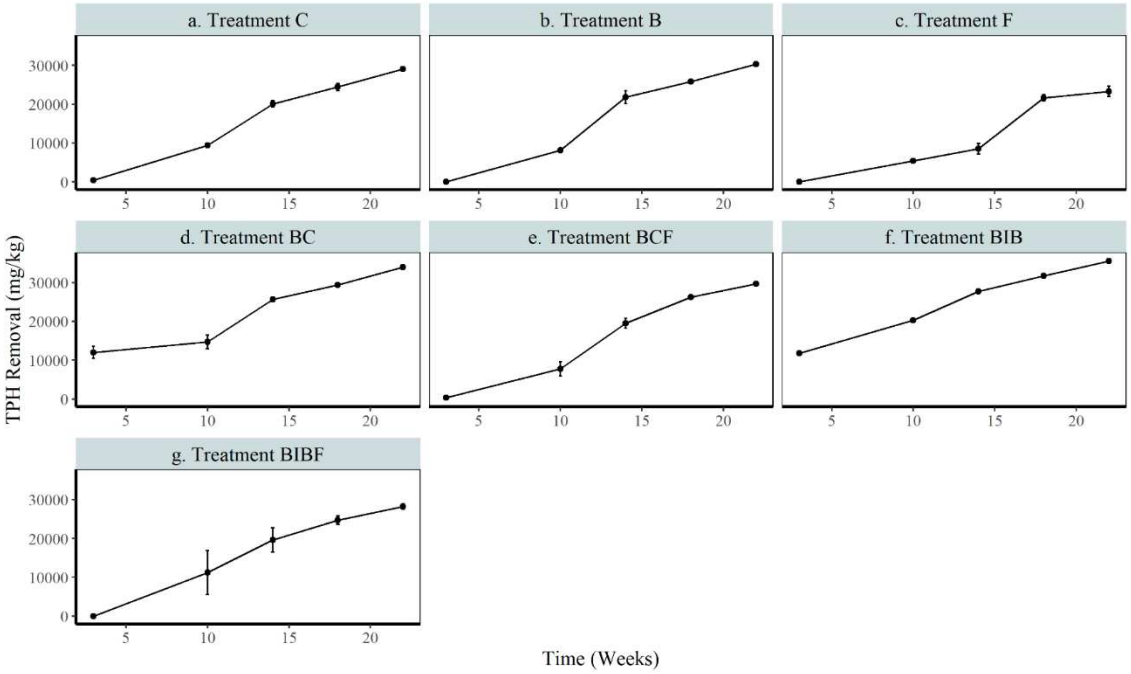


**Figure 2.** TPH removal in the different treatments over a 22-week incubation. Values represent the mean of replicate measurements, while the error bar represents the standard deviation of the mean. The hydrocarbon concentration at the beginning was 62,027 ± 1,209 mg/kg. C: Control (unamended soil); B: Bacteria alone added; F: 2% Fertiliser alone added; BC: 5% w/w Biochar; BCF: 5% w/w Biochar + 2% Fertiliser; BIB: Bacteria immobilised biochar; BIBF: Bacteria immobilised biochar + 2% Fertiliser.3.3.2 Impact of bacteria immobilised biochar on remediation of contaminated soil.

Bacteria were immobilised on biochar to improve the efficacy of biochar in the remediation of diesel-contaminated soil. Figure 2 d and f indicates that there was no significant difference ($p$<0.05) in hydrocarbon removal in soils amended with either bacteria immobilised biochar (BIB) or biochar (BC) at week 3. However, at 10 weeks, BIB was more effective in hydrocarbon removal than BC, achieving a significantly greater reduction of 5,533 mg/kg than the BC treatment ($p$<0.05). A recent study found a greater differences between both treatments in terms of residual hydrocarbon concentration from day 20 [21]. After week 10 in our current study, BIB remained significantly higher ($p$<0.05) than the biochar treatment until the end of the incubation (week 22). It is likely that BIB was more effective in hydrocarbon removal than the BC treatment because of the co-application of biochar with *Ochrobactrum* sp. Many strains of this bacteria degrade a range of contaminants such as polycyclic aromatic hydrocarbons, herbicides, crude oil, and phenols [53]. Xu, Yang, Wei, Huang, Wei and Lin [54] reported that the bioaugmentation of *Ochrobactrum* sp. resulted in higher PAH removal than the control treatment. However, adding the bacteria alone was largely non-beneficial, as this treatment did not result in significantly higher removal ($p$<0.05) relative to the control, except at week 22 (Figure 2 a and b). Therefore greater TPH removal in the BIB-amended soil could be due to the advantages of combining both remediation techniques (biochar and bioaugmentation); a proposed mechanism has been summarised in a previous review [55]. Briefly, biochar enhances the mass transfer of the contaminant from the soil to the immobilised biochar, reducing the contact distance between the immobilised bacteria and the contaminant [55]. The enhanced TPH removal observed in BIB is consistent with reports from previous works [18, 20, 21].

Interestingly, the addition of fertiliser to contaminated soil along with the BIB resulted in a significant reduction ($p$<0.05) in TPH removal compared with the BIB treatment, except at week 10 (Figure 2 f and g), implying that the fertiliser was non-beneficial; further when fertiliser alone was

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added to contaminated soil, a reduction in TPH removal was observed even relative to the untreated soil (Figure 2 a and c). To the best of our knowledge, there are lack of studies comparing the effect of fertiliser addition on the efficacy of BIB involving biosolids biochar. There may be a possibility that the 2% fertiliser addition may have led to a low soil C/N ratio, which further resulted in the slowdown in hydrocarbon removal. This assertion is supported by the results of the F and BCF treatment which showed non-beneficial effect on hydrocarbon removal compared to the control and BC treatment, respectively (Figure 2 a, c, d, and e). This observation stresses the need for care in the application of supplementary fertilisers.

3.3.2. Remediation kinetics and prediction

In line with the Environmental Protection Authority (EPA) Victoria Priority waste category classification [56], none of the treatments met the threshold for fill soil requirement after 22 weeks of incubation (1,000 mg/kg). However, treatment BIB and BC achieved the maximum level for category B (40,000 mg/kg) at week 14, while others, including the control, did not until week 18 or 22 [56]. Remediation kinetics was used to predict when the treatments would achieve the EPA Victoria soil threshold. It can be used to determine the concentration of the contaminants at any given time, which can determine when a threshold would be achieved [57]. The kinetics equation, $R^{2,}$ and half-life are shown in Table S2, and the $R^2$ of the different treatments varied between 0.84 – 0.99, which denotes that almost all the treatments fitted strongly well with the First Order Kinetics. Table S2 demonstrates that it would take BIB 131 days for half of the contaminants to be degraded, whereas the BC and C treatments would achieve that in 141 and 157 days, respectively. Predictions made with the kinetics showed that the EPA Victoria fill soil threshold would be achieved in the control, 23 and 14 weeks after BIB and BC treatments meets the threshold, respectively (Table S3). This suggests that the BIB would be more effective in hydrocarbon removal in the long run than the biochar or control treatment, reducing the time for the remediation significantly.

*3.4. FTIR analysis of the soil*

The changes in the chemical structure of the petroleum hydrocarbons in the soil as a function of time and treatment were assessed using FTIR. The changes in the functional groups or chemical structure before and after remediation can provide an insight on the degradation of contaminants [58]. The intensity of peaks (2853, 2923, and 2953 $cm^{-1}$), associated with $-CH_3$ and $-CH_2$ in aliphatic compounds decreased at week 22 in all treatments (Figure S1). This confirmed that the degradation of aliphatic compounds contributed to a reduction in the hydrocarbon concentration in the soil. A comparison of weeks 10 and 22, showed that the differences in the intensity of these peaks among these treatments were wider at week 10 than week 22 (Figure S1 and S2). This corroborates the results of the TPH removal, where greater differences in hydrocarbon removal among the three different treatments (C, BC, and BIB) was observed at week 10 compared to week 22 (Figure 2 a, d, and f). These results support the hypothesis that the lack of differences in hydrocarbon removal among the treatments at week 22, was because at this time, most of the aliphatic compounds (easily degradable fraction) were degraded and thus the majority of the remaining hydrocarbon requires more time to degrade.

*3.5. Quantification of functional genes*

Quantitative PCR was carried out on a 16S rRNA gene related to *Ochrobactrum* sp. (OCB) to understand the fate of the introduced autochthonous bacteria in the soil and to see if co-applying biochar with the bacteria contributed to differences in hydrocarbon removal between BC and BIB. At week 3, the copy number of this gene was higher in all treatments with the addition of the bacteria (B and BIB), confirming the success of the bioaugmentation. These findings are consistent with previous work where the copies of a gene targeting Mycobacterium *nidA* were higher in treatment with bioaugmentation at day 0 and 18 than those without bioaugmentation [59]. Interestingly in *soils amended with Ochrobactrum* sp., the number of gene copies initially increased but then decreased

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over time (Figure 3 b and d); *in soils* without bacterial amendment, copy numbers declined over time *(Figure 3 a and c)*. At week 3, a higher abundance of this gene was found in treatment B than BIB ($p$ <0.05). Partial immobilisation of all the bacteria cells to the biochar and the loss of the cells during the BIB preparation process may be responsible for the lower abundance in BIB at this time [59]. However, the copy number of this gene was significantly higher ($p$ <0.05) in BIB than in B subsequently, except at week 22, suggesting the benefit of immobilising the introduced microorganism *(Figure 3 b and d)*.

Overall, the numbers of OCB genes were significantly higher ($p$<0.05) in BIB than in the BC treatment at all sampling times, with exception of week 22. Previous studies found that the abundance of the introduced bacteria was higher in the biochar immobilised treatment than in the biochar treatment [20, 59]. For example, Song, Niu, Zhang and Li [20] observed that the relative abundances of Sphingomonas genus was significantly higher in the immobilised treatment than the biochar treatment ($p$<0.05). Although the gene targeted in this our current study was not only specific to Ochrobactrum, it is likely that the higher abundance of this gene may be due to the presence of *Ochrobactrum* sp. in the BIB treatments. This could have contributed to higher hydrocarbon removal observed in BIB treatments compared to BC. Ochrobactrum strains are reported to degradea arnge of contaminants, including crude oil [53]. For example, increased PAH removal was observed in a previous study amended with *Ochrobactrum* sp. relative to the control [54]. Bioaugmentation of nsoil with a hydrocarbon-degrading bacterium in a protective environment could give the BIB an increased advantage over the BC treatment, considering that the indigenous bacteria may not function to their full potential because of the resultant effect of the toxic environment and competition.

The gene encoding the total bacteria population (16S rRNA) was also quantified to understand the effect of remediation time and treatments on the bacteria population (Figure 3). The abundance of this gene in the various treatments ranged from 11.08 $\log_{10}$ to 12.5 $\log_{10}$ gene copies/g dry soil, with maximum values at either week 10 or 14 in each treatment (Figure 3).

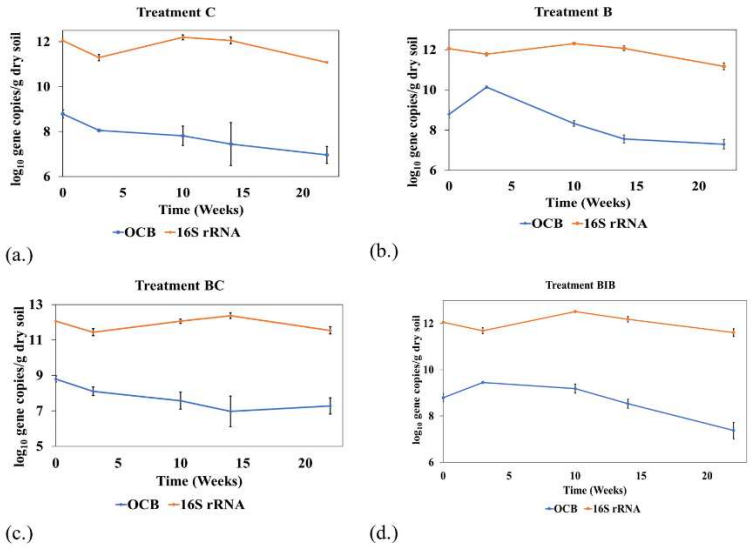


**Figure 3.** Number of copies of OCB ($\log_{10}$ gene copies/g dry soil) and 16S rRNA gene ($\log_{10}$ gene copies/g dry soil) in (a.) C; (b.) B; (c.) BC; and (d.) BIB over 22 weeks. Values represent the mean of triplicate measurements, except treatment BC at week 14 (duplicate readings only). The errors bar represents the standard deviation of the mean. C: Control (unamended soil); B: Bacteria alone added to soil; BC: 5% w/w Biochar; BIB: Bacteria immobilised biochar.

## 4. Conclusion

This work examined the effects of immobilising *Ochrobacterium* sp. on biosolids-derived biochar (BIB) for the remediation of Australian soil contaminated with diesel. Findings from this study

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revealed that biochar enhanced hydrocarbon removal, especially at the early stage. Further immobilisation of bacteria on biochar (BIB) led to a greater hydrocarbon removal, than the biochar application, which suggest the beneficial role of co-applying a hydrocarbon-degrading bacteria with biochar. However, the co-application of fertiliser slowed down the efficacy of BIB in hydrocarbon removal, which advocates for the need for caution and care when applying fertiliser. This study contributes to the understanding of the potential of biosolids-derived biochar in bacteria immobilisation and subsequent utilisation on the remediation of diesel-contaminated soil. Future studies should focus on how immobilisation studies involving biosolids-derived biochar could be further improved to achieve higher hydrocarbon removal, that is biochar modification, immobilisation optimisation, etc.

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