soil systems

MDPI

Article

Impact of Biosolids-Derived Biochar on the Remediation and Ecotoxicity of Diesel-Impacted Soil

Charles Chinyere Dike [1,2,*], Christian Krohn [1,2], Leadin S. Khudur [1,2], Alka Rani Batra [1,2,3], Mac-Anthony Nnorom [4], Aravind Surapaneni [2,5], Kalpit Shah [2,6] and Andrew S. Ball [1,2]

1 School of Science, RMIT University, Bundoora, VIC 3083, Australia
2 ARC Training Centre for the Transformation of Australia's Biosolids Resource, RMIT University, Bundoora, VIC 3083, Australia
3 Environment Protection Authority Victoria, Centre for Applied Sciences, Ernest Jones Drive, Macleod, VIC 3085, Australia
4 Centre for Environmental Health and Engineering (CEHE), School of Sustainability, Civil and Environmental Engineering, University of Surrey, Guildford GU2 7XH, UK
5 South East Water, 101 Wells Street, Frankston, VIC 3199, Australia
6 School of Engineering, RMIT University, Melbourne, VIC 3000, Australia
* Correspondence: charles.dike@student.rmit.edu.au

Abstract: This study aimed to investigate the impact of biosolids-derived biochar on the remediation of Australian soil contaminated with diesel while investigating the role of biochar in the remediation. To achieve the latter aim, sodium azide (NaN_3) was added to a separate biochar treatment (BN) to alter the bacterial community structure. Biochar (B) reduced detectable hydrocarbons by 2353 mg/kg compared to the control (C) treatment at week 24. However, the BN treatment reduced the hydrocarbon concentration by 3827 and 6180 mg/kg, relative to B and C, respectively. Soil toxicity significantly decreased at week 24 compared to the start of the remediation in B, but not in the control. Biochar and control treatments generally showed a similar bacterial community structure throughout the incubation, while the bacterial community structure in BN differed significantly. Biodegradation was found to play a significant role in hydrocarbon removal, as the variation in the bacteria community coincided with differences in hydrocarbon removal between B and BN. The increased removal of hydrocarbons in the BN treatment relative to B coincided with increased and reduced relative abundances of *Gordonia* and *JG30-KF-CM45* genera, respectively. This study showed that NaN_3 led to a transient and selective inhibition of bacteria. This study makes an important contribution towards understanding the use of NaN_3 in examining the role of biochar in the remediation of diesel-contaminated soil. Overall, we conclude that biochar has the potential to enhance the remediation of diesel-contaminated soil and that biodegradation is the dominant mechanism.

Keywords: degradation; *Gordonia*; *JG30-KF-CM45*; petroleum hydrocarbon; pyrochar; sewage sludge

Citation: Dike, C.C.; Krohn, C.; Khudur, L.S.; Batra, A.R.; Nnorom, M.-A.; Surapaneni, A.; Shah, K.; Ball, A.S. Impact of Biosolids-Derived Biochar on the Remediation and Ecotoxicity of Diesel-Impacted Soil. *Soil Syst.* **2024**, *8*, 40. https://doi.org/10.3390/soilsystems8020040

Academic Editor: Luciano Procópio

Received: 24 January 2024
Revised: 18 March 2024
Accepted: 29 March 2024
Published: 3 April 2024

1. Introduction

Soil contamination by petrogenic hydrocarbons represents one of the major legacies of the widespread use of crude oil throughout the 20th century. It is a major global environmental problem worldwide, including in Australia, Canada, and many other countries [1–3]. Exposure to this contaminant poses a threat to biota, from microbes to humans [4]. For example, humans and animals can be exposed to teratogenic, immunotoxic, carcinogenic, and other toxicological impacts when they come into contact with this class of contaminant [5]. These negative impacts and the global reach of this environmental problem confirm the urgent requirement to remediate petroleum hydrocarbon-contaminated soils. Different techniques exist to remediate hydrocarbon-contaminated soil [5]; the most desirable remediation approach should cost-effectively remove the contaminant without causing secondary

pollution. Some techniques fail in this regard; for example, thermal methods like incineration generate secondary waste products after remediation [6]. These shortcomings have created the opportunity to develop new or improve existing approaches. One amendment that has gained attention in the remediation of petroleum hydrocarbon-contaminated soil is biochar.

Biochar is a carbon-based product derived from the thermal decomposition of various organic biomass (including wastes) at high temperatures in an oxygen-limited environment [7]. Various studies have reported that biochar enhances the remediation of hydrocarbon-contaminated soil [8–14]. For example, Aziz et al. [10] reported a 110% increase in the removal of hydrocarbons from soil amended with biochar compared to the control treatment. In another study, biochar application led to an increase of 27% in hydrocarbon removal compared with the control treatment [14]. The source of the biochar that has been used in the remediation of petroleum hydrocarbon-contaminated soil differs but includes crop residues and biosolids (sewage sludge) [10,15–17]. The use of biosolids or sludge digestate in biochar production and subsequently in the remediation of contaminated soil is valuable from a circular economy perspective [9]. The sustainable management of sewage sludge (biosolids) represents one of the significant challenges of today's world due to increasing sludge generation [18]. In 2017, global biosolids production was 100–125 million tons; in 2025, production is estimated to reach 150–200 million tons [19]. Transforming biosolids into biochar reduces the amount of waste and biosolids-derived greenhouse gas emissions [20]. While efforts have been made to understand the potential of biosolids-derived biochar for the remediation of diesel-contaminated soil, no study has focused on soils with a total petroleum hydrocarbon (TPH) contamination greater than 3% [9,10]. There is a need to examine the effect of biosolids-derived biochar on the remediation of more contaminated diesel-contaminated soil (>5%); the efficacy of biochar in terms of the remediation of hydrocarbon-impacted soils has been shown to be influenced by different factors, including contaminant concentration [9,12,21].

The main mechanisms underlying the efficacy of biochar in the remediation of petroleum hydrocarbon-contaminated soil are the subject of debate [10,15,21,22]. Whilst, Mukome et al. [21] report that biodegradation is the main mechanism of hydrocarbon removal, Wei et al. [22] conclude that adsorption is likely the main route for hydrocarbon removal. One method that has been used in determining the mechanism involves exposing biochar-treated contaminated soil to sodium azide (NaN_3), with subsequent comparison to biochar-amended contaminated soil not exposed to NaN_3 [15]. NaN_3 is a selective inhibitor that targets Gram-negative bacteria [23] due to reduced cell wall thickness. NaN_3 inhibits microbial activity [15], allowing for the determination of biotic and abiotic contributions to hydrocarbon removal. However, it remains unclear whether exposure to petroleum hydrocarbon-contaminated soils containing biochar to NaN_3 results in microbial inhibition, as no evidence has been provided in past studies. This is in part due to the fact that NaN_3 is reported to have a selective effect on bacteria [24]. However, to date, the impact of the microbial community on the efficacy of the remediation of petroleum hydrocarbon-contaminated NaN_3-amended soil has not been reported. This study assesses the impact of biochar in the remediation of hydrocarbon-contaminated soil using NaN_3, while integrating both quantitative PCR and 16S rRNA sequencing to assess the impact of the inhibition. Understanding the mechanisms associated with the efficacy of biochar can provide information that can be used to optimise biochar-based remediation.

The use of chemical analytical instruments is the traditional way of assessing how much substance has been removed during the remediation of petroleum-hydrocarbon-contaminated soil [25]. While this can accurately determine the amount of contamination removed, it does not directly indicate the impact of the remaining contaminants and intermediates on the ecological system [26]. Previous studies have shown that the best treatment in terms of hydrocarbon removal is not the treatment exhibiting the lowest soil toxicity at the end of incubation [9,27]. Further, concerns relating to biochar toxicity

strengthen the need to integrate soil ecotoxicity testing into bioremediation studies [28]. Therefore, this study combines chemical analysis with ecotoxicity assessment.

This research is novel because it represents the first study to examine (i) the effect of biosolids-derived biochar on the remediation of diesel-contaminated soil with a high TPH concentration (>5%); and (ii) to integrate quantitative PCR and 16S rRNA sequencing while using NaN_3 to study the mechanism underlying the remediation of hydrocarbon-contaminated soil using biochar. This study seeks to determine: (i) if biosolids-derived biochar will enhance hydrocarbon removal and soil ecotoxicity in heavily diesel-contaminated Australian soil; and (ii) the main mechanism of biochar in the remediation of diesel-contaminated soil using NaN_3. The findings of this study will enhance the understanding of the effect of biosolid biochar on the remediation of Australian soil heavily contaminated with diesel and improve the way NaN_3 is used in studying the mechanism underpinning biochar's enhancement of the remediation of hydrocarbon-contaminated soil.

2. Materials and Methods

2.1. Soil Sample and Characterization

Uncontaminated pasture soil collected from Whittlesea, Melbourne, Australia, was used for this study [27]. The soil has a pH, total carbon, total nitrogen, and total phosphorus content of 7.6, 2.23%, 0.22%, and 313 mg/kg, respectively [27]. The soil texture was classified as clay loam soil.

2.2. Biochar Production and Characterization

The feedstock used for biochar production was biosolids from the Mount Martha water recycling plant operated by South East Water Corporation, Melbourne, Australia. The biochar was produced in a Barnstead Thermolyne 30400 muffle furnace at 900 °C. The basic properties of the biochar, including pH, surface area, pore volume, electrical conductivity, and proximate analysis, are shown in Table S1.

2.3. Soil Contamination and Bioremediation Experiment

Uncontaminated soil from Section 2.1 was spiked with above 5% diesel (Caltex, VIC, Australia) and mixed thoroughly. Diesel fuel is composed of approximately 64% aliphatic hydrocarbons, 35% aromatic hydrocarbons, and 1–2% olefinic hydrocarbons [29]. The contaminated soil was left in a fume hood for 24 h to allow for the dispersion of the volatile fraction. Soil was dispensed into 45 different plastic pots of similar diameter. The experimental setup included three (3) treatments, comprising 15 pots each of non-amended control (C), 5% (*w*/*w*) biochar (B), and 5% (*w*/*w*) biochar + 0.2% NaN_3 (BN) treatments. For BN treatments, each pot had 30 g of soil, while other treatment pots (B and C) had 50 g. Previous studies have reported increased hydrocarbon removal with biochar when applied at 5% *w*/*w* [16,30].

To achieve an initial moisture content of 13–15% (*w*/*w*) in all pots at week 0, Milli-Q deionised water was added to the B and C treatments, while a 0.2% NaN_3 solution was added to the BN treatment. The pots were incubated for 24 weeks at room temperature, and the contents were mixed at least once every week. The moisture content was maintained every week by adding 0.2% NaN_3 solution for the BN treatment and Milli-Q deionised water to treatments C and B, where necessary.

Three pots from each treatment group were destructively sampled at weeks 2, 4, 7, 13, and 24 for molecular and total petroleum hydrocarbon (TPH) analysis. Part of the sample used for TPH analysis was used for the determination of the soil physicochemical properties and ecotoxicity analysis for week 24. Samples were stored at 4 °C and −20 °C.

2.4. Total Petroleum Hydrocarbon (TPH) Analysis

RemScan technology (Ziltek, Edwardstown, Australia) was used to determine the total petroleum hydrocarbon content (C_{10}–C_{40}) of the soil [31]. Prior to analysis, soil samples

were air dried and sieved using a 2-mm sieve. The RemScan has been previously shown to correlate with traditional analysis (GCMS) [31].

2.5. Bacterial Community Analysis

2.5.1. DNA Extraction

DNA was extracted from 0.25 g of soil using DNeasy PowerSoil Pro kits (Qiagen, Venlo, Limburg, The Netherlands). DNA quality (A_{260}/A_{280}) and quantity were assessed using a Nanodrop Lite spectrophotometer (ThermoScientific, Waltham, MA, USA) and Qubit 4.0 fluorometer (ThermoScientific, Waltham, MA, USA), respectively. Quality assurance on the extracted DNA samples showed that the A_{260}/A_{280} concentrations were between 1.41–1.87 and 5.22–141 ng/µL, respectively. The extracted DNA samples were stored at −20 °C until further analysis.

2.5.2. Quantification of Total and Alkane-Degrading Bacteria

The abundance of genes encoding for the total (16S rRNA) and alkane-degrading (*alkB*) bacteria was determined using quantitative PCR in a QIAGEN Rotor-Gene machine (Qiagen, Venlo, Limburg, Netherlands). Quantitative PCR for both genes involved a 20 µL reaction volume, which comprised of KAPA SYBR®FAST qPCR Mastermix (10 µL), forward primer (0.4 µL), reverse primer (0.4 µL), PCR-grade water (8.2 µL), and DNA template (1 µL) [32]. More details are discussed in Text S2.

2.5.3. Bacterial Community Analysis via Next-Generation Sequencing

The V4 region of the 16S rRNA gene was amplified prior to sequencing, using primer pairs 341 F and 806 R as the forward and reverse primers, respectively [33]. Libraries were prepared using Nextera XT indices following Illumina guidelines [34], pooled to 6 pM, including 20% phiX, and sequenced on an Illumina MiSeq (2 × 300 bp) (Illumina Inc., San Diego, CA, USA).

Amplicon size was verified on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and via gel electrophoresis using amplicons and indexed samples. Following completion of the sequencing run, FastQ files were processed and denoised with DADA2 in Qiime2 as previously described, resulting in a median of 27,550 quality-filtered reads and a total of 3871 amplicon sequence variants (ASV) across 49 samples [35].

The 16S rRNA gene amplicon sequences obtained from this study were submitted to the NCBI Sequence Read Archive (SRA). The BioProject accession number is PRJNA990956.

2.6. Ecotoxicity Testing

Ecotoxicity assessment was carried out at the start and end of incubation using the Microtox test. A luminescent marine bacterium (freeze-dried *Aliivibrio fischeri*) obtained from Streamline Hydro Pty Ltd., Capalaba, (QLD, Australia) was used for ecotoxicity testing [33]. The test organism is a nonpathogenic bacterium that is responsive to a large number of toxic substances [36]. The freeze-dried bacteria were reconstituted using the medium provided by the supplier [37]. It was then exposed to an aqueous extract of diesel-contaminated soil [33]. Further details are discussed in Text S3.

2.7. Data Analysis

All analyses, unless otherwise stated, were carried out in replicates. One-way analysis of variance (ANOVA), with the Tukey, at $p < 0.05$ was used to assess the statistical significance of TPH removal, C/N ratio, PO_4, bacteria gene copies, and EC_{50} in Minitab software (Minitab, State College, PA, USA, https://www.minitab.com/en-us/products/minitab/).

3. Results and Discussion

3.1. Effects of Biochar on Hydrocarbon Removal from Contaminated Soil

The TPH concentration of the diesel-contaminated Victoria pasture soil at week 0 was 54,147 ± 1214 mg/kg. The hydrocarbon concentration was assessed in B, BN, and C at

different times over 24 weeks. The results showed that hydrocarbon removal from the contaminated soil increased in all treatments with time (Figure 1). A rapid hydrocarbon removal of 15–18% was observed in all treatments by week 2, followed by a slow but steady hydrocarbon removal. This pattern was similar to the findings of Koshlaf et al. [37], where rapid removal was observed in all treatments in the first 4 weeks, followed by a slow removal. In this present study, the hydrocarbon concentration in all three treatments (C, B, and BN) decreased from 54,147 to at least 27,820 mg/kg after 24 weeks of incubation, representing a hydrocarbon removal of at least 26,367 mg/kg from the soil (Figure 1). At the end of the incubation (week 24), the hydrocarbon removed in treatments amended with biochar (B and BN) was more than half that of the starting hydrocarbon concentration (Figure 1). This was not the case in the control, where the hydrocarbon removal was less than 50% at week 24. This suggests that biochar application alone or with NaN_3 showed higher hydrocarbon removal at week 24 than C.

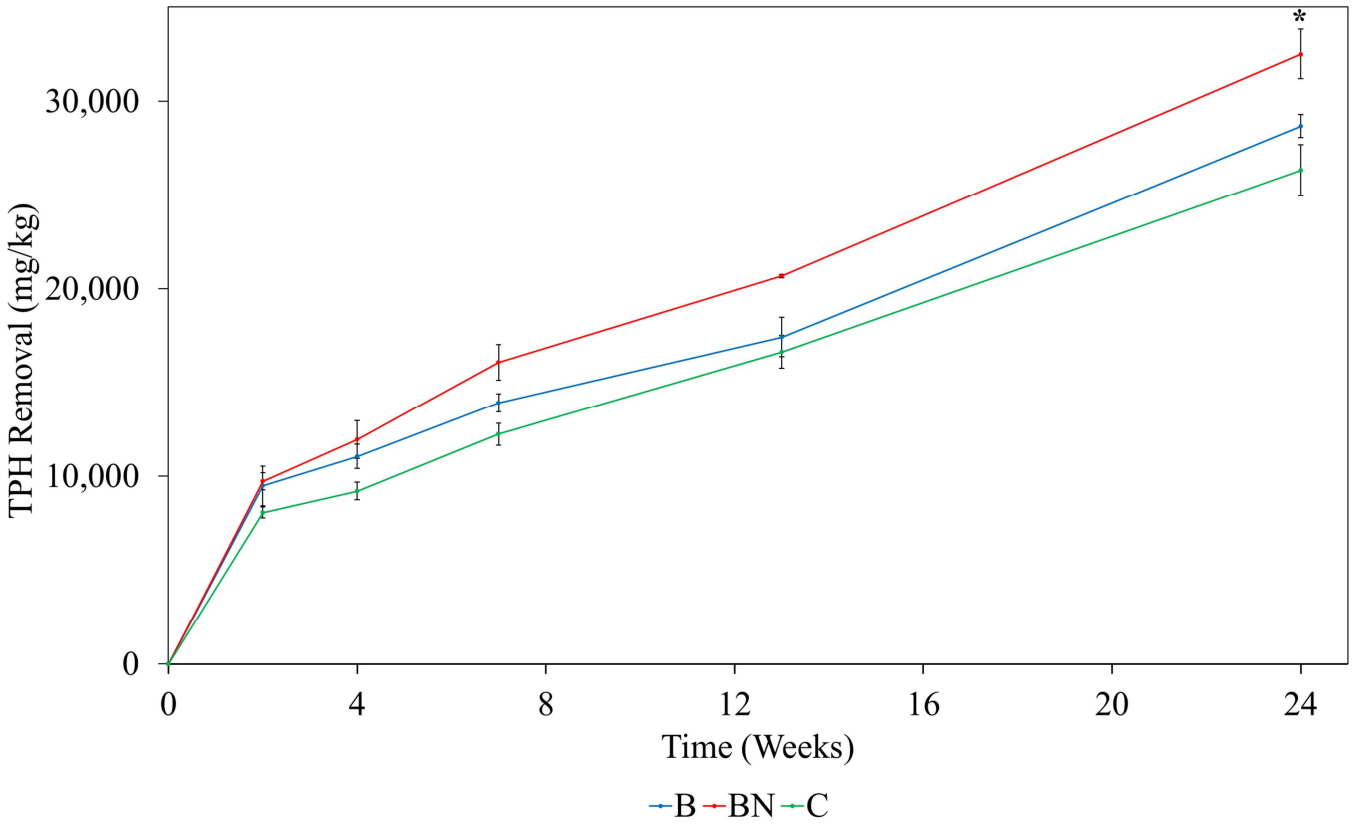

Figure 1. Total petroleum hydrocarbon (TPH) removal in the B, BN, and C treatments over 24 weeks for soil with a starting TPH concentration of 54,147 ± 1214 mg/kg at week 0. The error bars represent the standard deviation of the mean of three replicates. B: Diesel-contaminated soil + 5% *w*/*w* biochar. BN: Diesel-contaminated soil + 5% *w*/*w* biochar + 0.2% NaN_3. C: Diesel-contaminated soil (control). Asterisks (*) at week 24 show that treatments differ significantly at $p < 0.05$, based on a one-way ANOVA with Tukey test.

Figure 1 showed that between weeks 2 and 7 of incubation, the hydrocarbon removal was higher in the biochar treatment than the control by 1422–1833 mg/kg. After 24 weeks, the differences in hydrocarbon removal between both treatments increased to 2353 mg/kg (Figure 1). Increased hydrocarbon removal following biochar application has been reported in the literature due to biochar's positive role in improving soil properties and supporting soil microbes by providing shelter and protection [38]. For example, Wang et al. [16], who observed higher hydrocarbon removal in biochar treatment relative to the control, found that the phosphorus (P_2O_5) increased significantly at the end of the incubation compared

to the start of remediation in the biochar treatment, but not in the control. Previous papers have demonstrated that the application of biochar resulted in higher hydrocarbon removal than the control [8–10,14]. While our current result demonstrated the effectiveness of biosolids-derived biochar in the remediation of diesel-contaminated soil, the differences in hydrocarbon removal between the biochar and treatments were five times lower compared to a previous study [10]. The TPH concentration in our current study was 1.9 times higher than the previous study [10]. However, there is a likelihood that the differences in TPH concentration may not be fully responsible for these discrepancies, as an earlier paper showed that the application of biosolid biochar resulted in a similar difference in hydrocarbon removal between the biochar and control (2287 mg/kg) to that observed in the current study (2353 mg/kg), despite differences in incubation time and initial TPH concentration [9].

In the presence of NaN_3, biochar gave rise to significantly higher hydrocarbon removal compared to the control or the sole biochar treatment (without NaN_3) after 24 weeks of incubation ($p < 0.05$) (Figure 1). There are two possible reasons for the higher removal observed in the BN treatment. Firstly, the contaminated soil at week 0 had a higher C/N ratio by virtue of the addition of a very high amount of hydrocarbon to the soil (Table S2). Among all treatments at week 24, only BN showed a significantly ($p < 0.05$) lower C/N ratio than in week 0 (Table S2). Nitrogen was likely released due to azide reduction to the ammonium ion [39], lowering the C/N ratio of the contaminated soil. In addition, the higher phosphate concentration in the BN treatment may have contributed to the higher hydrocarbon removal (Table S2). Inorganic nutrients like phosphate can be utilised quickly when there is a large amount of organic carbon in the soil [40]. This is a result of increased metabolism by soil microbes [40]. There is a likelihood that the PO_4 in the soil in this current study will be altered since a large content (>5% TPH) of hydrocarbons was introduced to the soil. Biodegradation of the contaminated soil is limited in the presence of inorganic nutrient depletion [40]. Wang et al. [16] found that TPH removal increased as the phosphate (P_2O_5) level in the soil increased. The increase in phosphate may be due to the release of phosphate from dead microbial cells [39]. In addition, the presence of NaN_3 in the biochar-amended soil resulted in the selective inhibition of non-resistant microbes and the growth of NaN_3-resistant microbes. This could result in the promotion of beneficial hydrocarbonoclastic bacteria. The details of this will be discussed further in Section 3.4. The result of this study contradicts other works that reported that the co-application of NaN_3 with biochar resulted in lower [15,21] or non-significant hydrocarbon removal [41]. Aside from the fact that one of the studies applied ethanol alongside NaN_3 [21], several factors may be responsible for the different outcomes, including incubation conditions, biochar type, hydrocarbon class, soil microbial community, and soil properties.

Complying with the regulatory guidelines is important in any commercial remediation. At the end of the incubation, the three treatments achieved the EPA Victoria Category B waste requirement of 40,000 mg/kg [42], with BN being the first to fulfill this requirement. However, during the incubation, none of the treatments achieved the fill material maximum concentration of 1000 mg/kg. Kinetic analysis was used to estimate when the fill soil requirement would be achieved for the different treatments. Kinetics provides an avenue to predict the remediation time, as well as evaluate the contaminant concentration at a given time [43]. The kinetic curve, equation, and half-life of the reaction are shown in Figure S1. The data for the three treatments fit first-order kinetics (R^2 = 0.96–0.97). Other studies have also utilised the first order for their bioremediation kinetics [43,44]. The first-order kinetic curves showed that the biodegradation half-life ($t_{1/2}$) was lower in BN, followed by B and C. This implies that, based on the model, treatment BN will achieve the removal of half of the amount of hydrocarbon in the soil in a faster manner than other treatments. Based on estimation from the first-order kinetic equation [45], B and BN treatments would reach 993.3 mg/kg, a concentration lower than the maximum threshold of the EPA Victoria fill material maximum concentration of 1000 mg/kg, 16 and 44 weeks ahead of the control, respectively (Table S3) [42]. Achieving the EPA Victoria fill soil waste category earlier will

save cost and time of remediation, suggesting that applying biochar to the soil would benefit remediation.

3.2. Bacterial Gene Copies

The *alkB* and 16S rRNA gene copies were determined for all treatments at different sampling times to assess the population of the total bacteria and alkane-degrading bacteria, respectively (Figure 2). Figure 2a shows changes in the copy number of the *alkB* gene with time for all treatments. This gene is generally used to assess the potential for the biodegradation of alkanes [46]. The results indicated that the copy number of this gene was significantly higher ($p < 0.05$) at weeks 2 and 24 in all treatments, relative to week 0, except BN at week 2. The lack of changes in the abundance of *alkB* in treatment BN at week 2 relative to the start of remediation was due to the short-lived bacterial inhibition resulting from the presence of NaN_3. This observation aligns with the result of the bacterial community structure, where the community structure was similar in BN at week 2 compared to the soil at week 0 (Figure S3). Overall, this finding suggests that the hydrocarbons could have been degraded by the native hydrocarbonoclastic bacteria present in the soil. The strong positive correlation between the number of *alkB* gene copies and hydrocarbon removal further confirms the role of biodegradation in the removal of the contaminant in this study ($r = 0.74$, $n = 6$). A previous study also found a strong positive correlation between hydrocarbon removal and the number of copies of this gene [9].

The results showed that the number of 16S rRNA gene copies in all treatments did not significantly increase at weeks 2 and 24 compared to week 0 (Figure 2b). At week 2, BN was significantly lower than the B or C treatments because of the effect of NaN_3. In comparison to the control, biochar addition did not lead to an increase in bacterial gene copies, which demonstrates that biochar was not beneficial in increasing the bacterial population. This observation may be linked to the lack of differences in the soil properties between biochar and the control treatments (Table S2). Table S2 showed that important soil properties, soil pH, and C/N ratio did not differ between both treatments at week 24. Generally, soil microbes are sensitive to changes in soil properties [47], and if changes do not occur, there is a likelihood that the bacterial population will not change. Previous studies have also reported that biochar application did not alter the number of 16S rRNA gene copies [9,48]. The implication of this is that biochar addition to the soil, unlike other remediation techniques, did not cause disturbance to the bacterial population relative to the unamended control.

Overall, the results showed that the bacterial inhibition induced by NaN_3 is only short-lived, raising a question about one of the methods used in the literature to assess the major mechanism of biochar [15,41]. In such studies, one treatment is coapplied with biochar and NaN_3 (BN), while a separate treatment is amended with biochar alone (B). The assumption in these past studies is that NaN_3 inhibits bioactivity, but no evidence was provided to verify if the application of NaN_3 in hydrocarbon-contaminated soil amended with biochar was successful in causing complete inhibition. It would be more valuable to validate microbial inhibition before drawing any inferences about the mechanism in order not to exclude important information. This current study has addressed this gap by integrating quantitative PCR, which provides an avenue to check for bacterial inhibition.

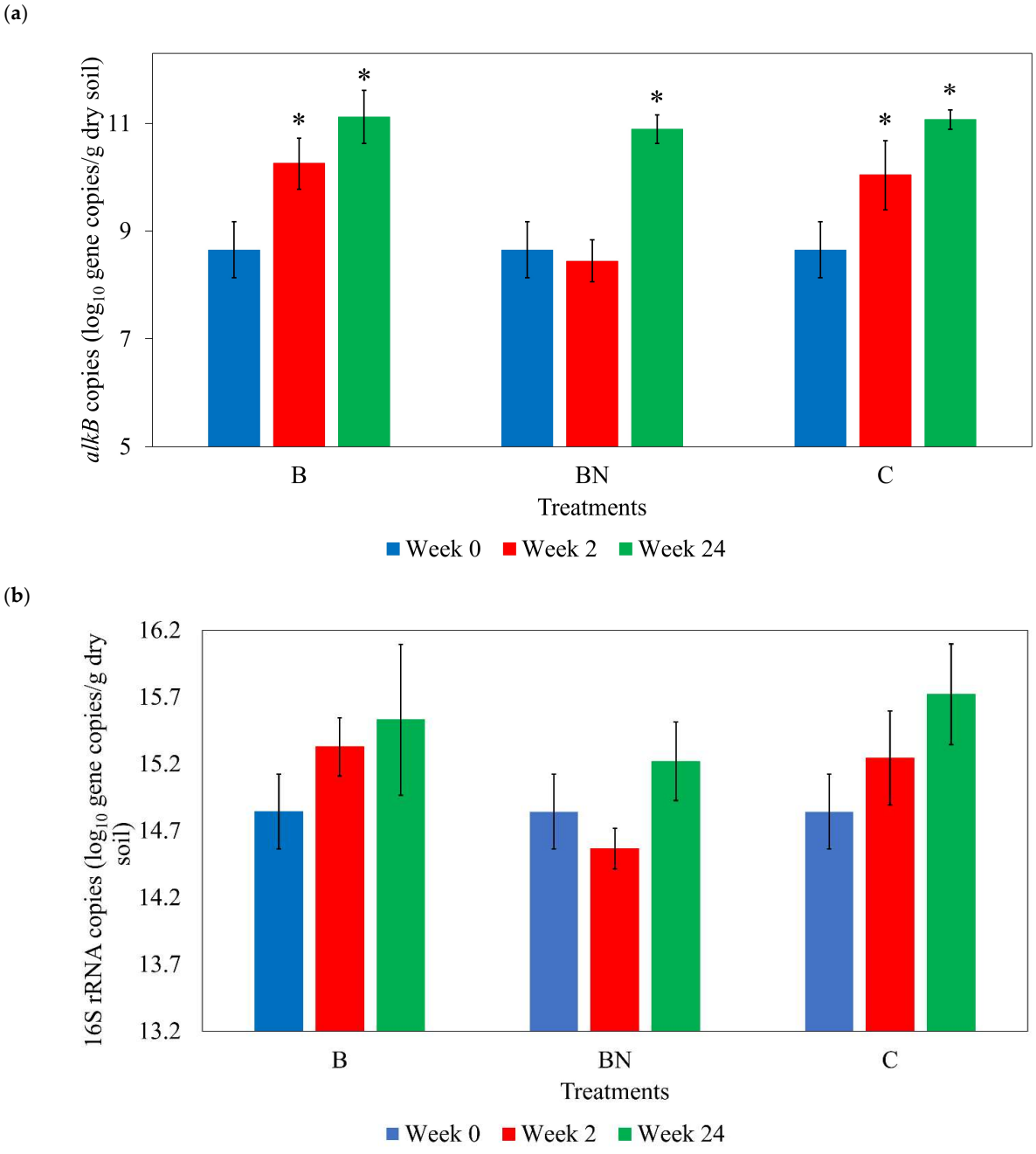

Figure 2. The effect of time and treatments on gene copy numbers of (**a**) *alkB*; and (**b**) 16S rRNA. Values represent the means of at least two replicates, while the error bar represents the standard deviation of the mean. Asterisks (*) show that treatments differ significantly at sampling time relative to week 0 ($p < 0.05$), based on one-way ANOVA with Tukey test. B: Diesel-contaminated soil + 5% (w/w) biochar. BN: Diesel-contaminated soil + 5% (w/w) Biochar + 0.2% NaN_3. C: Diesel-contaminated soil (control).

3.3. Bacterial Diversity

The diversity of the bacteria within a community was studied using alpha diversity [22]. The dynamics of bacterial richness, Shannon diversity, and evenness in the contaminated soil as a function of time and treatments are shown in Figure S2. The result of this study showed that all three alpha diversity indices were lower at week 24 relative to week 0. As an example, the species richness in B, BN, and C treatments at week 24 was approximately 310, 140, and 300, respectively (Figure S2). At week 2, only BN showed similarly or close similarity to the initial-contaminated soil. The observation of the BN

treatment at week 2 agrees with the result of the 16S rRNA gene copies, where no difference was found between week 2 and week 0 (Figure 2b). This may have been due to the fact that at week 2, NaN_3 acted as a preservative and thus prevented the overgrowth of any bacterial group [49,50]. At Week 24, treatment BN generally had a lower richness, diversity, and evenness compared to B, presumably because of NaN_3 activity as a selective inhibitor [51]. Previous studies have found that the NaN_3 addition resulted in lower richness (Chao1 estimator) [52] or Shannon index [53]. Figure S2 shows that the application of biochar resulted in higher species richness and Shannon diversity at both sampling times than the control. This observation suggests that biochar benefited a wider range of bacteria relative to the control. For example, in week 24, only 123 and 158 genera were present in the control and biochar treatments, respectively (Table S4). This implied that the addition of biochar made the soil conducive to more bacterial genera than the control without biochar. An earlier study reported higher Shannon diversity, ACE, and Chao in biochar treatments relative to the control at days 45 and 120 [8].

3.4. Bacterial Community Structure and Abundance

The results of the sequencing revealed that Actinobacteriota (23–81%), Firmicutes (8–32%), Proteobacteria (3–26%), Chloroflexi (1–20%), Planctomycetota (0.08–5%), Verrucomicrobiota (0.03–5%), Bacteroidota (0.008–5%), Acidobacteriota (0.17–4%), Sumerlaeota (0.014–3%), Patescibacteria (0.01–2%), Myxococcota (0.05–1%), Gemmatimonadota (0.008–1%), and Nitrospirota (0.01–1%) had a relative abundance of 1% and above in at least one of the treatments (Figure 3). The relative abundances of these phyla accounted for at least 99.5% of the total bacterial community. Among them, Actinobacteriota was the most dominant in all treatments at different sampling times, except for the contaminated soil at week 0 and the BN at week 2. Actinobacteriota is one of the largest and most diverse bacterial phyla and has many genera with hydrocarbon-degrading abilities [54–56]. This phylum was identified as the most dominant phylum in hydrocarbon-contaminated soils in a previous study [12]. It is apparent from Figures 3 and S3 that changes in the community structure were observed in all treatments after week 0 at both phylum and genus levels.

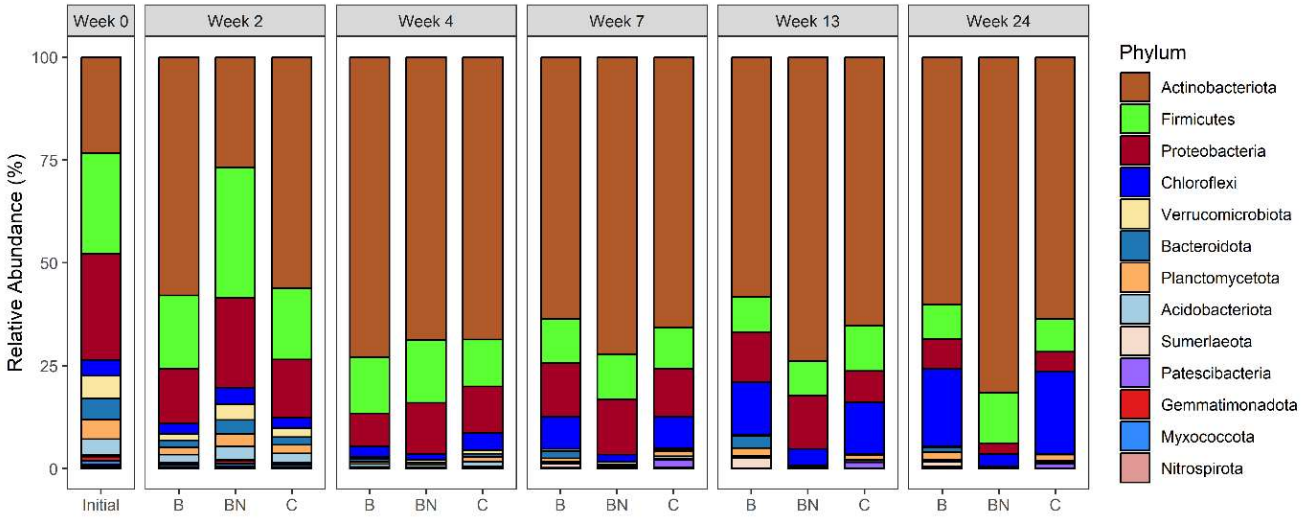

Figure 3. Top 13 bacteria phylum at treatments B, BN, and C as a function of sampling times. Initial: Diesel-contaminated soil at week 0. B: Diesel-contaminated soil + 5% (*w*/*w*) biochar. BN: Diesel-contaminated soil + 5% (*w*/*w*) biochar + 0.2% NaN_3. C: Diesel-contaminated soil (control).

At the genus level, the relative abundances of some of the genera generally increased after week 0 (*Rhodococcus*, *JG30-KF-CM45*, and *Norcardiodes*), whereas others decreased (*Bacillus*, *Rhizocola*, and *Candidatus_Udaeobacter*) (Figure S3). The number of genera decreased from 281 in the contaminated soil at week 0 to 80–252 after week 0, depending

on treatment and time (Table S4). The dynamics of the bacterial community structure can be due to the changes in hydrocarbon concentration and type over time [57]. Another reason, which is particular to the amended treatments only (B and BN), is the introduction of amendments to the soil. The soil bacterial community is sensitive to changes in the soil's physicochemical properties [47], which could take place due to the above mentioned scenarios. As can be seen in Table S2, the C/N ratio was different between the contaminated soil at week 0 and the treatments at week 24, though only statistically significant in the BN treatment. The soil C/N ratio controls important soil parameters, nitrogen immobilization and mineralisation [58].

The data in Figure 3 indicates that the application of biochar in most cases did not cause a significant change in the bacterial community structure relative to the control at the different sampling times at the phylum level; even at the genus level, both treatments were similar (Figure S3). These findings corroborated the lack of differences observed in the Pielou evenness between both treatments (Figure S2). Other reports have shown that the application of biochar did not cause a significant difference in the bacterial community relative to the control [22,59,60]. For example, Bao et al. [59] found that the relative abundance and community structure of bacteria in the control and biochar treatments were similar. This absence of major differences in the bacterial community structure may be because both treatments did not differ significantly in most soil properties (pH, C/N, PO_4) (Table S2). Soil pH has been reported in previous studies to influence soil bacterial diversity and community composition [61,62]; one way that pH may influence the microbial community is by modulating soil nutrient availability [63]. Another soil property, the C/N ratio, affects nitrogen mineralization and immobilisation [58].

Figure 3 showed that, apart from week 2, the bacterial community differed from the initial contaminated soil at all sampling times, which demonstrates the lack of complete inhibition of NaN_3. The bacterial communities of BN and B were dissimilar at the different sampling times, especially after week 4; a lower number of bacterial phyla accounted for the top 1% bacterial phylum in the BN treatment (Figure 3). Similarly, compared to BN, the total relative abundances of the Gram-negative (GN) and Gram-positive (GP) bacteria were higher and lower in the biochar treatment after week 4, respectively. The relative abundance of Actinobacteriota, a GP bacterial phylum, was higher in BN than in B after week 4 (Figure 3). Conversely, the relative abundance of Chloroflexi, a GN bacterial phylum, was lower in the BN treatment than in the B treatment after week 2. At the genus level, the relative abundance of *JG30-KF-CM45*, a Chloroflexi phylum, was higher in B than in treatment BN after week 2 (Figure S3). In contrast, the *Gordonia* genus from the Actinobacteriota phylum was seen in treatment BN after week 2 but was found in B only at week 24, with a relative abundance of 0.01% (Figure S3). This genus has been reported in previous hydrocarbon-contaminated compost amended with biochar [64,65] and soil with bacteria-immobilised biochar and soil amended with digestate [66]. The presence of sodium azide in treatment BN was likely responsible for the apparent differences between both treatments. Sodium azide is a selective bacteria inhibitor that targets GN bacteria [23], although some can survive in its presence, as seen in this study (Figures 3 and S3). This is the first time the effect of NaN_3 on bacterial community structure in petroleum hydrocarbon-contaminated soil amended with biochar has been reported. However, a previous non biochar study reported that NaN_3 had a selective effect on bacteria [51].

Another aim was to determine how differences in the autochthonous bacterial community between B and BN treatments could have been responsible for the variation in hydrocarbon removal. Although there could be other explanations, two genera could provide insight into how the differences in the bacterial community structure affected hydrocarbon removal (*Gordonia* and *JG30-KF-CM45*) (Figures S3 and S4). At week 2, when the genus *Gordonia* was absent in treatment BN, there was no discernible difference in hydrocarbon removal between both treatments (Figure S4a). As the difference in the relative abundance of this genus increased with time after week 4, the difference in hydrocarbon removal between these two treatments increased (Figure S4a). A strong positive correlation

was found between the difference in the relative abundance of this genus and the difference in hydrocarbon removal between both treatments (r = 0.89, n = 6). This genus contains several species that are reported to degrade various hydrocarbons and are present in contaminated sites across the world, such as Korea, Mexico, China, Russia, Kazakhstan, and Indonesia [54,67].

Another genus that deserves attention is the Gram-negative *JG30-KF-CM45*. As shown in Figure S4b, there was no difference in the relative abundance of the *JG30-KF-CM45* genus between B and BN treatments at week 2, which coincides with the lack of difference in TPH removal between both treatments at the same sampling time. As the difference in the relative abundance of *JG30-KF-CM45* between both treatments increased over time, the difference in hydrocarbon removal increased (Figure S4b). A strong negative correlation was found between the difference in hydrocarbon removal and the difference in the relative abundance of *JG30-KF-CM45* (r = −0.96, n = 6). Although in a previous study involving a phytomicrobial electrochemical system, *norank_f__JG30-KF-CM45* was associated with hydrocarbon degradation [68], there is a scarcity of literature that implicates *JG30-KF-CM45* in hydrocarbon removal. A previous study found that PAH removal was lower in treatments with a higher relative abundance of Chloroflexi phylum and *Chloroflexi_unclassified* genus [59]. *JG30-KF-CM45* belongs to the Chloroflexi phylum [68], and the *Chloroflexi_unclassified* genus may be related to *JG30-KF-CM45*.

In summary, while *Gordonia* was beneficial in promoting hydrocarbon removal, *JG30-KF-CM45* was detrimental. Additionally, the results of the sequencing demonstrate that the changes in the bacterial community between B and BN coincided with differences in hydrocarbon removal and thus show that biodegradation was the main mechanism for hydrocarbon removal in this study.

3.5. Ecotoxicity

The toxicity of the soil was measured at the beginning and the end of incubation using the Microtox test. The toxicity of the soil at week 24 was at least 2.6 times lower in all treatments, relative to the beginning (Figure 4), confirming a decrease in soil toxicity following remediation, consistent with a previous study [60]. However, only treatment B showed significantly lower toxicity at week 24, relative to week 0 ($p < 0.05$). Although the sole biochar treatment was not the best treatment in this work in terms of hydrocarbon removal, lower soil toxicity was achieved in this treatment at week 24, relative to other treatments (Figure 4). Previous works have also shown the lack of a relationship between both parameters [9,27]. A number of reasons for these observations have been highlighted previously [9], although, in the current study, the component of one of the treatment agents (NaN_3) strongly contributed to this observation.

At week 24, the soil was two times less toxic than the control, suggesting that biochar application was beneficial in reducing soil toxicity. The reduced soil toxicity following biochar application is consistent with previous works [9,60]. During the biodegradation of hydrocarbon contaminants, intermediate metabolites like fatty acids and aldehydes are formed [69]. Biochar reduces soil toxicity because it can immobilise the toxic metabolites formed during bioremediation [60]. The result of an earlier study showed that the toxicity of the soil was lower when biochar was applied on day 80, relative to when applied at day 0 [60]. They speculated that when biochar was added at the early stage, the petroleum hydrocarbon was absorbed at the site for immobilisation, thus not leaving enough space for the metabolites. However, when it was added at later stages, there were more available sites for the toxic substance to immobilise, which could have led to lower toxicity, which suggests the role of adsorption. Biochar's role in adsorption/immobilisation is facilitated by its unique properties, including its porous nature and surface functional groups [70]. A previous study found that soil toxicity decreased as the pyrolysis temperature used to produce the biochar increased, with the surface area also increasing with temperature [9].

In the presence of NaN_3, biochar (BN) showed higher toxicity compared to the sole biochar. The higher toxicity of BN, relative to B, was due to the selective effect of NaN_3

on bacteria [51]. As seen in Table S4, Figures 3 and S2, NaN_3 addition in BN resulted in a reduction in soil bacterial diversity as well as the number of phylum and genera that can survive in the soil. Further, the test organism for the ecotoxicity testing (*Aliivibrio fischeri*) is a Gram-negative bacterium, and this group of bacteria is more susceptible to NaN_3. A previous study had earlier demonstrated that sodium azide showed toxicity when exposed to the test organism [71].

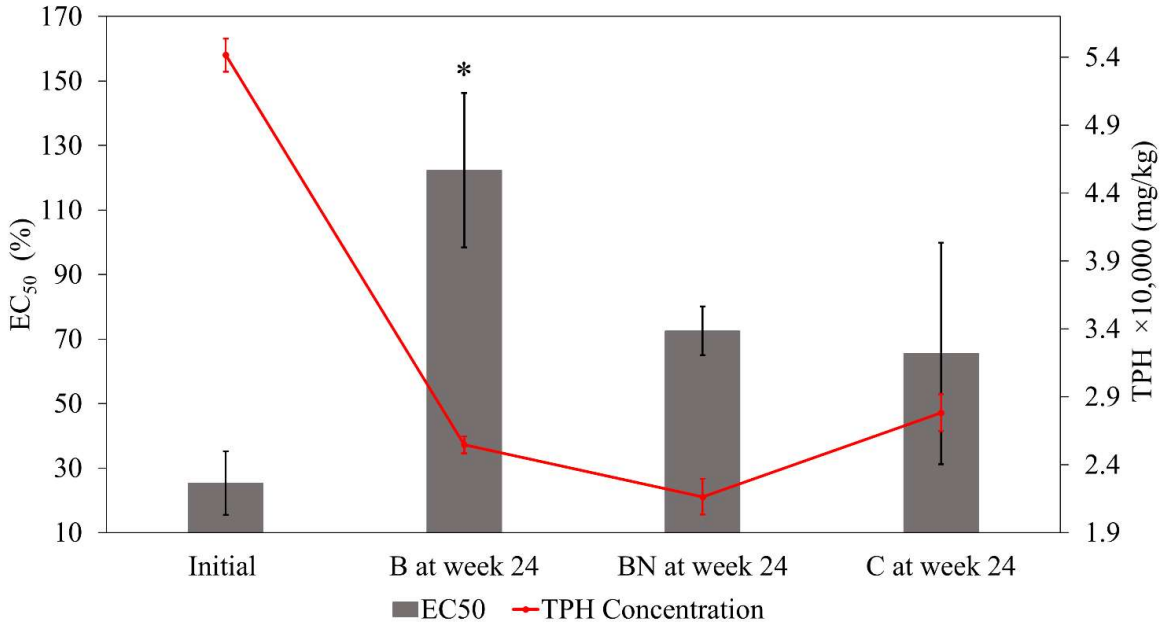

Figure 4. Effective concentration (EC_{50}) versus TPH concentration of the contaminated soil at week 0 as well as the different treatments (B, BN, and C) at week 24. Values represent the mean of the three replicates, while the error bar represents the standard deviation of the mean. Asterisks (*) show that treatments differ significantly at week 24 relative to week 0 ($p < 0.05$), based on one-way ANOVA with Tukey test. Initial: Diesel-contaminated soil at week 0. B: Diesel-contaminated soil + 5% (w/w) biochar. BN: Diesel-contaminated soil + 5% (w/w) biochar + 0.2% NaN_3. C: Diesel-contaminated soil (control).

4. Conclusions

This study examined the impact of biosolids-derived biochar on the remediation and ecotoxicity of heavily diesel-contaminated soil while assessing the major mechanisms of biochar in remediation. The results showed that biochar enhanced hydrocarbon removal and reduced soil toxicity. This study also showed that NaN_3 results in a short-lived and selective inhibition of bacteria. The efficacy of biochar in enhancing hydrocarbon removal was influenced by the differences in the bacterial community following NaN_3 addition relative to the sole biochar treatment, thus demonstrating that biodegradation contributed greatly to the removal of the hydrocarbon. Specifically, the differences in the relative abundances of *Gordonia* and *JG30-KF-CM45* genera were related to variations in hydrocarbon removal in BN relative to the biochar treatment. The findings of this study will help improve the way NaN_3 is used in studying the major mechanism of biochar in the remediation of hydrocarbon-contaminated soil. This study is limited in that it was carried out under laboratory conditions and thus may not wholly reflect outcomes from field studies. Future studies should integrate complete physicochemical characterisation as well as identification and quantification of degradation products during the remediation of diesel-contaminated Australian soil amended with biochar.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/soilsystems8020040/s1, Text S1: Soil Physicochemical Properties; Text

S2: Quantification of total and alkane-degrading bacteria [32,37,72–75]; Text S3: Ecotoxicity Testing [9]; Text S4: Kinetic Analysis [45,76]; Table S1: Properties of the biosolids-derived biochar used in this study; Table S2: Soil properties before and after remediation; Table S3: Time for treatments to achieve the EPA Victoria Fill threshold (1000 mg/kg), based on the kinetic analysis; Table S4: Number of genera in B, BN, and C at different sampling times; Figure S1: First-order kinetics curves fitting the degradation of B, BN, and the C treatments, with their respective equation, R^2, and half-life ($t_{1/2}$).; Figure S2: Alpha Diversity (Richness, Shannon diversity, Pielou Evenness) of the contaminated soil at week 0 as well as the different treatments (B, BN, and C) at weeks 2, and 24.; Figure S3: Relative abundances of top eleven genus for all treatments at different sampling times. (a.): Gordonia; (b.): Norcardiodes; (c.): Rhodococcus; (d.): JG30-KF-CM45; (e.): Bacillus; (f.): Unknown; (g.): Streptomyces; (h.): Rhizocola; (i.): Promicromonospora; (j.): Candidatus_Udaeobacter; (k.): Nocardia; Figure S4: Differences between Treatment BN and B regarding TPH removal and relative abundances of (a.) *Gordonia*, (b.) *JG30-KF-CM45* genus.

Author Contributions: Conceptualization, C.C.D. and A.S.B.; Formal analysis, C.C.D.; Investigation, C.C.D., C.K. and L.S.K.; Methodology, C.C.D., C.K., L.S.K. and A.S.B.; Supervision, A.S. and K.S.; Visualization, C.C.D., A.R.B., and M.-A.N.; Writing—original draft, C.C.D.; Writing—review and editing, C.C.D., C.K., L.S.K., A.R.B., A.S. and A.S.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be available on request.

Acknowledgments: CCD acknowledges RMIT University and the ARC Training Centre for the Transformation of Australia's Biosolids Resource for the RMIT Scholarship Stipend and Top-Up Scholarship, respectively. CCD thanks Jampaiah Deshetti, South East Water, and Kamrun Nahur for biochar characterization, biosolids provision, and biochar characterization, respectively.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wang, Z.; Yang, C.; Yang, Z.; Hollebone, B.; Brown, C.E.; Landriault, M.; Sun, J.; Mudge, S.M.; Kelly-Hooper, F.; Dixon, D.G. Fingerprinting of petroleum hydrocarbons (PHC) and other biogenic organic compounds (BOC) in oil-contaminated and background soil samples. *J. Environ. Monit.* **2012**, *14*, 2367–2381. [CrossRef] [PubMed]
2. Asquith, E.A.; Geary, P.M.; Nolan, A.L.; Evans, C.A. Comparative bioremediation of petroleum hydrocarbon-contaminated soil by biostimulation, bioaugmentation and surfactant addition. *J. Environ. Sci. Eng. A* **2012**, *1*, 637–665.
3. National Oil Spill Detection and Response Agency. *Nigerian Oil Spill Monitor*; National Oil Spill Detection and Response Agency: Abuja, Nigeria, 2022.
4. Ince, M.; Ince, O.K. Introductory Chapter: Sources, Health Impact, and Environment Effect of Hydrocarbons. In *Hydrocarbon Pollution and its Effect on the Environment*; Ince, M., Ince, O.K., Eds.; IntechOpen: London, UK, 2019; p. 1.
5. Ossai, I.C.; Ahmed, A.; Hassan, A.; Hamid, F.S. Remediation of soil and water contaminated with petroleum hydrocarbon: A review. *Environ. Technol. Innov.* **2020**, *17*, 100526. [CrossRef]
6. 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]
7. Shackley, S.; Sohi, S.; Brownsort, P.; Carter, S.; Cook, J.; Cunningham, C.; Gaunt, J.; Hammond, J.; Ibarrola, R.; Mašek, O. *An Assessment of the Benefits and Issues Associated with The Application of Biochar to Soil*; Department for Environment, Food and Rural Affairs, UK Government: London, UK, 2010.
8. Chaudhary, D.K.; Bajagain, R.; Jeong, S.-W.; Kim, J. Insights into the biodegradation of diesel oil and changes in bacterial communities in diesel-contaminated soil as a consequence of various soil amendments. *Chemosphere* **2021**, *285*, 131416. [CrossRef] [PubMed]
9. Dike, C.C.; Khudur, L.S.; Hakeem, I.G.; Rani, A.; Shahsavari, E.; Surapaneni, A.; Shah, K.; Ball, A.S. Biosolids-derived biochar enhances the bioremediation of diesel-contaminated soil. *J. Environ. Chem. Eng.* **2022**, *10*, 108633. [CrossRef]
10. Aziz, S.; Ali, M.I.; Farooq, U.; Jamal, A.; Liu, F.-J.; He, H.; Guo, H.; Urynowicz, M.; Huang, Z. Enhanced bioremediation of diesel range hydrocarbons in soil using biochar made from organic wastes. *Environ. Monit. Assess.* **2020**, *192*, 56. [CrossRef] [PubMed]
11. Saeed, M.; Ilyas, N.; Bibi, F.; Shabir, S.; Jayachandran, K.; Sayyed, R.; Shati, A.A.; Alfaifi, M.Y.; Show, P.L.; Rizvi, Z.F. Development of novel kinetic model based on microbiome and biochar for in-situ remediation of total petroleum hydrocarbons (TPHs) contaminated soil. *Chemosphere* **2023**, *324*, 138311. [CrossRef] [PubMed]

12. Song, L.; Niu, X.; Zhang, N.; Li, T. Effect of biochar-immobilized Sphingomonas sp. PJ2 on bioremediation of PAHs and bacterial community composition in saline soil. *Chemosphere* **2021**, *279*, 130427. [CrossRef] [PubMed]
13. Guo, S.; Liu, X.; Tang, J. Enhanced degradation of petroleum hydrocarbons by immobilizing multiple bacteria on wheat bran biochar and its effect on greenhouse gas emission in saline-alkali soil. *Chemosphere* **2022**, *286*, 131663. [CrossRef]
14. Yousaf, U.; Khan, A.H.A.; Farooqi, A.; Muhammad, Y.S.; Barros, R.; Tamayo-Ramos, J.A.; Iqbal, M.; Yousaf, S. Interactive effect of biochar and compost with Poaceae and Fabaceae plants on remediation of total petroleum hydrocarbons in crude oil contaminated soil. *Chemosphere* **2022**, *286*, 131782. [CrossRef] [PubMed]
15. Kong, L.; Gao, Y.; Zhou, Q.; Zhao, X.; Sun, Z. Biochar accelerates PAHs biodegradation in petroleum-polluted soil by biostimulation strategy. *J. Hazard. Mater.* **2018**, *343*, 276–284. [CrossRef] [PubMed]
16. Wang, Y.; Li, F.; Rong, X.; Song, H.; Chen, J. Remediation of petroleum-contaminated soil using bulrush straw powder, biochar and nutrients. *Bull. Environ. Contam. Toxicol.* **2017**, *98*, 690–697. [CrossRef] [PubMed]
17. Agarry, S.E.; Oghenejoboh, K.M.; Solomon, B.O. Kinetic modelling and half life study of adsorptive bioremediation of soil artificially contaminated with bonny light crude oil. *J. Ecol. Eng.* **2015**, *16*, 1–13. [CrossRef]
18. Spinosa, L.; Molinari, L. Standardization: A Necessary Support for the Utilization of Sludge/Biosolids in Agriculture. *Standards* **2023**, *3*, 385–399. [CrossRef]
19. Mohajerani, A.; Lound, S.; Liassos, G.; Kurmus, H.; Ukwatta, A.; Nazari, M. Physical, mechanical and chemical properties of biosolids and raw brown coal fly ash, and their combination for road structural fill applications. *J. Clean. Prod.* **2017**, *166*, 1–11. [CrossRef]
20. Ghorbani, M.; Konvalina, P.; Walkiewicz, A.; Neugschwandtner, R.W.; Kopecký, M.; Zamanian, K.; Chen, W.-H.; Bucur, D. Feasibility of biochar derived from sewage sludge to promote sustainable agriculture and mitigate GHG emissions—A review. *Int. J. Environ. Res. Public Health* **2022**, *19*, 12983. [CrossRef]
21. Mukome, F.N.D.; Buelow, M.C.; Shang, J.; Peng, J.; Rodriguez, M.; Mackay, D.M.; Pignatello, J.J.; Sihota, N.; Hoelen, T.P.; Parikh, S.J. Biochar amendment as a remediation strategy for surface soils impacted by crude oil. *Environ. Pollut.* **2020**, *265*, 115006. [CrossRef]
22. Wei, Z.; Wang, J.J.; Gaston, L.A.; Li, J.; Fultz, L.M.; DeLaune, R.D.; Dodla, S.K. Remediation of crude oil-contaminated coastal marsh soil: Integrated effect of biochar, rhamnolipid biosurfactant and nitrogen application. *J. Hazard. Mater.* **2020**, *396*, 122595. [CrossRef]
23. AL-Khafaji, J.K.T. Preparation of modified selective medium for isolation of Enterococcus faecalis in pure culture from heavy sources (rapid diagnosis). *Med. J. Babylon* **2021**, *18*, 340–342.
24. Aguirre-Garrido, J.F.; Martínez-Abarca, F.; Montiel-Lugo, D.; Hernández-Soto, L.M.; Ramírez-Saad, H. Metagenomic analyses uncover the differential effect of azide treatment on bacterial community structure by enriching a specific Cyanobacteria present in a saline-alkaline environmental sample. *Int. Microbiol.* **2020**, *23*, 467–474. [CrossRef]
25. Maila, M.P.; Cloete, T.E. The use of biological activities to monitor the removal of fuel contaminants—Perspective for monitoring hydrocarbon contamination: A review. *Int. Biodeterior. Biodegrad.* **2005**, *55*, 1–8. [CrossRef]
26. Xu, Y.; Lu, M. Bioremediation of crude oil-contaminated soil: Comparison of different biostimulation and bioaugmentation treatments. *J. Hazard. Mater.* **2010**, *183*, 395–401. [CrossRef] [PubMed]
27. Khudur, L.S.; Shahsavari, E.; Miranda, A.F.; Morrison, P.D.; Nugegoda, D.; Ball, A.S. Evaluating the efficacy of bioremediating a diesel-contaminated soil using ecotoxicological and bacterial community indices. *Environ. Sci. Pollut. Res.* **2015**, *22*, 14809–14819. [CrossRef]
28. Kuppusamy, S.; Thavamani, P.; Megharaj, M.; Venkateswarlu, K.; Naidu, R. Agronomic and remedial benefits and risks of applying biochar to soil: Current knowledge and future research directions. *Environ. Int.* **2016**, *87*, 1–12. [CrossRef]
29. Gad, S.C. Diesel Fuel. In *Encyclopedia of Toxicology*, 3rd ed.; Wexler, P., Ed.; Academic Press: Cambridge, MA, USA, 2014; pp. 115–118.
30. Zhang, B.; Zhang, L.; Zhang, X. Bioremediation of petroleum hydrocarbon-contaminated soil by petroleum-degrading bacteria immobilized on biochar. *RSC Adv.* **2019**, *9*, 35304–35311. [CrossRef] [PubMed]
31. Khudur, L.S.; Ball, A.S. RemScan: A tool for monitoring the bioremediation of Total Petroleum Hydrocarbons in contaminated soil. *MethodsX* **2018**, *5*, 705–709. [CrossRef]
32. Shahsavari, E.; Aburto-Medina, A.; Taha, M.; Ball, A.S. A quantitative PCR approach for quantification of functional genes involved in the degradation of polycyclic aromatic hydrocarbons in contaminated soils. *MethodsX* **2016**, *3*, 205–211. [CrossRef] [PubMed]
33. Beker, S.A.; Khudur, L.S.; Krohn, C.; Cole, I.; Ball, A.S. Remediation of groundwater contaminated with dye using carbon dots technology: Ecotoxicological and microbial community responses. *J. Environ. Manag.* **2022**, *319*, 115634. [CrossRef]
34. Amplicon, P.; Clean-Up, P.; Index, P. *16s Metagenomic Sequencing Library Preparation*; Illumina: San Diego, CA, USA, 2013.
35. Krohn, C.; Jin, J.; Wood, J.L.; Hayden, H.L.; Kitching, M.; Ryan, J.; Fabijański, P.; Franks, A.E.; Tang, C. Highly decomposed organic carbon mediates the assembly of soil communities with traits for the biodegradation of chlorinated pollutants. *J. Hazard. Mater.* **2021**, *404*, 124077. [CrossRef]
36. James, R.; Dindal, A.; Willenberg, Z.; Riggs, K. *Environmental Technology Verification Report: Strategic Diagnostics Microtox Rapid Toxicity Testing System*; Batelle and U.S. Environmental Protection Agency: Columbus, OH, USA, 2003.

37. Koshlaf, E.; Shahsavari, E.; Haleyur, N.; Osborn, A.M.; Ball, A.S. Impact of necrophytoremediation on petroleum hydrocarbon degradation, ecotoxicity and soil bacterial community composition in diesel-contaminated soil. *Environ. Sci. Pollut. Res.* **2020**, *27*, 31171–31183. [CrossRef]
38. Dike, C.C.; Shahsavari, E.; Surapaneni, A.; Shah, K.; Ball, A.S. Can biochar be an effective and reliable biostimulating agent for the remediation of hydrocarbon-contaminated soils? *Environ. Int.* **2021**, *154*, 106553. [CrossRef]
39. Skipper, H.D.; Westermann, D.T. Comparative effects of propylene oxide, sodium azide, and autoclaving on selected soil properties. *Soil Biol. Biochem.* **1973**, *5*, 409–414. [CrossRef]
40. Kebede, G.; Tafese, T.; Abda, E.M.; Kamaraj, M.; Assefa, F. Factors influencing the bacterial bioremediation of hydrocarbon contaminants in the soil: Mechanisms and impacts. *J. Chem.* **2021**, *2021*, 9823362. [CrossRef]
41. Kong, L.; Song, B.; Zhang, T.; Gao, K.; Liu, J. Effects of soil organic matter on biochar application in developing the biodegradation potentials of polycyclic aromatic hydrocarbons (PAHs). *Appl. Soil Ecol.* **2021**, *167*, 104046. [CrossRef]
42. EPA Victoria. Waste Disposal Categories—Characteristics and Thresholds. Available online: https://www.epa.vic.gov.au/about-epa/publications/1828-2 (accessed on 17 March 2024).
43. Tazangi, M.H.; Ebrahimi, S.; Nasrabadi, R.G.; Naeeni, S.A.M. Kinetic Monitoring of Bioremediators for Biodegradation of Gasoil-Polluted Soil. *Water Air Soil Pollut.* **2020**, *231*, 418. [CrossRef]
44. Agarry, S.E.; Aremu, M.O.; Aworanti, O.A. Kinetic modelling and half-life study on enhanced soil bioremediation of bonny light crude oil amended with crop and animal-derived organic wastes. *J. Pet. Environ. Biotechnol.* **2013**, *4*, 137. [CrossRef]
45. Kim, S.H.; Woo, H.; An, S.; Chung, J.; Lee, S.; Lee, S. What determines the efficacy of landfarming for petroleum-contaminated soils: Significance of contaminant characteristics. *Chemosphere* **2022**, *290*, 133392. [CrossRef]
46. Schloter, M. Effects of different compost amendments on the abundance and composition of alkB harboring bacterial communities in a soil under industrial use contaminated with hydrocarbons. *Front. Microbiol.* **2014**, *5*, 67345.
47. Dasgupta, D.; Brahmaprakash, G. Soil microbes are shaped by soil physico-chemical properties: A brief review of existing literature. *Int. J. Plant Soil Sci.* **2021**, *33*, 59–71. [CrossRef]
48. Galitskaya, P.; Akhmetzyanova, L.; Selivanovskaya, S. Biochar-carrying hydrocarbon decomposers promote degradation during the early stage of bioremediation. *Biogeosciences* **2016**, *13*, 5739–5752. [CrossRef]
49. Noonin, C.; Peerapen, P.; Thongboonkerd, V. Contamination of bacterial extracellular vesicles (bEVs) in human urinary extracellular vesicles (uEVs) samples and their effects on uEVs study. *J. Extracell. Biol.* **2022**, *1*, e69. [CrossRef]
50. Thongboonkerd, V.; Saetun, P. Bacterial overgrowth affects urinary proteome analysis: Recommendation for centrifugation, temperature, duration, and the use of preservatives during sample collection. *J. Proteome Res.* **2007**, *6*, 4173–4181.
51. Lichstein, H.C.; Soule, M.H. Studies of the effect of sodium azide on microbic growth and respiration: I. The action of sodium azide on microbic growth. *J. Bacteriol.* **1944**, *47*, 221–230. [CrossRef] [PubMed]
52. Bruckberger, M.C.; Bastow, T.P.; Morgan, M.J.; Gleeson, D.; Banning, N.; Davis, G.; Puzon, G.J. Biodegradability of polar compounds formed from weathered diesel. *Biodegradation* **2018**, *29*, 443–461. [CrossRef]
53. Bruckberger, M.C.; Morgan, M.J.; Walsh, T.; Bastow, T.P.; Prommer, H.; Mukhopadhyay, A.; Kaksonen, A.H.; Davis, G.; Puzon, G.J. Biodegradability of legacy crude oil contamination in Gulf War damaged groundwater wells in Northern Kuwait. *Biodegradation* **2019**, *30*, 71–85. [CrossRef] [PubMed]
54. Kämpfer, P. Actinobacteria. In *Handbook of Hydrocarbon and Lipid Microbiology*; Timmis, K.N., Ed.; Springer: Berlin/Heidelberg, Germany, 2010; pp. 1819–1838.
55. Tischler, D.; Van Berkel, W.J.; Fraaije, M.W. Actinobacteria, a source of biocatalytic tools. *Front. Microbiol.* **2019**, *10*, 800. [CrossRef] [PubMed]
56. Prince, R.C.; Amande, T.J.; McGenity, T.J. Prokaryotic hydrocarbon degraders. In *Taxonomy, Genomics and Ecophysiology of Hydrocarbon-Degrading Microbes*; McGenity, T., Ed.; Springer: Cham, Switzerland, 2019.
57. Greer, C.; Whyte, L.; Niederberger, T. Microbial communities in hydrocarbon-contaminated temperate, tropical, alpine, and polar soils. In *Handbook of Hydrocarbon and Lipid Microbiology*; Timmis, K.N., Ed.; Springer: Berlin/Heidelberg, Germany, 2010.
58. Brust, G.E. Management strategies for organic vegetable fertility. In *Safety and Practice for Organic Food*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 193–212.
59. Bao, H.; Wang, J.; Zhang, H.; Li, J.; Li, H.; Wu, F. Effects of biochar and organic substrates on biodegradation of polycyclic aromatic hydrocarbons and microbial community structure in PAHs-contaminated soils. *J. Hazard. Mater.* **2020**, *385*, 121595. [CrossRef]
60. Qin, G.; Gong, D.; Fan, M.-Y. Bioremediation of petroleum-contaminated soil by biostimulation amended with biochar. *Int. Biodeterior. Biodegrad.* **2013**, *85*, 150–155. [CrossRef]
61. Wu, Y.; Zeng, J.; Zhu, Q.; Zhang, Z.; Lin, X. pH is the primary determinant of the bacterial community structure in agricultural soils impacted by polycyclic aromatic hydrocarbon pollution. *Sci. Rep.* **2017**, *7*, 40093. [CrossRef]
62. Rousk, J.; Bååth, E.; Brookes, P.C.; Lauber, C.L.; Lozupone, C.; Caporaso, J.G.; Knight, R.; Fierer, N. Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J.* **2010**, *4*, 1340–1351. [CrossRef] [PubMed]
63. Rousk, J.; Brookes, P.C.; Bååth, E. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. *Appl. Environ. Microbiol.* **2009**, *75*, 1589–1596. [CrossRef] [PubMed]
64. Lv, Y.; Bao, J.; Li, S.; Liu, D.; Dai, D.; Qv, M.; Zhu, L. Biochar amendment of aerobic composting for the effective biodegradation of heavy oil and succession of bacterial community. *Bioresour. Technol.* **2022**, *362*, 127820. [CrossRef] [PubMed]

65. Xu, S.; Zhan, J.; Li, L.; Zhu, Y.; Liu, J.; Guo, X. Total petroleum hydrocarbons and influencing factors in co-composting of rural sewage sludge and organic solid wastes. *Environ. Pollut.* **2022**, *319*, 120911. [CrossRef] [PubMed]
66. Gielnik, A.; Pechaud, Y.; Huguenot, D.; Cébron, A.; Riom, J.-M.; Guibaud, G.; Esposito, G.; van Hullebusch, E.D. Effect of digestate application on microbial respiration and bacterial communities' diversity during bioremediation of weathered petroleum hydrocarbons contaminated soils. *Sci. Total Environ.* **2019**, *670*, 271–281. [CrossRef] [PubMed]
67. Sowani, H.; Kulkarni, M.; Zinjarde, S. Harnessing the catabolic versatility of Gordonia species for detoxifying pollutants. *Biotechnol. Adv.* **2019**, *37*, 382–402. [CrossRef] [PubMed]
68. Zhang, X.; Li, R.; Song, J.; Ren, Y.; Luo, X.; Li, Y.; Li, X.; Li, T.; Wang, X.; Zhou, Q. Combined phyto-microbial-electrochemical system enhanced the removal of petroleum hydrocarbons from soil: A profundity remediation strategy. *J. Hazard. Mater.* **2021**, *420*, 126592. [CrossRef] [PubMed]
69. Chen, F.; Li, X.; Zhu, Q.; Ma, J.; Hou, H.; Zhang, S. Bioremediation of petroleum-contaminated soil enhanced by aged refuse. *Chemosphere* **2019**, *222*, 98–105. [CrossRef] [PubMed]
70. Al Masud, M.A.; Shin, W.S.; Sarker, A.; Septian, A.; Das, K.; Deepo, D.M.; Iqbal, M.A.; Islam, A.R.M.T.; Malafaia, G. A critical review of sustainable application of biochar for green remediation: Research uncertainty and future directions. *Sci. Total Environ.* **2023**, *904*, 166813. [CrossRef] [PubMed]
71. Klapötke, T.M.; Scharf, R.; Stierstorfer, J.; Unger, C.C. Toxicity assessment of energetic materials by using the luminescent bacteria inhibition test. *Propellants Explos. Pyrotech.* **2021**, *46*, 114–123. [CrossRef]
72. Schafer, H.; Muyzer, G. Denaturing gradient gel electrophoresis in marine microbial ecology. *Methods Microbiol.* **2001**, *30*, 425–468.
73. Pérez-de-Mora, A.; Engel, M.; Schloter, M. Abundance and diversity of n-alkane-degrading bacteria in a forest soil co-contaminated with hydrocarbons and metals: A molecular study on alkB homologous genes. *Microb. Ecol.* **2011**, *62*, 959–972. [CrossRef] [PubMed]
74. Khudur, L.S.; Gleeson, D.B.; Ryan, M.H.; Shahsavari, E.; Haleyur, N.; Nugegoda, D.; Ball, A.S. Implications of co-contamination with aged heavy metals and total petroleum hydrocarbons on natural attenuation and ecotoxicity in Australian soils. *Environ. Pollut.* **2018**, *243*, 94–102. [CrossRef] [PubMed]
75. Shahsavari, E.; Adetutu, E.M.; Anderson, P.A.; Ball, A.S. Plant residues—a low cost, effective bioremediation treatment for petrogenic hydrocarbon-contaminated soil. *Sci. Total Environ.* **2013**, *443*, 766–774. [CrossRef]
76. Sun, T.; Miao, J.; Saleem, M.; Zhang, H.; Yang, Y.; Zhang, Q. Bacterial compatibility and immobilization with biochar improved tebuconazole degradation, soil microbiome composition and functioning. *J. Hazard. Mater.* **2020**, *398*, 122941. [CrossRef]