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Biosolids-derived biochar enhances the bioremediation of diesel-contaminated soil

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ABSTRACT

Biochar, a low-cost carbonaceous product, is gaining relevance in the remediation of hydrocarbon-contaminated soil. Current literature has shown that biochar studies have been carried out under different conditions. Although some attempts have been made to assess the effect of varying conditions in biochar-based remdediation studies, no work has assessed the effect of biochar pyrolysis temperature, biochar dose, and fertiliser dose altogether on the efficacy of biosolids-derived biochar in the remediation of diesel-contaminated soil, despite the fact that the influence of these parameters on the efficacy of the remediation process are likely to be significant. This study aimed to investigate the effect of biosolids-derived biochar on the remediation and ecotoxicity of diesel-contaminated soil, as well as to assess the influence of biochar pyrolysis temperature, biochar dose, and fertiliser addition on soil remediation. After 12 weeks, the contaminated soil amended with biochar produced at 900 °C and applied at 10% together with 1% fertiliser had a TPH concentration lower than the EPA Victoria maximum threshold for Category D waste (5000 mg/kg); in contrast, the TPH concentration in the control exceeded this threshold. Further, soil ecotoxicity at week 12 was lower in most of the biochar treatments. The *alk*B gene copy numbers increased at week 12 in almost all treatments. Hydrocarbon removal and soil ecotoxicity was affected by the studied factors. This study demonstrated the potential of biosolids biochar as a low-cost treatment to enhance the bioremediation of diesel-contaminated soils, while showing the importance of the treatment conditions on the biochar efficacy in remediation.

1. Introduction

Globally, crude oil has been and will in the near future remain the dominant energy source [1]; global oil consumption (million tonnes) increased on average 2% p.a. between 1965 and 2020 [2]. Further, crude oil-derived products remain major energy sources for many domestic and industrial activities and raw materials for many industries [3,4]. However, the increasing and widespread use of petroleum hydrocarbons has resulted in the accidental release of significant quantities of petroleum hydrocarbons into the environment [5,6]; in many countries, petroleum hydrocarbon represents the most common soil contaminant [7,8]. For example, petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and benzene, toluene, ethylbenzene, xylene accounts for at least 50% of the active and suspected contaminant on surface soil or soil medium in the Federal contaminated sites inventory in Canada [8]. Their presence in the soil poses a significant risk to soil ecosystem processes, soil biota, and human health [9]. Human and animal exposure to hydrocarbon contaminant can result to teratogenic, hepatotoxic, carcinogenic, cardiotoxic, mutagenic, immunotoxic, and haemotoxic effects [9]. The impact of exposure to these contaminants and the increased risk of oil spills suggests that the development of new and the improvement of existing remediation techniques is paramount.

In recent years, the use of biochar has gained relevance in the remediation of hydrocarbon-contaminated soil [10]. Biochar is a carbonaceous material obtained from the thermochemical conversion (usually by pyrolysis) of organic resources such as agricultural and forestry residues, biosolids, and manure) in an oxygen deficient environment [11]. The role of biochar in remediation can be attributed to its

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ability to serve as a biostimulator, or a sorbent [10,12]. As a biostimulator, biochar modifies soil physicochemical properties, supplies nutrients and provides shelter to microbes, thus enhancing bioremediation, while as a sorbent, it reduces the amount of contaminant in the soil by uptake of contaminant by the biochar [10,13]. Biochar used for remediation has been produced from various feedstock, such as biosolids, plant residues, compost and manure. Biosolids is a nutrient (nitrogen, phosphorus and micronutrient) rich feedstock; its utilisation for biochar production and subsequently in remediation affords an opportunity for resource recovery [14]. The conversion of biosolids to biochar and its application in soil remediation provides a valuable alternative for the wastewater industry in managing their problematic waste [15]. Biosolids management currently presents a challenge to the wastewater industry because of the emerging contaminant concerns, high processing cost and the large volumes of biosolids produced globally [14]. For example, the average annual amount of biosolids produced in Australia is 407 MT/yr [16].

A number of reports have focused on the application of biochar to the soil using a range of conditions [10,17]. Reports have highlighted the impact of changes in application, production, soil, and petrogenic contamination-related factors on the efficacy of the biochar treatment [18–27]. However, no single study has investigated the influence of biochar pyrolysis temperature, biochar application rate and fertiliser addition altogether on the efficacy of biosolids derived biochar in terms of the remediation of diesel-contaminated soil. Yet pyrolysis temperature, biochar application rate and fertiliser addition are all factors likely to significantly affect the efficacy of biochar for the remediation of diesel-contaminated soil. Pyrolysis temperature is known to significantly impact the properties of the biochar which, in turn will influence its ability to perform a distinct function like hydrocarbon remediation [28,29]. Further, the influence of biochar on the soil's biological, physical and chemical properties will be dependent on the amount of biochar added to the soil [30,31] and the presence of additional fertiliser. Rather than assessing these factors one at a time, the assessment of multiple factors in one study is important since the influence of a particular factor can be dependent on the condition of another. For example, Tazangi et al. [22] observed that the particle size of biochar did not influence hydrocarbon removal at low biochar dose, but at a higher dose, significantly influenced remediation.

Studying the influence of many factors affecting a process using the traditional one factor at a time (OFAT) technique is time-consuming and expensive [32,33]. The use of design of experiment (DOE) techniques like factorial, Taguchi and response surface methodology can overcome these challenges [33,34]. Estimation of the effect of any factor affecting a process is more precise in DOE than OFAT; in addition the interaction between factors can also be studied [35]. The Taguchi DOE methodology is a fractional factorial-based technique that utilises an orthogonal array to assess the influence of a number of factors using a reduced number of experiments [36,37]. Although this DOE methodology has been applied in different areas of biology/biotechnology, including bioremediation studies on hydrocarbon-contaminated soils, to the best of the authors' knowledge, this paper is the first to utilise this DOE technique in petroleum hydrocarbon-contaminated soils amended with biochar [37–40]. The Taguchi methodology was employed to assess the influence of biochar pyrolysis temperature, biochar application dose, and fertiliser application dose on soil remediation.

Assessment of the concentration of the target contaminant in soil is the conventional way of monitoring the bioremediation process [41]. This approach, however, is insufficient to evaluate soil toxicity because a reduction in the concentration of the soil contaminant does not necessarily reflect a reduction in soil toxicity as it does not assess the impact of any by-products produced during the degradation process [42,43]. In addition, toxicity concerns regarding the application of biochar confirm the importance of assessing soil ecotoxicity in biochar-based remediation studies [44]. These suggest the need to integrate the quantification of residual hydrocarbon concentration with ecotoxicological testing and bacteria population quantification.

In addition to being the first study to assess the influence of pyrolysis temperature, application dose and fertiliser addition on the efficacy of the remediation of diesel-contaminated soil using biosolids-derived biochar, to the best of our knowledge this report is the first to examine the effect of biochar on the remediation of Australian soil contaminated with petroleum hydrocarbon. This is surprising considering that Australia has been identified as one of the most active countries in terms of biochar research [45,46]. Biochar have previously been shown to be effective in the remediation of hydrocarbon-contaminated soils in other countries including China, Nigeria, Pakistan, Russia, and South Korea [23,47–50]. Examining biochar effectiveness from an Australian soil perspective will be a valuable contribution to the global pool of biochar studies.

This study aimed at investigating the effect of biosolids-derived biochar on the remediation and ecotoxicity of diesel-contaminated soil, as well as the influence of biochar pyrolysis temperature, application dose and fertiliser dose on bioremediation efficacy of biochar. The specific objectives of this study were to assess (i) the effect of biosolids-derived biochar application on the remediation and ecotoxicity of diesel-contaminated soil; (ii) the effect of biosolids-derived biochar pyrolysis temperature, its application dose and fertiliser addition on remediation and ecotoxicity; and (iii) the effect of biosolids-derived biochar application on both the total soil bacterial and the alkane-degrading (*alk*B) community.

2. Materials and methods

2.1. Soil sample and characterisation

Clean pasture soil obtained from Whittlesea, Melbourne, Australia was used for this study [51]. The properties of the soil have been previously described. Briefly the clay texture soil contains 2.3% carbon, 0.22% nitrogen, 0.03% phosphorus, with a pH of 7.6 [51]. The soil was sieved with a 4 mm mesh before use.

2.2. Biochar production and characterisation

The biochar was produced from biosolids sourced from Mount Martha municipal wastewater recycling plant, South East Water Corporation, Melbourne, Australia. The biosolids derived from this plant have been characterised in a previous study; the key properties are shown in Table S1 [52]. Biochar was produced in a muffle furnace (Barnstead Thermolyne Furnace 30400) at three different temperatures (350, 500, and 900 ^{o}C) and at a residence time of 3 h. The characterisation and properties of the biochar are presented in Table S1.

2.3. Remediation microcosm and experimental design

The clean pasture soil was artificially contaminated by spiking with diesel (Caltex, Melbourne, Australia) at 2% (w/w). The soil was left in a fume hood for 12 h. Aliquots (150 g) of the diesel-contaminated soil were dispensed into microcosms (L1-L10), while clean soil (150 g) without any diesel contaminant or amendment (biochar or fertiliser) was dispensed in microcosm (L11). The fertiliser used was Yates Thrive all-purpose soluble fertiliser (NPK: 25:5:8.8) purchased from Bunnings, Melbourne, Australia. A total of 11 treatments in triplicate were prepared in this study, which comprised of an uncontaminated control (L11), diesel-contaminated control (L10), and nine different biochar treatments (L1-L9) (Table S2). In the various biochar treatments, the biochar pyrolysis temperature (350, 500, and 900 ^{o}C), biochar application dose (2%, 5%, and 10% w/w), and fertiliser application dose (0%, 1%, and 2% w/w) were varied (Table S2). The Taguchi methodology was employed to design the nine different biochar treatments, using the L9 orthogonal array.

All microcosms were maintained at room temperature in a fume

hood for the first day and in an incubator at room temperature for the remaining days (until week 12). The content of the microcosms was mixed manually weekly. On day 0, MilliQ water was added to all treatments, and the moisture content maintained at around 3–10 (wt%) throughout the incubation. Microcosms were sampled at weeks 2, 4, 6, and 12.

2.4. Total petroleum hydrocarbon (TPH) analysis

The assessment of the TPH (C_{10} – C_{40}) concentration of the soil was carried out using a RemScan (Ziltek Pty. Ltd., Australia) [53]. The RemScan uses a diffuse reflectance (mid)-infrared Fourier transform (DRIFT) spectrometry in measuring the soil TPH [51]. The RemScan is a non-extractible TPH assessment device, which has been previously reported to correlate strongly ($R^2 = 0.998$) with the GC MS analysis, and has been previously calibrated using soil used in this study [53]. In a preliminary study, TPH in both contaminated soil and biochar-amended contaminated soil was measured on day 0 with the RemScan. No considerable difference between TPH concentrations in both treatments was recorded confirming the suitability of RemScan to determine TPH concentration in the absence and presence of biochar. Prior to the analysis of TPH in the soil using the RemScan, soil samples (30 g) were air-dried for at least 10 h in a fume hood, ground, and sieved with a 2 mm mesh.

2.5. DNA extraction and quantification of 16 S rRNA and alkB gene

Soils sampled on day 0, week 2, and week 12 were used for molecular analysis in the diesel contaminated treatments (L1-L10), along with day 0 and week 12 samples of the uncontaminated soil (L11). DNA was extracted using the DNeasy PowerSoil PRO Kit (Qiagen, USA) and stored at −20 °C until required. Quantification of bacterial 16 S rRNA and *alk*B gene copies was performed by qPCR using the Qiagen Rotor-Gene machine (Qiagen, USA) [54]. A 20 µL reaction was used, consisting of 10 µL qPCR mastermix (SensiFAST SYBR No-ROX Kit, Bioline), 8.2 µL PCR-grade water, 1 µL DNA template, 0.4 µL forward primer and 0.4 µL reverse primer [54]. The primer used for the 16 S rRNA was 341-F (5′CCTACGGGAGGCAGCAG3′) and 518-R (5′ATTACCGCGGCTG CTGG3′), while alkB-f (5′AAYACIGCICAYGARCTIGGICAYAA3′) and alkB-r (5′GCRTGRTGRTCIGARTGICGYTG3′) was used as *alk*B gene primers [43,55] (see Text S2).

2.6. Ecotoxicological testing

Ecotoxicological assessment was carried out on the diesel-contaminated soil on day 0 and week 12 using the Microtox test. The Microtox test involves the exposure of a luminescent marine bacterium (*Allivibrio fischeri*) to soil aqueous extracts [43]. The acute microtox reagent (*A. fischeri*) was obtained from Streamline Hydro Pty Ltd. (Queensland, Australia) and reconstituted and equilibrated prior to use [43]. The aqueous extract was prepared by adding 1 g of 2 mm sieved soil and 9 mL of MilliQ water in a centrifuge tube. The mixture was agitated in a shaker for 24 h at 140 rpm and manually shaken for 10 min. The sample was centrifuged twice, and the supernatant used for the analysis. The diluent used in this study was 2% NaCl, while a 22% NaCl solution was used to adjust the osmotic pressure [56]. Bacterial luminescence was measured using a Microtox® Model 500 Analyser. The Effective Concentration 50 (EC_{50}) at 15 min, which is the concentration that causes a reduction in 50% of the luminescence at 15 min was calculated using the instrument software [51].

2.7. Statistical and Taguchi analysis

One-way analysis of variance (ANOVA), with the Tukey Post HOC, at $p < 0.05$ was used to assess statistical significance of TPH removal or concentration among treatments in Minitab software (Minitab, US). Correlation analysis was carried out using Microsoft Excel. The analysis of the Taguchi Data was carried out using Minitab Software (Minitab, US). Apart from the orthogonal array, Taguchi utilises the main effect, signal-to-noise (S/N) ratios, and ANOVA [57]. The main effect is used to study the contribution of each factor affecting the response variable, calculated by averaging the response for each level [37,58]. For the S/N ratio, "*the larger is better*" was used since the maximum response value is desirable here. The equation for the S/N ratio is shown in equation (1) [59].

$$S/N = -10\log\left[\frac{1}{n}\sum_{i=1}^{n}\frac{1}{yi^{2}}\right] \quad (1)$$

where, n and yi represent the number of experiments and the nth observations of the response value, respectively [57,59].

The ANOVA, the F-ratio, p-value and percentage contribution can be used to assess the qualitative and quantitative statistical significance [60]. While the F-ratio and p-value only shows if a significance exist (qualitative evaluation), the percentage contribution gives an idea of the quantitative evaluation of the factorial effects of the factors [60]. The percentage contribution demonstrates the relative strength of a factor to decrease variation [61]. A factor with higher percentage contribution will have a larger influence on the response [62]. The percentage contribution was calculated using equation (2) [57].

$$Percentage\quad Contribution\quad (\%) = \left(\frac{Sum\quad of\quad squares}{Total\quad sum\quad of\quad squares} \times 100\right)\% \quad (2)$$

3. Results and discussion

3.1. Effect of biochar on hydrocarbon removal

The removal of petroleum hydrocarbon is shown in Fig. 1. Petroleum hydrocarbon decreased from 16220 mg/kg to 4960–9080 mg/kg after 12 weeks of incubation in all contaminated treatments (Table S3). The removal efficiency at the end of incubation in the diesel-contaminated control (L10) and the different biochar treatments (L1-L9) was 54% and 44–69%, respectively. This showed that in comparison to the diesel-contaminated control, biochar application enhanced or inhibited hydrocarbon removal, depending on the treatment condition. Highest removal (69%) was observed in treatments with biochar produced at 900 °C and applied at 10% together with 1% fertiliser (L9); lowest removal (44%) occurred in treatment with biochar produced at 500 °C applied at 5% with 2% fertiliser (L5) (Table S3). At week 12, the TPH concentration in the best biochar treatment (L9) was lower than the EPA Victoria maximum threshold for Category D waste (5000 mg/kg), which can be reused onsite or in a project site (Fig. 1d) [63,64]. However, the TPH concentration in the control exceeded the maximum threshold for Category D waste at week 12 by 51% (Fig. 1d). Generally, hydrocarbon removal was greater in biochar-amended treatments up to week 6, although not statistically significant in all cases ($p < 0.05$) (Fig. 1a-c, Table S3). During hydrocarbon remediation, the readily degradable hydrocarbons are decomposed rapidly by microbes, leaving behind the recalcitrant fraction which are degraded slowly, if at all [65–67]. In this study most of the readily degradable hydrocarbons in the biochar treatments could have been degraded by week 6. A study with a similar incubation time also observed that biochar enhanced remediation only up to week 6 [68]; similarly reports utilising longer incubation period (180 days) observed that biochar application caused a considerable difference in hydrocarbon removal after the early stage [48,69]. The longer duration of incubation in these studies could have provided more time to degrade the recalcitrant molecules.

Notably, among all biochar treatments, a rapid and significant hydrocarbon removal was observed in L6, L8, and L9 at week 2 ($p < 0.05$) with TPH removal in these treatments higher than the control by 277.88–675.28% (Table S3). The TPH removal in L6, L8, and L9

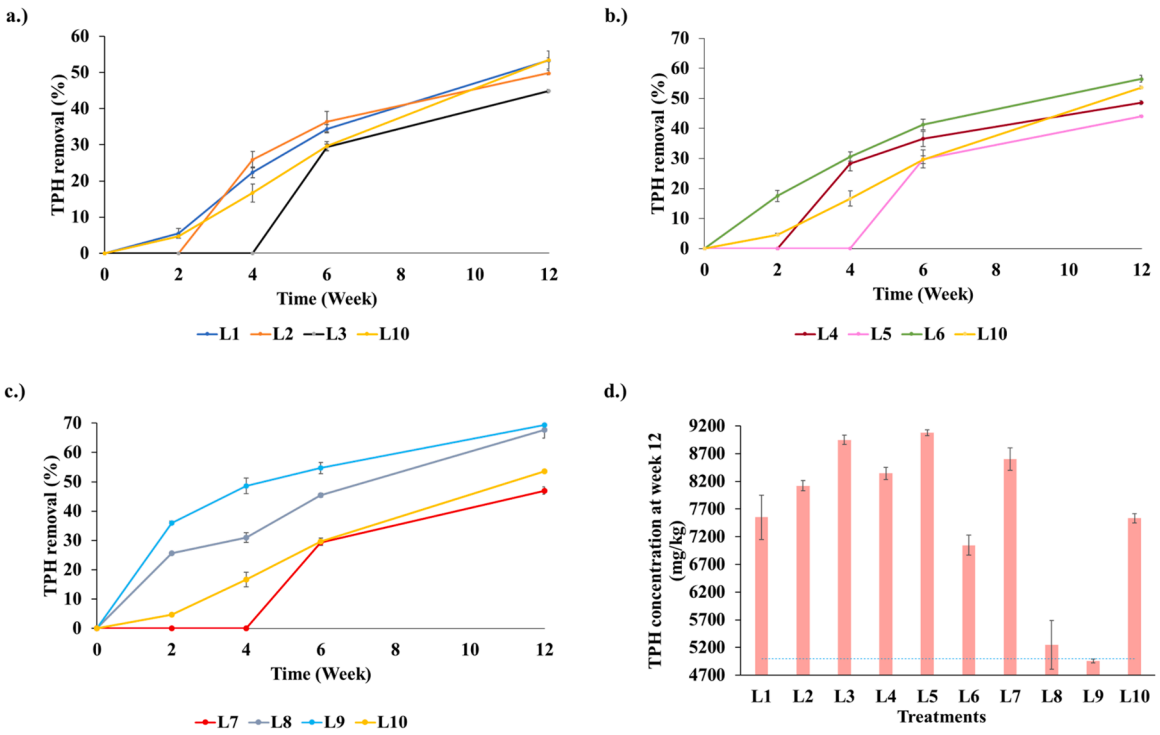


Fig. 1. TPH removal over 12 weeks for a.) L1-L3; b.) L4-L6; c.) L7-L9 in comparison to L10 (control); d.) Residual TPH concentration for treatments L1-L10 at week 12. With exception to week 0 in Figs. 1a-1c, values are the mean of three replicates in the four figures, while the error bar represents the standard deviation of the mean. The blue dotted line in the Fig. 1d represents the maximum TPH threshold value for Category D soil waste (5000 mg/kg), based on EPA Victoria Guidelines. L1: 350/2/0; L2: 350/5/1; L3: 350/10/2; L4: 500/2/1; L5: 500/5/2; L6: 500/10/0; L7: 900/2/2; L8: 900/5/0; L9:900/10/1; L10: Diesel contaminated control; The key in biochar treatments are interpreted as pyrolysis temperature/biochar application dose/fertiliser application dose as per Table S2.

remained significantly higher ($p < 0.05$) than the control up to week 6, but not as high as in week 2. After 12 weeks, only L8 and L9 significantly improved hydrocarbon removal relative to the diesel-contaminated control (Table S3); other biochar treatments resulted in significantly lower or insignificant removal in comparison to the control (L1-L7) (Table S3). This contrasting observation mirrors the inconsistent reports in the literature on biochar efficiency in the remediation of hydrocarbon-contaminated soils. Some authors have found that biochar application resulted in higher hydrocarbon removal, while others observed a lower or insignificant removal in comparison to the control [18,19,22,23,48,68,70–72]. The inconsistences in biochar efficacy in our study is linked to the variation in the biochar production and application conditions. The biochar in L8 and L9 treatments was produced at a higher temperature (900 °C) and applied at higher doses (5% and 10%) in conjunction with lower or no supplementary fertiliser (0–1%). The biochar used in these two treatments (L8 and L9) was rich in ash (mineral nutrients) and possessed a higher capacity to support microbes, while the low or no fertiliser application resulted in soil with a higher C/N ratio (Tables S1 and S4). This resulted in higher hydrocarbon removal in L8 and L9 compared to other biochar treatments (L1-L7). Previous reports have also linked the ineffectiveness of biochar with the properties of biochar [20,73,74]. García-Delgado et al. [74] reported that the application of biochar with a very high C/N ratio, composed of largely aromatic carbon with low biodegradability resulted in low bacterial and fungal development.

Interestingly, there were some anomalous findings in week 2 and 4, worthy of discussion. The residual hydrocarbon concentration in all biochar treatment co-applied with fertiliser was higher at week 2 compared to day 0, except for L9 (Table S3). At week 4, the hydrocarbon concentration remained higher than the starting TPH concentration only in biochar treatments co-applied with 2% fertiliser dose (L3, L5 and L7). This increase in hydrocarbon concentration compared to the beginning is consistent with other biochar-related remediation studies [19,75]. One report suggested that the splitting of some hydrocarbon chains fraction to chains with smaller carbon numbers, soil matrix retention and non-homogeneity of the samples was responsible for this abnormal observation [19]. The biochar treatments in our study where these anomalies were observed have a common denominator, which is low C/N ratio (Table S4). Previous studies have found these anomalies in biochar treatments co-applied with nutrient source [19,75]. Further studies are required to explore the linkage between the low C/N ratio and increase in hydrocarbon concentration.

3.2. *Optimum condition and regression model for hydrocarbon removal in biochar amended soil*

The optimum condition for each factor was at the level with the maximum S/N ratio for each factor [76]. Thus, optimum removal can be achieved by applying biochar produced at 900 °C and applied at 10% (w/w) without any fertiliser (Table S5). At this condition, the TPH removal after 12 weeks was predicted to be 70.53%.

The results of this study were modelled through regression as shown in Eq. (3). The model had an R^2 of 88.92%, which indicates that the studied factors contributed 89% of the variation in the TPH removal efficiency [37]. The ANOVA for the regression model showed that fertiliser application dose and biochar pyrolysis temperature was significant at $p < 0.05$, while the biochar application dose was not significant (Table S6).

$$Average\ \ TPH\ \ removal\ \ (\%) = 41.90 + 0.02325x + 0.881y - 6.97z \quad (3)$$

where, x, y, and z are biochar pyrolysis temperature (°C), biochar application dose (% w/w), and fertiliser application dose (% w/w), respectively.

3.3. Influence of treatment/process condition on biochar efficacy in TPH removal

Taguchi analysis confirmed that the studied factors influenced hydrocarbon removal (Fig. 2), with fertiliser application dose being the most important, followed by biochar pyrolysis temperature and biochar application dose (Ranking in Table S5). The ANOVA table showed that only the fertiliser application dose and pyrolysis temperature was significant at $p < 0.1$ (Table S7). The percentage contribution demonstrates the relative sensitivity of a parameter to change [77] and can be used to explain the quantitative statistical relevance of these factors (Table S7). The ranking implies that a small variation in the fertiliser application dose can greatly reduce hydrocarbon removal relative to similar variation in other factors [78]. The negative impact of fertiliser application in this study is likely connected to their role in influencing the soil C/N ratio, a key parameter in soil remediation. The results of the correlation analysis between the calculated C/N ratio at the beginning and the hydrocarbon removal at the end further provides support for the importance.

3.3.1. Influence of biochar pyrolysis temperature on hydrocarbon removal

The removal of TPH increased as the biochar pyrolysis temperature increased, with a mean response of 49.40%, 49.71%, and 61.35% observed at biochar pyrolysis temperature of 350, 500, and 900 °C, respectively (Fig. 2). Increasing the pyrolysis temperature increased the ash content, surface area, and pore volume of the biochar; whereas increasing the temperature generally decreased the labile carbon ($\frac{Volatile\ \ matter}{Volatile\ \ matter+Fixed\ \ Carbon}$, O/C, and H/C ratio) of biochar (Table S1). For example, increasing the temperature from 350 to 900 °C increased the surface area from 2.25 to 108.75 m^2/g; similarly, the ash content increased from 40.72% to 63.60%. In contrast, the O/C and H/C ratio decreased by 34% and 81%, respectively from 350 to 900 °C. These changes in biochar properties with pyrolysis temperature may be responsible for the influence of biochar pyrolysis temperature on hydrocarbon removal. Ash content is an indication of the inorganic nutrient status of biochar and has been reported to correlate negatively with residual PAH concentration ($p < 0.05$ for PAHs with 3–6 rings, except for PHE) [27]. Surface area and pore volume play a role in soil aeration, nutrient retention, water retention, as well as providing a means of colonisation and habitat for soil microbes [79,80]. This could in turn influence soil microbes. For example, the relative abundance of petroleum degrading microbial communities at the genus level in a previous study increased with biochar pyrolysis temperature [49]. Furthermore, the labile C in biochar represents the amount of C readily available to soil microbes and this can determine if the soil microbes will depend on the hydrocarbon contaminant in the soil as a major C substrate [81,82].

The observation that the hydrocarbon removal increased with biochar pyrolysis temperature is consistent with other studies [25,27]. However, other authors observed that a decrease or irregularity or no significant effect on hydrocarbon remediation with pyrolysis temperature [25,27,49,83]. In another study, the biodegradation ratio of phenanthrene decreased with pyrolysis temperature in the presence of low-temperature biochar (100–400 °C) and increased with temperature in the presence of high-temperature biochar (500–700 °C) [84].

3.3.2. Influence of biochar application dose on hydrocarbon removal

The results of the study showed that the mean TPH removal increased as the biochar application dose increased (Fig. 2). The mean TPH removal increased from 49.65% to 53.87% when the biochar dose increased from 2% to 5%. A further increase of biochar dose to 10% increased the mean TPH removal to 56.94%. This increase with biochar dose could be related to the availability of more nutrients to soil microbes, and/or more sites for microbial colonisation, water retention, and nutrient retention. This increase of hydrocarbon removal with increasing biochar application dose is generally consistent with previous reports [22–24]; however, Song et al. [85] reported that the PAH removal decreased with application dose in soil amended with biochar produced at 600 °C.

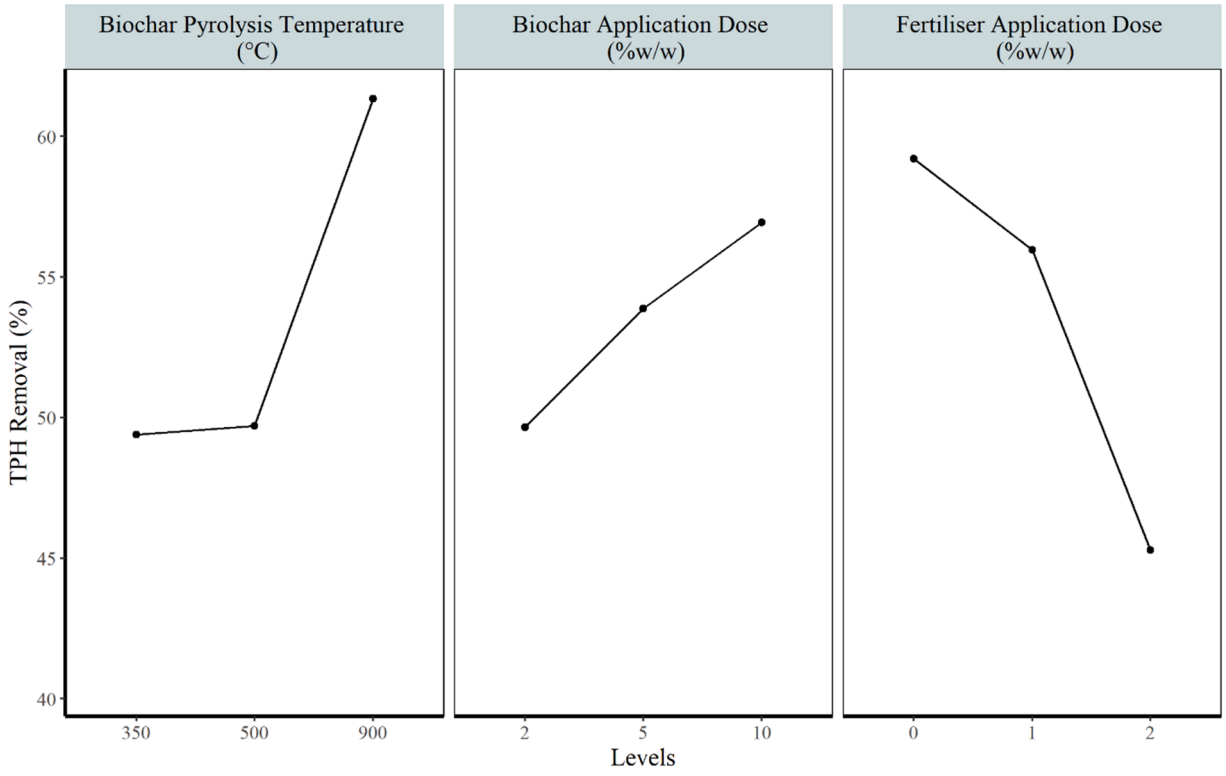


Fig. 2. Plot of the mean effect of the studied factors.

3.3.3. Influence of the addition of fertiliser on hydrocarbon removal

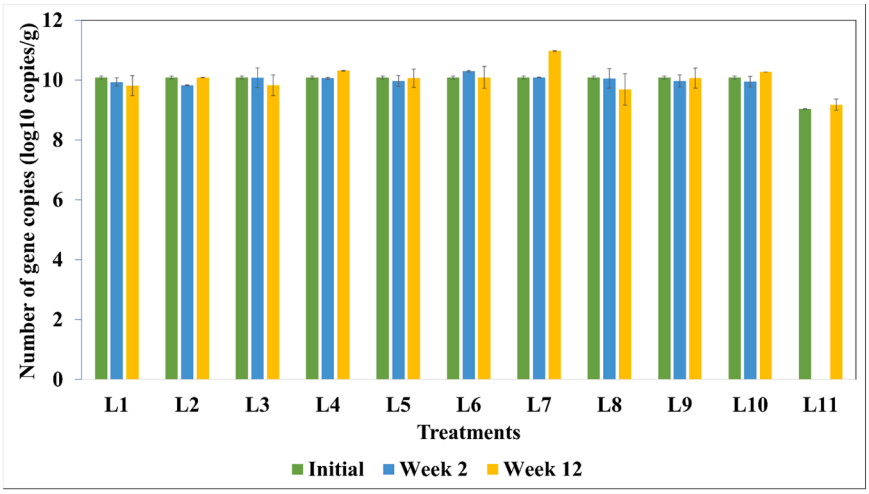
In this study, the mean response of TPH removal decreased from 59.21% to 55.97%, following an increase in the fertiliser application dose from 0% to 1% (w/w) (Fig. 2, Table S5). An increase in the fertiliser application dose to 2% (w/w) decreased the mean response of TPH to 45.28%, demonstrating that co-applying biochar with fertiliser reduces hydrocarbon removal, especially at high fertiliser application doses. This decrease may be due to ammonia toxicity to soil microbes that resulted from the low C/N ratio and excess nitrogen in soils amended with fertiliser (Table S4) [86,87]. The findings of our study on the effect of co-applying biochar with fertiliser on the remediation of hydrocarbon-contaminated soil conflict with the results of previous studies [18,26]. In these previous studies, biochar was derived from bulrush straw and sugarcane residue. Compared to these feedstocks, biosolids-derived biochar has a lower C and higher N content, which is due to the properties of the parent feedstock material (biosolids); therefore, applying biochar without fertiliser will not cause a shift in the C/N ratio, relative to the use of biochar derived from bulrush straw and sugarcane residue. However, in another study involving wood brick biochar and fertiliser in four different C/N/P ratios, no considerable difference in hydrocarbon removal was observed between the sole biochar treatment (3.17%) and the different biochar and fertiliser co-applied treatment (2.27–3.72%) [88].

3.4. Bacterial and alkane degrading gene quantification

The copy number of 16 S rRNA and the alkane degrading (*alk*B) gene in the various treatments at different times were assessed (Fig. 3). The copy number of 16 S rRNA genes at week 2 and week 12 did not differ considerably from day 0 in most cases (Fig. 3a). The general lack of a considerable change in the gene copy number at week 2 and 12 from day 0 suggests that the remediation process did not negatively impact the total bacterial population. Furthermore, the total bacteria population did not vary considerably in all biochar treatments (L1-L9) from the diesel-contaminated control (L10). This is similar to a previous observation that reported that biochar did not cause a change in gene copy numbers [68]. However, other studies found that the gene copies were significantly higher in biochar treatments than the control in PAH-contaminated soil [25,89]. The effect of biochar cannot be generalised since the properties of biochar, soil initial physicochemical, and microbial community structure vary from study to study and this variation can lead to differences in biochar effect [68].

Fig. 3b shows that the copy numbers of *alk*B gene at different time points. The gene copy number increased by at least 8% in all treatments without fertiliser (L1, L6, and L8) at week 2 compared to day 0, while a decrease or no change was observed in all biochar treatments co-applied with fertiliser (L2-L5, L7, and L9). This decrease in L2-L5, and L7 correlated with an absence of hydrocarbon degradation in these treatments at week 2, while the increase observed in L1, L6, and L8 correlate

a.)



b.)

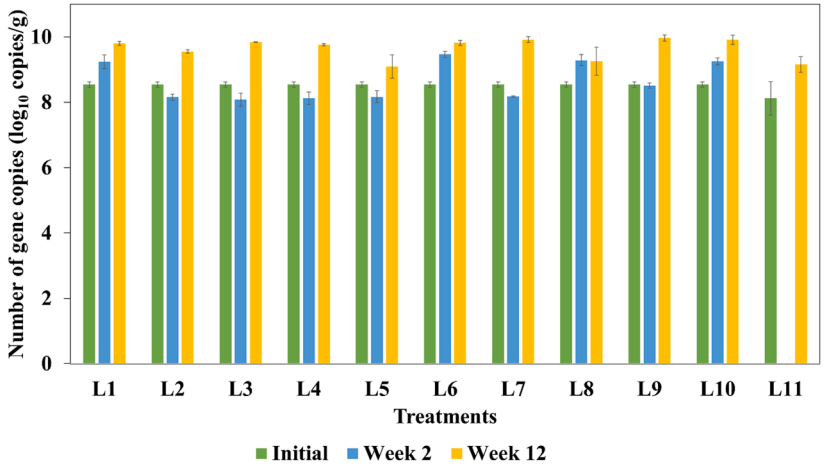


Fig. 3. Time Variation in the number of gene copies of a.) 16 S rRNA and b.) *alk*B in $\log_{10}$ gene copies/g soil for the different treatments. Values are the mean of at least two replicates, while the error bar denotes the standard deviation of the mean. L1: 350/2/0; L2: 350/5/1; L3: 350/10/2; L4: 500/2/1; L5: 500/5/2; L6: 500/10/0; L7: 900/2/2; L8: 900/5/0; L9: 900/10/1; L10: Diesel Contaminated control; L11: Uncontaminated control. The key in biochar treatments is interpreted as pyrolysis temperature/biochar application dose/fertiliser application dose as per Table S2.

with the presence of hydrocarbon degradation at this time point (Fig. 1). The number of gene copies increased by > 10% in all biochar treatments at week 12, relative to day 0, except in L5 and L8, where less than 10% increase was observed. (Fig. 3b). The removal of TPH correlated strongly with the copy number of *alk*B gene (r = 0.78, n = 20), confirming a strong relationship between hydrocarbon removal and alkane-degrading population. This showed that alkane degradation took place during the remediation and contributed to the decrease in the residual hydrocarbon concentration. Other authors have also observed a positive correlation between hydrocarbon removal and the copy numbers of this gene [90,91]. Furthermore, the *alk*B gene copy numbers at week 12 in biochar treatments did not differ from the control in almost all biochar treatments. This is consistent with the reports of other authors who did not observe a significant difference in biochar or other biostimulating biomass compared to the control [43,92]. Gene copies were lower in L5 than the diesel-contaminated control at week 12; a previous study observed lower gene copies in the biochar treatment relative to the control [92]. The lower number of gene copies in treatment L5 at week 12 in comparison to the control and the minimal increase at week 12 with time (relative to day 0) observed in L5 could be due to lower hydrocarbon degradation (Figs. 1, 3b).

3.5. Ecotoxicological assessment

The Microtox test was used to assess soil ecotoxicity before and after remediation (Fig. 4). The EC_{50} increased substantially by at least 148% in all diesel-contaminated treatments after 12 weeks of incubation, confirming that the soil toxicity decreased after remediation, with the highest and lowest toxicity at week 12 found in L1 and L3, respectively (Fig. 4). There was no relationship between residual hydrocarbon concentration and the EC_{50} at week 12, which means that soil toxicity was not directly a reflection of the hydrocarbon concentration in the soil. Delille et al. [93] speculated that the degradation of less toxic compounds occurs first, while a large part of toxic residues remained for a longer time in the soil. Other reasons include the changes in hydrocarbon bioavailability during bioremediation and the complex interaction between the soil and the contaminants [94]. The presence of biochar cannot be ignored since biochar could change the fate of contaminants in the soil. Additionally, intermediate metabolites such as fatty acids and aldehydes are more hydrophilic than hydrocarbons and since the toxicity assay used in our study involved aqueous extraction, the intermediate metabolites will be more readily extracted than the hydrocarbon [95,96]. Previous studies have shown that ecotoxicity was not a reflection of the hydrocarbon concentration [51,94,97].

The EC_{50} in almost all the biochar treatments was higher than the diesel-contaminated control after 12 weeks of incubation, confirming that biochar application reduced the toxicity of the soil relative to the control (Fig. 4). The reduced toxicity following biochar application can be attributed to the immobilisation of soil contaminants [98]. Qin et al. [69] demonstrated that biochar can sorb the toxic intermediates as lower toxicity was observed when biochar was applied at day 80, relative to day 0. The decrease in toxicity with the increase in application dose/pyrolysis temperature observed in our study supports the fact that immobilisation via sorption was responsible for the reduced toxicity in biochar treatments (Fig. S2). This is due to the increase in the pore volume and amount of immobilisation sites with increasing pyrolysis temperature and application dose, respectively (Table S1). Previous reports have found that biochar application was both beneficial [69,85] and detrimental [74] in terms of soil toxicity, relative to non-biochar treatments. Among the biochar treatments in this study, the EC_{50} was influenced by all factors examined in our study, with fertiliser application dose being the most important, followed by the biochar application dose and pyrolysis temperature (Figs. S2, S3 and Table S8).

4. Conclusion

This study focused on investigating the effects of biosolids-derived biochar on the remediation and ecotoxicity of diesel-contaminated soil, as well as the influence of biochar pyrolysis temperature, application dose and fertiliser dose on bioremediation efficacy of biochar. The effect of these factors on the efficacy of biosolids-derived biochar was assessed using the Taguchi method. This represents the first study to examine the efficacy of biochar to remediate a petroleum hydrocarbon-impacted Australian soil. Additionally, this is the first to assess the impact of pyrolysis temperature, biochar application dose and fertiliser addition on the efficacy of biosolids-derived biochar to remediate diesel-contaminated soil. The study showed that generally, the addition of biochar enhanced the removal of petroleum hydrocarbons from contaminated soils. At the end of incubation (12 weeks), the TPH concentration in the best biochar treatment was lower than the EPA Victoria maximum threshold for Category D waste (5000 mg/kg); in contrast the non-amended control exceeded this threshold by 51%. Soil ecotoxicity was also generally lower in most of the biochar treatments than the control. Taguchi analysis showed that biochar pyrolysis temperature, biochar application dose and fertiliser dose affected hydrocarbon removal. Correlation analysis revealed that hydrocarbon removal was related to the number of *alk*B gene copies confirming the role of alkane degrading microbes in the bioremediation process. Overall, this study confirms the potential of biosolids derived biochar in enhancing bioremediation and reducing soil toxicity. This study further demonstrates the need for the biosolids-derived biochar production and application conditions to be selected carefully. Further work, examining the

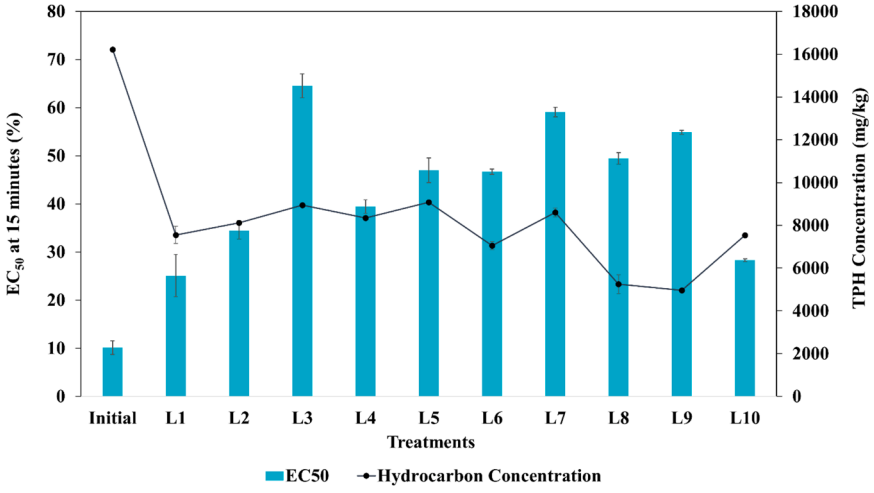


Fig. 4. EC_{50} at 15 min versus TPH concentration in the initial contaminated soil at day 0 and various treatments (L1-L10) at week 12. Values of the EC_{50} and TPH concentration are the mean of at least two replicates, while the error bar denotes the standard deviation of the mean. L1: 350/2/0; L2: 350/5/1; L3: 350/10/2; L4: 500/2/1; L5: 500/5/2; L6: 500/10/0; L7: 900/2/2; L8: 900/5/0; L9: 900/10/1; L10: Diesel Contaminated control. The key in biochar treatments is interpreted as pyrolysis temperature/biochar application dose/fertiliser application dose as per Table S2.

influence of soil type and environmental conditions will provide further insights into the potential application of biosolids-derived biochar for the remediation of petroleum hydrocarbon-contaminated soils. Future studies will focus on providing a mechanistic understanding on how biosolids derived biochar enhance hydrocarbon remediation.

CRediT authorship contribution statement

Charles Chinyere Dike: Conceptualization, Formal analysis, Methodology, Investigation, Writing – original draft and acquisition, Visualization, Writing – review & editing. **Leadin S. Khudur**: Methodology, Investigation, Writing – review & editing. **Ibrahim Gbolahan Hakeem**: Methodology, Investigation, Writing – review & editing. **Alka Rani**: Investigation, Writing – review & editing. **Esmaeil Shahsavari**: Conceptualization, Methodology, Writing – review. **Aravind Surapaneni**: Supervision, Writing – review & editing. **Kalpit Shah**: Supervision, Writing – review & editing. **Andrew S. Ball**: Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2022.108633.

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