SUPPLEMENTARY MATERIAL

Impact of biosolids-derived biochar on the remediation and ecotoxicity of diesel-impacted soil

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Text S1: Soil Physicochemical Properties

The physicochemical properties of the soil, namely, organic carbon, inorganic carbon, pH, electrical conductivity (EC), phosphate (PO_4), and total N (TN), were determined at the beginning (week 0) and the end of incubation (week 24). Organic and inorganic C was assessed using RemScan technology (Ziltek, South Australia, Australia) as described in Section 2.4. Soil (1 g) and Milli-Q water (20 ml) were agitated at 150 rpm for 90 min, followed by centrifugation at 9500 rpm for 5 min before analysis of other properties (pH, PO_4, EC, and TN). pH and EC were determined using a pH meter (Hanna Instruments, Rhode Island, USA) and a LAQUAtwin-EC-11 compact conductivity meter (Horiba Scientific, Kyoto, Japan), respectively. Total N and PO_4 were determined using the persulfate and acid persulfate digestion methods of the Hach reagent kit, respectively (Hach, Colorado, USA).

Text S2: Quantification of total and alkane-degrading bacteria

The primers used for the 16S rRNA gene were 341-F (5′CCTACGGGAGGCAGCAG3′) and 518-R (5′ ATTACCGCGGCTGCTGG3′) [1], while *alkB*-f (5′AAYACIGCICAYGARCTIGGICAYAA3′) and *alkB*-r (5′ GCRTGRTGRTCIGARTGICGYTG3′) were used for the *alkB* gene [2]. The 16S rRNA and alk*B* genes were amplified using the cycling conditions previously described in [3] and [4], respectively. Serial dilutions of cleaned PCR products for both genes, within 10^{-1} to 10^{-6} were used to prepare a standard curve [5]. Copy numbers were generated by correlating the Cycle Threshold value to the prepared standard curve [5]. Gene copies were reported as $\log_{10}$ gene numbers g^{-1} dry soil [6].

Text S3: Ecotoxicity Testing

To prepare the aqueous extract of contaminated soil, a mixture of 1 g of 2 mm sieved air-dried soil and 9 ml of Milli-Q water was agitated in a shaker for 24 h at 140 rpm, followed by manual mixing [7]. The mixture was centrifuged twice to obtain a supernatant, which was used as the aqueous extract. The osmotic pressure was adjusted using a 22% NaCl solution, while 2% NaCl was used as the diluent [4]. The luminescence of the bacteria following exposure to the aqueous extract was measured using a Microtox® Model 500 Analyser (Modern Water Inc., Delaware,

USA). The effective concentration 50 (EC_{50}) at 15 min was calculated and used for the ecotoxicity assessment.

Text S4: Kinetic Analysis

Kinetic graph and correlation analysis was carried out using Microsoft Excel (Microsoft, Washington, USA).

The bioremediation of hydrocarbon was fitted with First-order kinetics [8]. The equation is stated in (1)

$$C_t = C_0 . \exp(-kt) \tag{1}$$

Where C_t is the concentration at the time t (mg/kg), C_0 is the initial concentration (mg/kg), k is the first-order kinetic constant (day^{-1}), and t is the time (day) [8].

The half-life (DT_{50}) was calculated using (2) [9]

$$DT_{50} = \ln 2 / k \tag{2}$$

Where k is the first-order kinetic constant (day^{-1}).

Table S1: Properties of the biosolids-derived biochar used in this study

Properties	Biochar
Surface area (m^2/g)[a]	65.6
Total pore volume (cm^3/g)[a]	0.071
Average pore diameter (nm)[a]	3.82
pH	10.28 ± 0.17
Electrical conductivity (µS/cm)	606.00 ± 15.04
Proximate analysis (wt% d.b)	
Moisture content (%)	0.76 ± 0.079
Volatile matter (%)	4.036 ± 0.30
Fixed carbon (%)	13.03 ± 1.67
Ash content (%)	82.18 ± 2.05

With exception to ([a]), values are the mean of replicates, while the error bar represents the standard deviation of the mean.

Table S2: Soil properties before and after remediation

Treatments	PO$_4$ (mg/L)	C/N ratio	pH	Electrical conductivity (µS/cm)
Initial	0.74 ± 0.078	87.52 ± 16.42	7.19 ± 0.087	80.83 ± 3.75
B at week 24	1.06 ± 0.064	49.35 ± 12.62	7.36 ± 0.17	123.33 ± 3.21
BN at week 24	2.86 ± 0.078*	34.16 ± 3.44*	8.79 ± 0.039	193.00 ± 0.87
C at week 24	0.99 ± 0.13	55.86 ± 12.95	7.20 ± 0.025	67.17 ± 2.52

Values are the mean of replicates and the standard deviation of the mean. Initial: Diesel-contaminated soil at week 0; B at week 24: Diesel-contaminated soil + 5% (w/w) biochar at week 24; BN at week 24: Diesel-contaminated soil + 5% (w/w) biochar + 0.2 % NaN$_3$ at week 24; C at week 24: Diesel-contaminated soil (control) at week 24. Asterisk (*) shows the C/N ratio and PO$_4$ differs at week 24 relative to week 0 at $p<0.05$ using one-way ANOVA with Tukey.

Table S3: Time for treatments to achieve the EPA Victoria Fill material maximum concentration (1000 mg/kg), based on the kinetic analysis

	Time (day)	TPH conc at that time (mg/kg)
B	999.61	993.3
BN	799.69	993.3
C	1110.68	993.3

The prediction was done using the Equation of the first-order kinetics $C_t = C_0 \cdot \exp(-kt)$, where C_t is the concentration at the time t (mg/kg), C_0 is the initial concentration (mg/kg), k is the half-life (day^{-1}), and t is the time (day) [8]. 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).
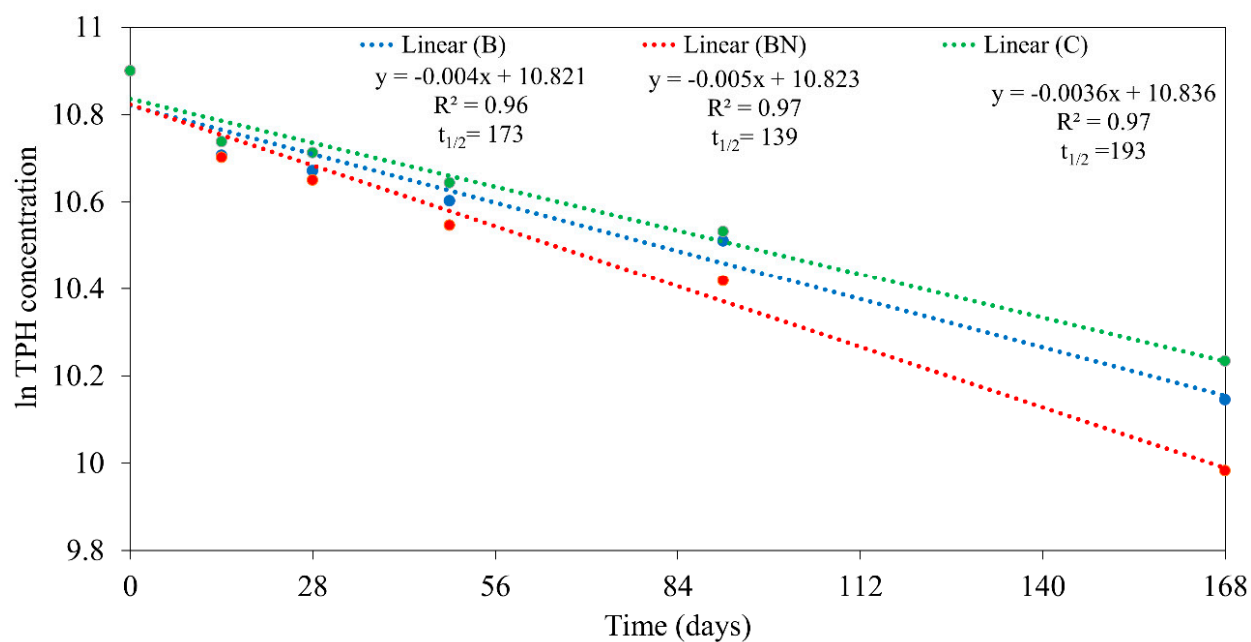
Table S4: Number of genera in B, BN, and C at different sampling times

Treatments	0	2	4	7	13	24
B	281	209	143	132	115	158
BN	281	252	139	111	80	100
C	281	180	153	122	90	123

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).

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 ($t_{1/2}$).

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).
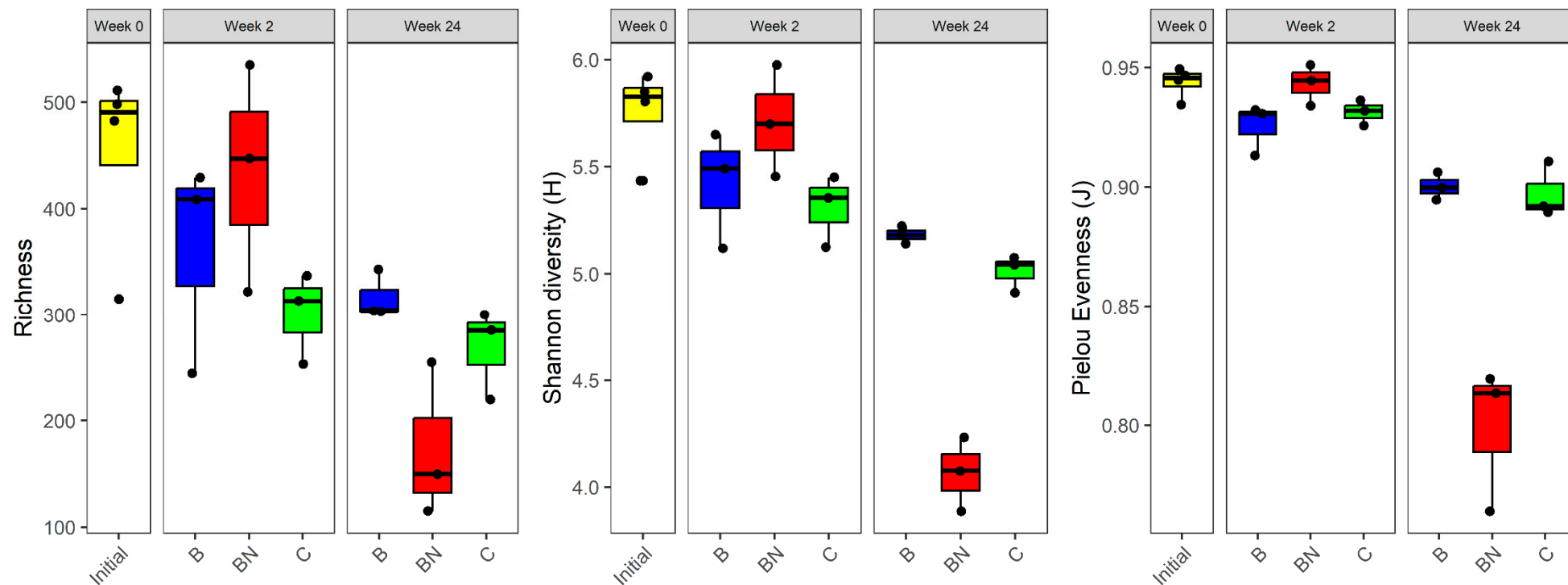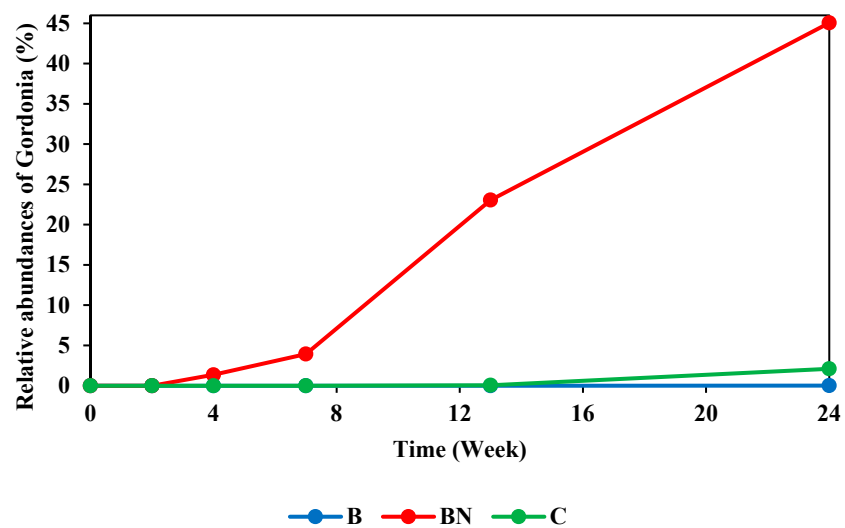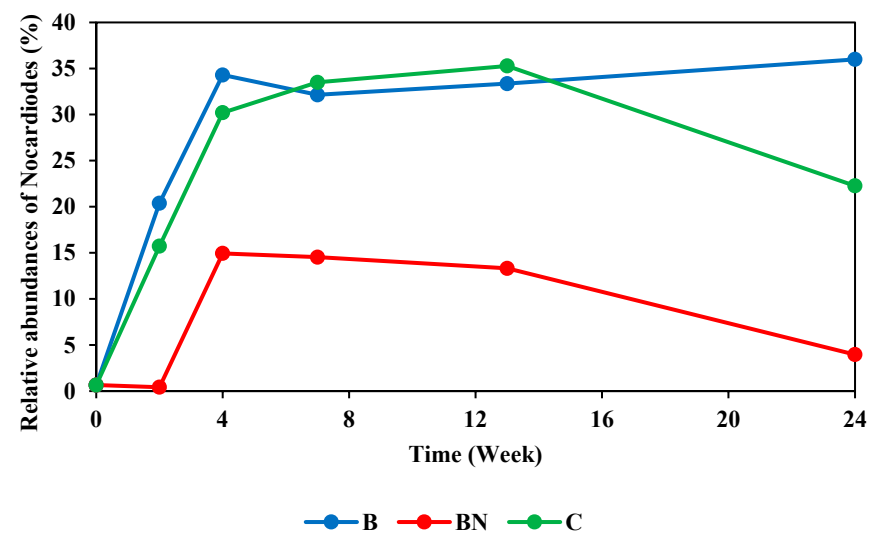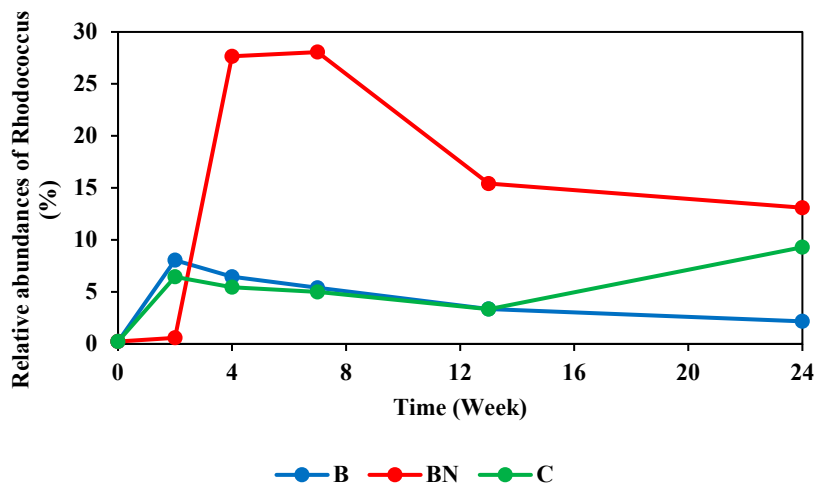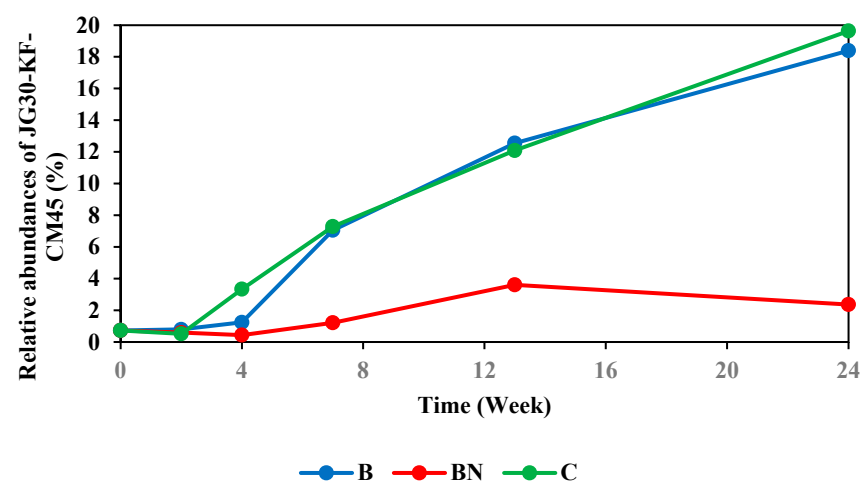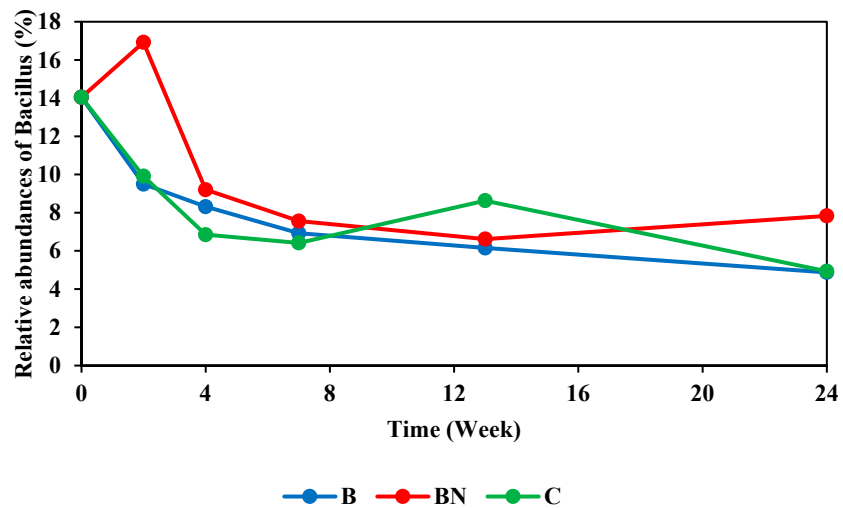
Figure S2: Alpha Diversity (Richness, Shannon diversity, Pielou Evenness) of the contaminated soil at week 0 as well as the different treatments (B, BN, and C) at weeks 2, and 24.

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).
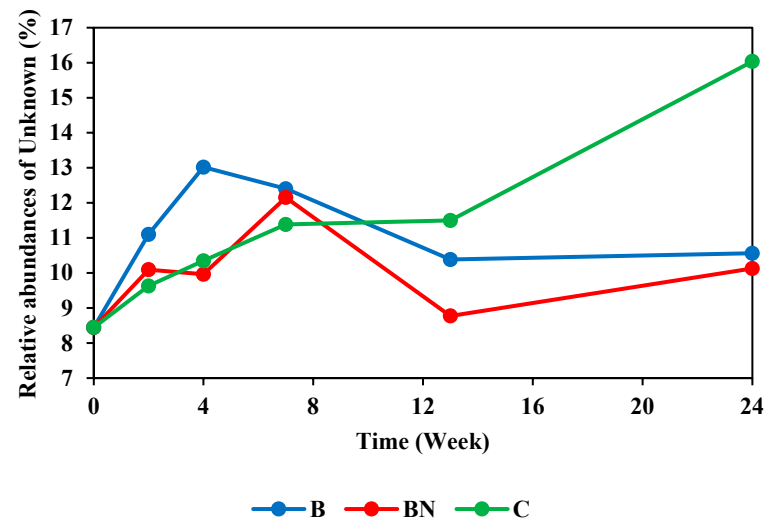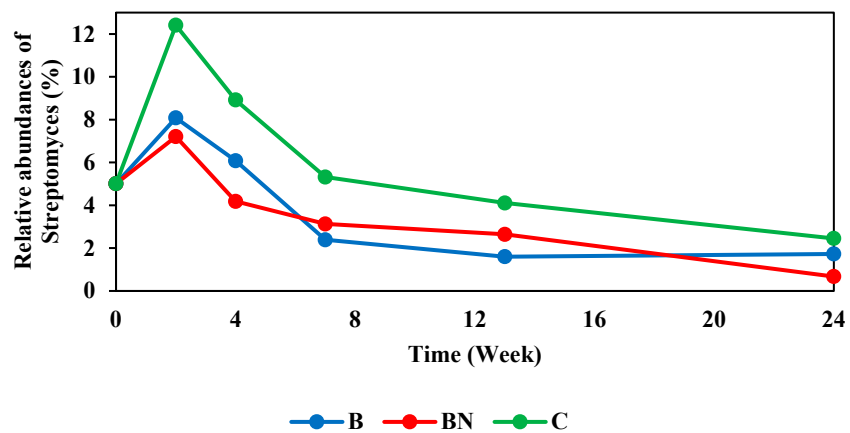
a.)
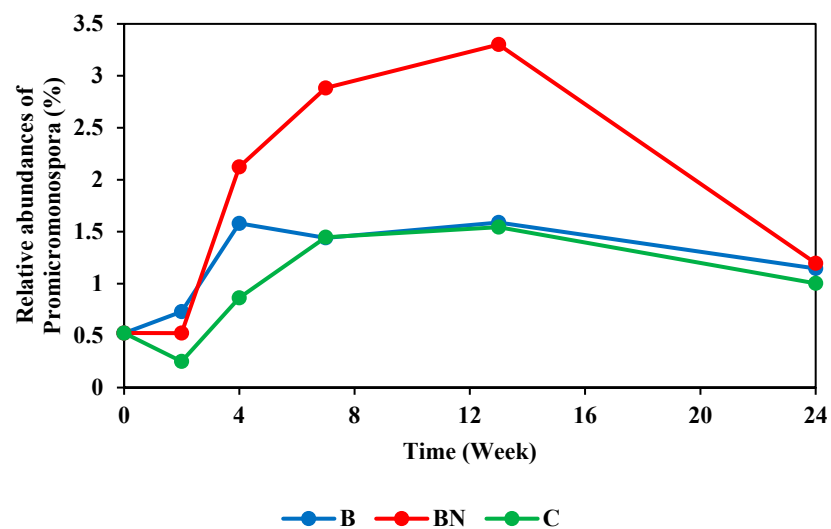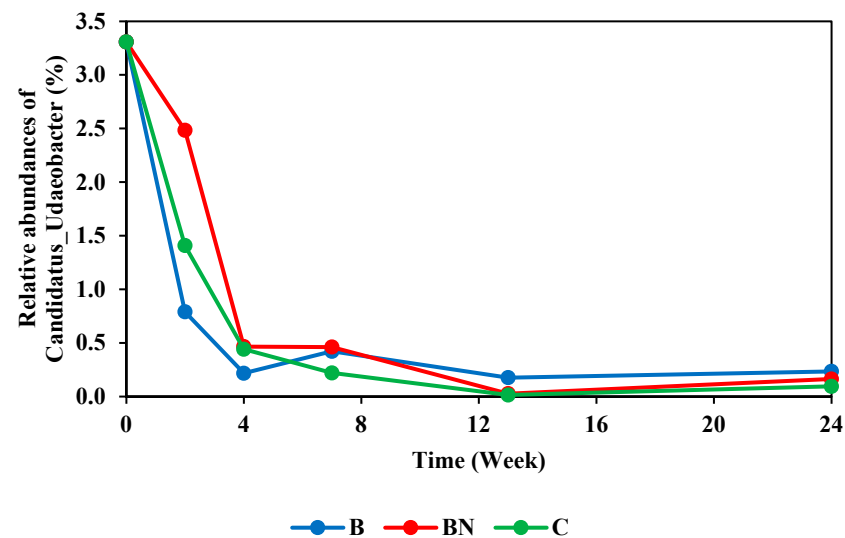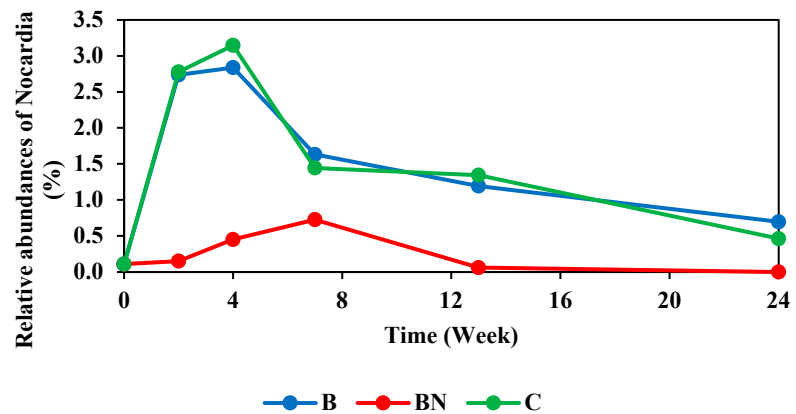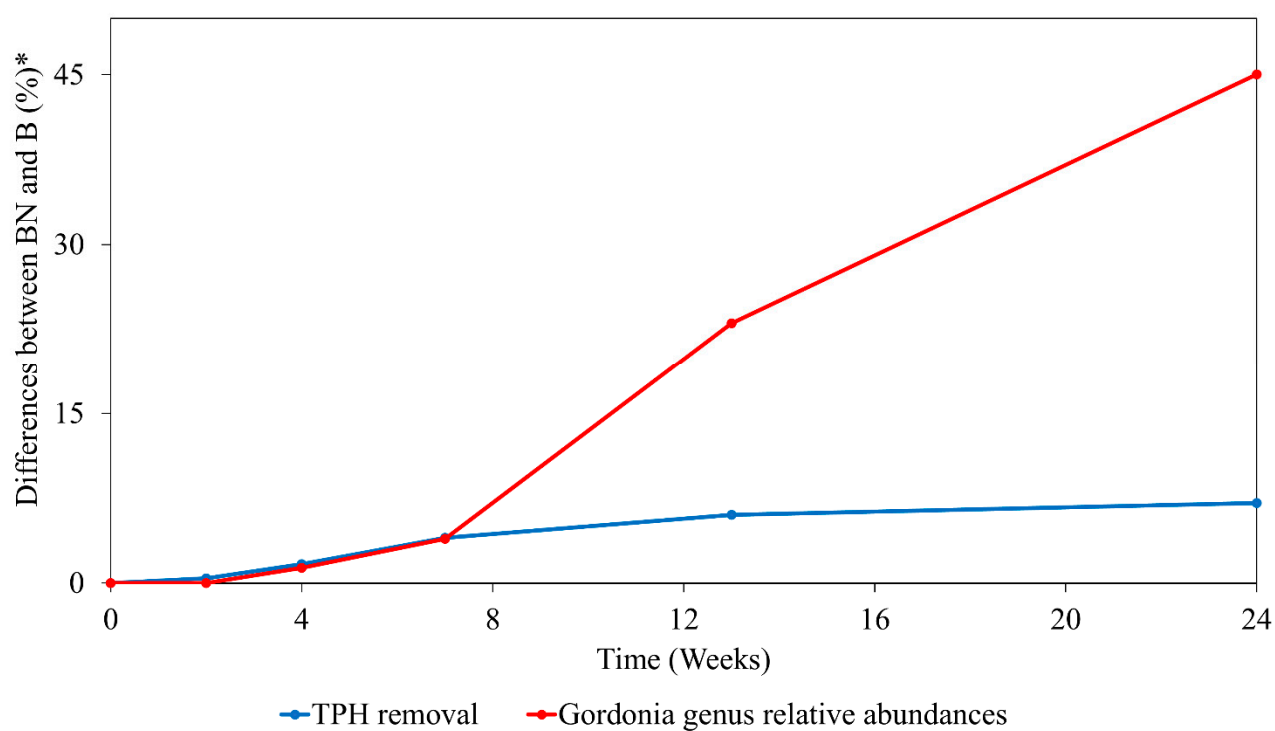
b.)

c.)

d.)

e.)

f.)

g.)

h.)

i.)

j.)

k.)

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.

B: Diesel-contaminated soil + 5 %(w/w)biochar; BN: Diesel-contaminated soil + 5 % (w/w) biochar + 0.2 % NaN3; C: Diesel-contaminated soil (control).
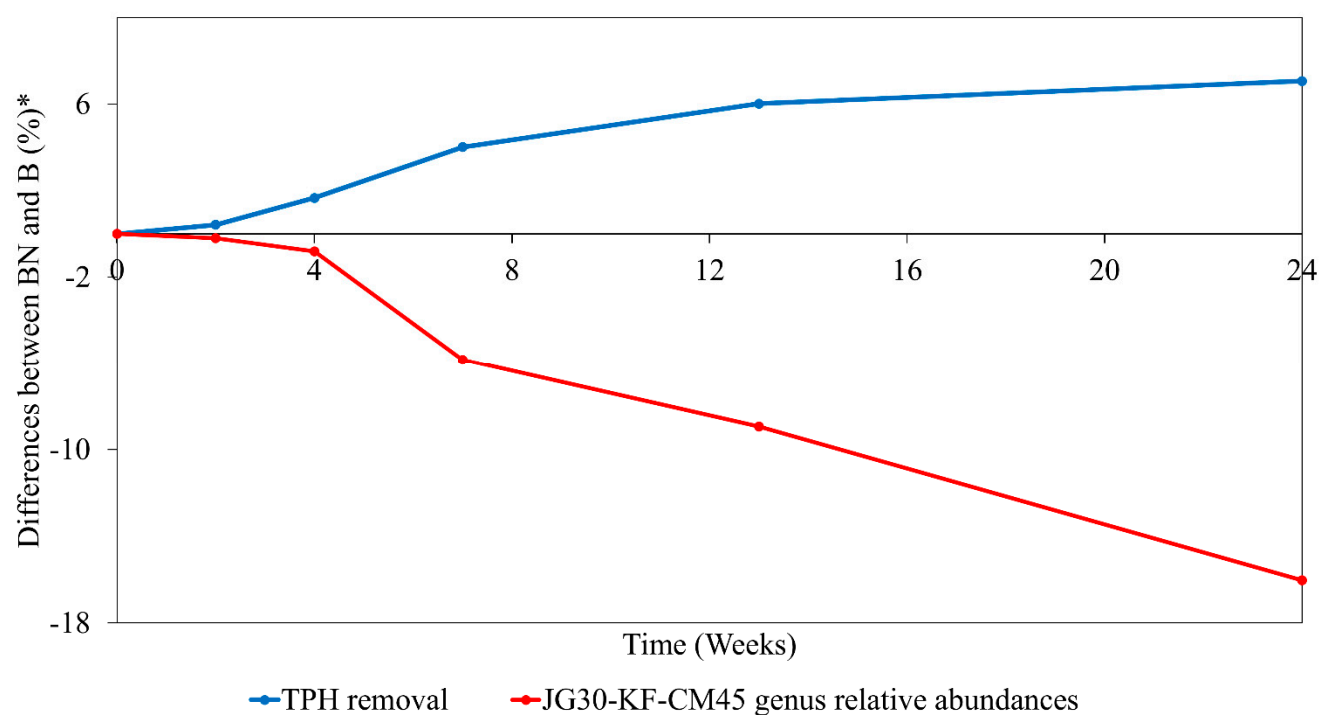
(a.)

(b.)

Figure S4: Differences between Treatment BN and B regarding TPH removal and relative abundances of a.) *Gordonia*, b.) *JG30-KF-CM45* genus.

*: Difference= Relative abundance or percentage hydrocarbon removal of BN - Relative abundance or percentage hydrocarbon removal of B.

References

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