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BIOCHAR-MEDIATED REMEDIATION IMPACTS ON NITROGEN CYCLING BACTERIA AND AMMONIA MONOOXYGENASE ACTIVITY IN CRUDE OIL POLLUTED SOIL

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ABSTRACT

This study adopted an ecosystem services approach to pollution management by investigating the impact of biochar-mediated remediation on soil nitrogen, abundance of nitrogen cycling bacteria and the activity of ammonia monooxygenase (AMO) enzyme in petroleum-polluted soil using two biochar types applied at two treatment levels with monitoring over 15 weeks. The corn cob-derived biochar (CDB), generally, had a stronger restorative effect on soil ammonium nitrogen, nitrate and total organic nitrogen concentrations than the bone-derived biochar (BDB). Both biochar types had a more robust impact on restoration of *Nitrosomonas*, *Nitrobacter* and *Azotobacter* abundance (with the re-establishment of pre-pollution levels) than on *Rhizobium* and *Pseudomonas aeruginosa*. Biochar amendment restored the activity of AMO enzyme in the soil by week 15. The CDB (72.4% – 73.7%) showed more effective total petroleum hydrocarbon (TPH) elimination capacity than the BDB (51.1% – 57.7%). Biochar amendments exhibited great potential for restoration of nitrogen cycling while facilitating remediation of petroleum-polluted soils.

Keywords: Ammonia monooxygenase; Biochar; Ecosystem services approach; Nitrogen cycle; Petroleum; Remediation.

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1. INTRODUCTION

Biodiversity is fundamental to a wholly functional ecosystem. Any environmental stressor that diminishes ecosystem biodiversity will inadvertently impact negatively on ecosystem function and ecosystem services delivery. Microorganisms are the key providers of ecosystem services like decomposition, mineralisation, inorganic nutrient cycling and contaminant removal and are, therefore, invaluable players in any ecosystem [1]. Nutrient cycling is classed as both a regulatory ecosystem service and a supporting service [2,3]. Nitrogen cycling may be considered a vital ecosystem service as it drives primary production in the soil ecosystem. It is the major means by which atmospheric nitrogen is made available to plants and terrestrial organisms [4]. The cycle is the chief regulator of the inorganic nitrogen concentration in soil. Hydrocarbon pollution triggers imbalances in the cycle normally due to changes in the abundance and activities of bacteria concerned with certain stages of the cycle [5]. These imbalances typically bring about over-production of nitrates with resultant leaching into groundwater. Consumption of nitrate tainted groundwater has been linked to stillbirths and cancer risk [6,7]. The production of the greenhouse gases, nitric oxide and nitrous oxide, from soil is also governed by the nitrogen cycle [8].

The autotrophic nitrogen cycle is simplified into three main steps – nitrogen fixation, nitrification and denitrification – driven, for the most part, by specialised groups of soil microorganisms, mainly bacteria. The cycle begins with the transformation of atmospheric nitrogen to ammonia in the soil (nitrogen fixation), the ammonia undergoes nitrification where it is first converted to nitrites during the process of ammonification and then to nitrates via nitrite oxidation. The nitrates are then reduced back to gaseous forms of nitrogen which then evolve from the soil (denitrification). While nitrogen fixation and nitrification are accomplished by highly specialised microbial groups, a wide variety of heterotrophs have the capacity for denitrification so the process is not as impacted by environmental changes as the other stages [9]. Nitrification has been recognised as the most sensitive part of the nitrogen cycle and the stage at which disruption would normally occur in the presence of stressors. The recovery period for this disruption is upwards of 20 years for total recovery in non-aquatic ecosystems. Ammonification is the rate limiting step in the nitrification process and is

catalysed in part by the ammonia monooxygenase enzyme [10]. The microorganisms involved in the process are considered 100 – 1000 times more sensitive to environmental pollution than typical heterotrophic bacteria [5,11]. Furthermore, there is an intricate interrelationship between nitrification and denitrification such that disruption in nitrification may limit nitrogen removal from the ecosystem [12].

Accumulation of nitrogen in the environment presents a problem because while nitrogen is essential for ecosystem function, it becomes a formidable pollutant when its concentration rises above natural ranges. Nitrogen levels in the environment have a strong influence on the viability of other ecosystem services [13], thus, it is a pollutant with the capacity to essentially shutdown the ecosystem. It has been grouped as part of a new class of modern environmental pollutants whose deleterious effects do not result from primary toxicity but from modifications to ecosystem functional dynamics [14]. For these reasons, observation of ammonification potential is normally recommended in the routine monitoring for soil quality [15]. This highlights the significance of ammonification and the ammonia monooxygenase enzyme, AMO responsible for production of the hydroxylamine (NH_2OH) intermediate during ammonification. Additionally, nitrification has been highlighted as a measure of the effectiveness of remediation in edaphic systems and nitrifying bacteria are employed as bio-indicators in ecotoxicity studies [13,16].

Due to the impact of petroleum pollution on the ecosystem and its knock-on effect on human health and safety, an ecosystem services approach to pollution management has been proposed. This novel approach has been adopted in other fields related to climate change, human systems management, ecosystems and biodiversity [3,17]. With the ecosystems services-centric approach, remediation activities will not focus on the removal of the pollutant alone but measure and monitor the impact on ecosystem services to ensure that disrupted or impaired services are reinstated in the process and that the structures responsible for these services are not eliminated; for microorganisms, it would be essential to establish that the relevant species are present and occur at numbers where they can effectively meet ecosystem demands. The MEA [2] stress that nitrogen cycling assessment is integral to any pollution management system design.

Biochar is a carbon-rich solid produced by heating organic materials in the absence of oxygen or at very low oxygen levels. It is characterised by high chemical and biological stability and is loaded with functional groups. It is a material particularly suited to remediation because of its relatively high adsorption, cation exchange capacity and nutrient delivery capacity [18,19]. Biochar is known to improve soil porosity, water holding capacity and fertility and has been hailed as good for climate change because of its propensity for carbon sequestration in soil [18,20]. Biochar was reported to significantly improve the abundance and quality of soil bacteria and fungi in temperate forest soils [21,22]. Ippolito *et al*. [23] maintain that application of biochar restores soil health and boosts yield. Notwithstanding its many advantages, only limited studies employ the exemplary sorbent capabilities of biochar in organic contaminant removal from environmental media, and still fewer studies consider its ability to positively impact on nitrogen cycling bacteria during the remediation process.

This study adopted an ecosystem services approach to remediation by assessing the changes in soil nitrogen levels and the response of selected bacterial drivers of the nitrogen cycle to the application of biochar to petroleum polluted soil to facilitate natural attenuation. The response of the selected nitrogen cycling bacteria was investigated by monitoring variations in their abundance and the activity of ammonia monooxygenase enzyme in the soil during the 15-week study.

2. MATERIALS AND METHODS

Collection of Samples

Sandy loam soil from a farmland in Port Harcourt, Nigeria was used in this study. Samples were collected from up to 15 cm depth at different points using a hand trowel. The soil samples were homogenised and then a 2 mm mesh sieve was used to remove stones and other large particles. Soil samples were collected from six equidistant points within a 3 m^2 area and merged to form composites.

Two types of feedstock were used to produce the biochar used in the study. White corn cobs without the kernel were gathered from traders in the local market while the long bones from cows (White Fulani cattle variant) were obtained from a randomly selected local abattoir in Port Harcourt, Nigeria. Bonny light crude oil was used for the study.

Production of Biochar

The corn cobs and bones were rinsed off to remove impurities, air dried and then heated at 500°C for 2 hours in a muffle furnace (SIOMM, model SXL 1700C, Shanghai, China). The biochar produced was reduced to nanoscale and sterilised in an autoclave at 121 °C for 15 minutes before application.

Morphological Characterisation of the Biochar Samples

The crystalline phases of the samples were determined by X-ray diffraction (XRD) analysis with continuous scanning using a copper anode while the surface microstructure of the biochar sample was observed using a scanning electron microscope (SEM) equipped with an EDX analyser.

For SEM, a known weight of 0.5 g of the biochar sample was coated with an Au/Pd film and the micrograph images obtained using a secondary electron detector on SEM Quanta FEG 450 (APOLLO X – EDX). The pattern obtained from the XRD analysis was interpreted using the standards outlined by Morris *et al*. [24].

Physicochemical characterisation of Biochar Samples

Analysis was replicated and mean values for each parameter determined. The biomass yield was calculated from the biochar samples after cooling using the formula below:

$$\text{Yield (\%)} = \frac{Wf}{Wi} \times 100$$

Where: W_i – Weight of organic biomass (g); W_f – Weight of biochar produced after pyrolysis (g)

The carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) contents of biochar samples were determined by dry combustion using PerkinElmer 2400 CHNS/O analyser (PerkinElmer, Shelton, CT, USA). The oxygen (O) content was calculated by mass difference [25]. The molar H/C, O/C, (O+N)/C and (O+N+S)/C atomic ratios were calculated. The ash content was determined by combusting biochar samples at 550 °C for 6 h in open crucibles on the basis of dry weight. The electrical conductivity (EC) and pH of biochar samples were measured in deionized water at 1:5 solid/solution ratio. About 2 g of the biochar sample was blended with 10 mL deionized water. The suspension was allowed to stand for 30 min before measuring pH and EC using a benchtop combination meter.

The moisture content was determined by heating 2 g of the biochar sample in a hot air oven (DHG-9023A, Hinotek, China) at 105 °C until a constant weight was attained. The water loss (moisture content) was resolved as the difference in weight between the sample before heating and after a constant weight was reached. Samples were cooled in a desiccator before weighing. The modified barium chloride compulsive exchange method was used to determine the cation exchange capacity (CEC) of the biochar samples [26]. The calcium carbonate ($CaCO_3$) content of the samples was estimated from alkalinity levels which were determined using the modified titration method of Yuan *et al*. [27].

Design of the Remediation Experiment

The two types of biochar (based on feedstock type) were used at two different treatment levels. Each laboratory microcosm comprised 1000 g soil spiked with 10 % w/v of Bonny light crude oil and the various treatments as depicted in Table 1.

All treatments were set up in two replicates and incubated at room temperature. Sterile distilled water was added regularly to maintain the moisture content at 60 % water holding capacity. Soil nitrogen content, TPH levels, nitrogen cycling bacterial abundance and ammonia monooxygenase activity via hydroxylamine levels were determined at regular intervals for 15 weeks after a 1 week resting period. Three forms of soil nitrogen were studied – ammonium nitrogen, nitrate and total organic nitrogen.

Table 1. Experimental Setup for Remediation Study

Treatment	Description
Control 1 A (Unpolluted Control)	1 kg Soil alone
Control 1 B (Oiled Control)	1 kg Crude Oil Contaminated Soil alone
Treatment 2 A	1 kg Contaminated Soil + 10 % w/w BDB
Treatment 2 B	1 kg Contaminated Soil + 15 % w/w BDB
Treatment 3 A	1 kg Contaminated Soil + 10 % w/w CDB
Treatment 3 B	1 kg Contaminated Soil + 15 % w/w CDB

BDB – Bone-derived Biochar; CDB – Corn cob-derived Biochar

Determination of Soil Nitrate

The levels of nitrate nitrogen in the soil were determined by the phenol disulphonic acid

method [28].

Determination of Soil Ammonium Nitrogen

Soil ammonium nitrogen concentration was measured by the nesslerisation method [29].

Determination of Soil Total Organic Nitrogen

Total Kjeldahl nitrogen content was established using the Kjeldahl digestion method of Brenmer [30]. The total organic nitrogen was then determined as the difference between total nitrogen and ammonium nitrogen.

Isolation and Enumeration of Selected Nitrogen Cycling Bacteria

The nitrogen cycling bacteria monitored were the nitrogen fixing bacteria, *Rhizobium* sp. and *Azotobacter* sp.; the nitrifying bacteria, *Nitrosomonas* sp. (ammonification) and *Nitrobacter* sp. (nitrite oxidation) and the denitrifier, *Pseudomonas aeruginosa*. *Rhizobium* sp. and *Azotobacter* sp. were isolated using yeast extract mannitol agar and Ashby's medium respectively while Winogradsky medium phase I, Winogradsky medium phase II and cetrimide agar were employed in the isolation of *Nitrosomonas* sp., *Nitrobacter* sp. and *Pseudomonas aeruginosa* respectively.

About 0.5g of soil was added to 50 ml sterile normal saline in an Erlenmeyer flask and shaken thoroughly. The mixture was then serially diluted and 1 ml aliquots of selected dilutions were aseptically plated unto the relevant media using the pour plate technique. The plates were incubated for 48 h at 37 °C for *P. aeruginosa* and at 30 °C for the other test bacteria for up to 7 days. Visible colonies were numbered using an automated digital colony counter (Maya Laboratory Equipment, China) and expressed as colony forming units per gram of soil sample. Only plates with counts between 30 and 300 were considered. Discrete colonies were purified by sub-culturing twice via the streaking plate technique. The pure isolates were then transferred to slants for use in the confirmatory tests.

Confirmatory Tests for Bacterial Isolates

The identities of the nitrogen cycling bacteria isolated using the specialised media were confirmed via cell morphology and cultural and biochemical characteristics as recommended by Holt *et al*. [31] and Cheesbrough [32]. Some of the tests employed to confirm the identities of the isolates: Gram's staining, spore staining, urease production, lysine utilisation, nitrate

reduction, hydrogen sulphide production, citrate utilisation, motility, Methyl Red, Voges Proskauer reaction, ornithine utilisation, gelatine liquefaction, triple sugar iron test, phenylamine deamination, indole production, starch utilisation, catalase reaction, oxidase production and fermentation of simple and complex sugars.

Assay for Ammonia Monooxygenase (AMO) Activity

The variation in hydroxylamine (NH_2OH) concentration with time was considered a measure of the AMO activity. Using the modified assays of Vepsäläinen *et al*. [33] and Šnajdr *et al*. [34], the enzyme content in the soil was extracted using 100 mM phosphate buffer (pH 7.0) containing barium (II). The homogenised mix of soil and the buffer was agitated on an orbital shaker at 100 rpm for 2h. The mixture was centrifuged at 4000 rpm and the supernatant desalted. The hydroxylamine content in the supernatant was measured using the gas chromatography method of Liu *et al.* [35]. The results were standardised with the dry weight (dw) of the soil sample.

Determination of Total Petroleum Hydrocarbons (TPH) Using Gas Chromatography-Flame Ionisation Detection

The total petroleum hydrocarbons content of soil during the study was monitored using a gas chromatograph fitted with a flame ionisation detector (Agilent 6890N, USA) via liquid-liquid extraction method as per Protocol 3560 [36]. The samples were extracted with dichloromethane and eluted using pentane.

Statistical Analysis of Data

All tests were replicated and the data expressed as mean values relative to standard deviation. The relationship between the abundance of the different nitrogen cycling groups and TPH concentration at $\rho \leq 0.05$ was defined using Pearson's Product Moment Correlation co-efficient. Analysis of variance was used to highlight differences in the abundance of the selected nitrogen cycling bacteria from one treatment level to the other at 95% confidence interval. Analysis was done using Microsoft Excel® 2016 and SPSS®.

3. RESULTS AND DISCUSSION

Morphological and Physicochemical Qualities of the Biochar Sample

The x-ray diffraction pattern of the bone derived biochar sample is shown in Figure 1 while

the scanning electron micrographs of the two types of biochar used are shown in Plate 1. The strongest peak was seen at 32.13° 2ɵ. The intensity of the strongest peak was at 697 counts/s and the biochar was confirmed to be polycrystalline, largely amorphous and carbon-rich. The surface of the biochar was rough and somewhat porous which would ideally provide suitable surface area for adsorption of the hydrocarbon pollutant and attachment of degrading microorganisms.

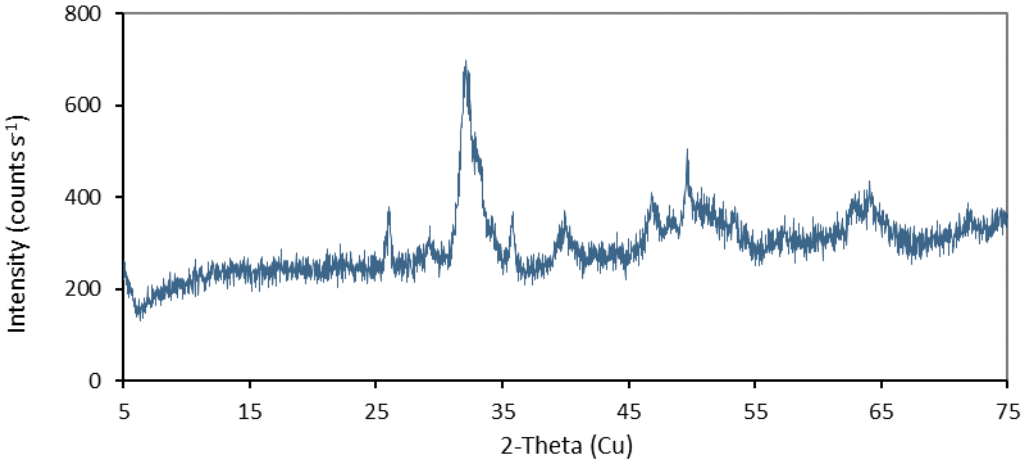
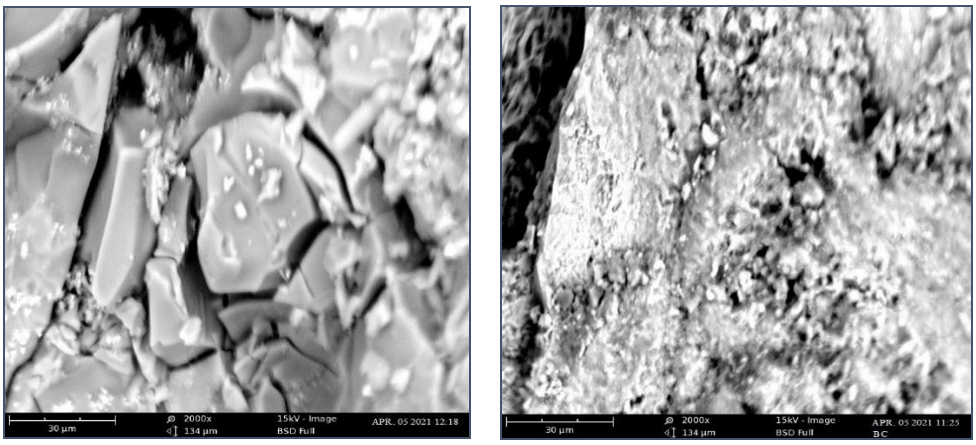


Fig.1. X-ray diffraction pattern for the biochar (BDB) sample showing peaks



(a) **(b)**

Plate 1. Scanning electron micrographs of biochar obtained from cow bones (a) and corn cobs (b)

Based on the physicochemical characteristics summarised in Table 1, both types of biochar were stable as they had organic carbon contents of over 65 %, hydrogen to organic carbon ratios of below 0.7 and oxygen to carbon ratios of less than 0.4 in accordance with stipulated international standards [37]. They also fulfilled the outlined requirements for pyrogenic matter

to be termed biochar [38,39].

Studies show that feedstock yield decreases with increasing pyrolysis temperature and the yield at temperatures of 450 °C and above is usually around 18 % – 25 % though, akin to this present study, values of up to 35 % and over have been reported [40–42]. The calcium carbonate ($CaCO_3$) content obtained indicated that the bone derived biochar (BDB) had better liming potential than the corn cob derived biochar (CDB). As found in the current study, the pH levels for biochar obtained by other studies ranged from 7.0 to 10.5 while the reported cation exchange capacities (CEC) from other researchers varied widely between 5 cmol/kg and 162 cmol/kg. This range may, therefore, be considered typical of biochar [18,20].

Table 2. Physicochemical characteristics of biochar samples (mean values) used in the study

Physicochemical Parameter	Bone-derived Biochar	Corn Cob-derived Biochar
Yield (%)	34.65 ± 0.81	30.90 ± 5.73
pH	8.22 ± 1.15	8.91 ± 0.37
Electrical Conductivity (µS/cm)	712.51 ± 100.17	431.77 ± 60.79
Ash Content (%)	5.05 ± 0.44	10.44 ± 2.03
Moisture Content (%)	4.69 ± 0.81	2.33 ± 0.42
Total Nitrogen (%)	2.08 ± 0.49	2.16 ± 0.06
Sulphur (%)	1.35 ± 0.09	0.69 ± 0.08
Carbon (%)	75.11 ± 0.88	78.56 ± 4.77
Hydrogen (%)	2.77 ± 0.15	2.42 ± 0.58
Nitrogen (%)	0.41 ± 0.007	0.58 ± 0.27
Oxygen (%)	19.46 ± 0.58	10.40 ± 3.27
Calcium carbonate, $CaCO_3$ (%)	6.63 ± 0.62	5.86 ± 1.15
H/C ratio	0.037 ± 0.001	0.031 ± 0.008
O/C ratio	0.259 ± 0.005	0.132 ± 0.031
H/C_{org}	0.45 ± 0.08	0.41 ± 0.007
CEC (cmol/kg)	99.64 ± 6.54	114.10 ± 1.66

CEC – cation exchange capacity; C_{org} – organic carbon content

The differences in values for pH and CEC between the current study and other similar studies could be due to the differences in feedstock materials. Tomcyzk *et al.* [20] stated that the type of biomass used for biochar production plays an important role in its observed physicochemical properties as the source biomass determines the functional groups in the

biochar structure which, in turn, strongly influence the pH and CEC. The CEC is an important parameter when the remediation potential of biochar is being considered as it determines the surface charge and ultimately, the adsorption characteristics and affinity for nutrients and pollutants alike. The largely negatively charged biochar surface attracts organic pollutants that have mostly aromatic functional groups [43,44]. The CDB predictably had a higher CEC than the BDB as biochar from plant–derived feedstock has been shown to have greater CEC than biochar from other feedstock sources [45]; in addition, a higher ash content as seen in the CDB in the current study typically results in a higher CEC [46].

Variations in Soil Nitrogen Content and pH during the study

There was little difference between the nitrate, ammonium nitrogen and total organic nitrogen levels at the start and at the end of the study except in the polluted control (Control 1B) as represented in Figure 2. This implied that the biochar amendments helped to restore pre-pollution levels of the parameters studied in the soil. Biochar increased nitrate and ammonium nitrogen levels in the amended soils compared to the polluted control but only had negligible effect on total organic nitrogen (TON) concentrations. The corn cob-derived biochar (CDB), generally, had a stronger restorative effect on soil ammonium nitrogen, nitrate and TON concentrations than the bone-derived biochar (BDB). For soil nitrate, the lower treatment level of 10 % w/w proved more effective at restoring soil nitrate content for both CDB and BDB. The enhanced levels of nitrate in the treated soils at the end of the study may suggest increased activity of nitrifying bacteria leading to increased nitrate production.

The results obtained from the current study correspond with the conclusions of Mierzwa-Hersztek *et al*. [47] in their study on biochar from poultry waste. It was ascertained that the biochar increased overall soil nitrogen content. The feedstock material may be the pivotal influence here. The results in the current study also corroborate the findings of Wertz *et al*. [48] that following the introduction of petroleum into an environment, the sensitive nitrifying bacteria will initially show a lull in activity and then greatly increased activity levels as the ecosystem players either adapt to the pollutant or as the pollutant levels drop. The increased activity ultimately yields higher concentrations of nitrates in the soil.

The reduced organic nitrogen content in the polluted soil could be due to growth suppression

of soil microbes exposed to the contaminants and the attendant interruption of organic nitrogen biosynthesis in these cells. Moreover, the active mineralisation of organic nitrogen to ammonium ions due to biochar application may also be responsible for the depressed concentrations of organic nitrogen in treated soil during the study (Figure 2). The pH levels obtained tended towards neutral with the application of biochar; the effect was similar for both types of biochar employed in the study. Studies by Shetty and Prakash [49] and Bista *et al*. [50] in which biochar was confirmed to reduce soil acidity support the findings of the present study.

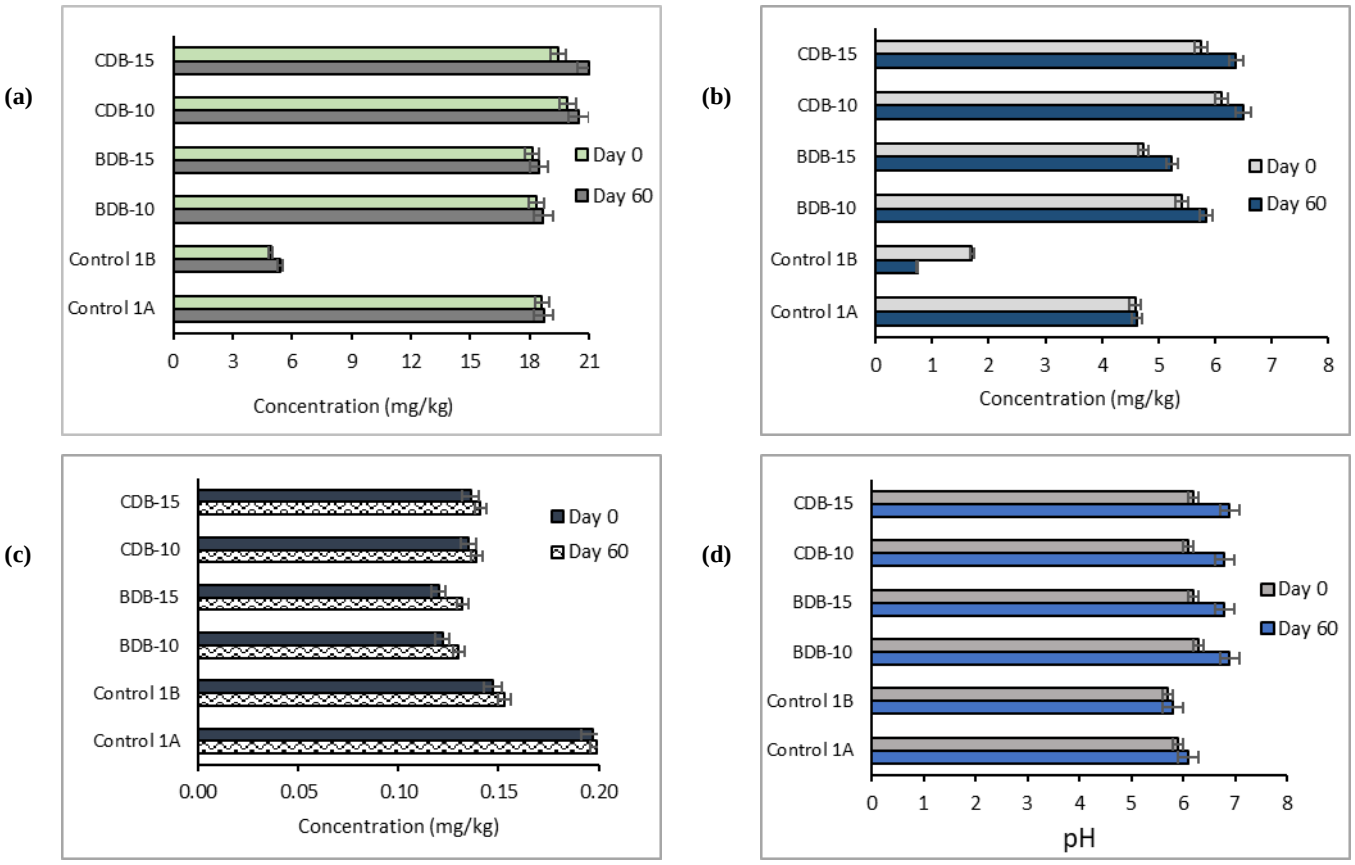


Fig.2. Changes in the concentrations of soil ammonium nitrogen (a), nitrate (b), total organic nitrogen (c) and pH (d) during the study

Bars represent standard deviation; BDB – bone derived biochar; CDB – corn cob derived biochar

Response of the Soil Nitrogen Cycling Community

The responses of the nitrogen fixing bacteria, nitrifying bacteria and the denitrifier, *P. aeruginosa* are depicted in Figures 3, 4 and 5 respectively. The biochar had a more robust impact on restoration of nitrifying bacterial counts (*Nitrosomonas* and *Nitrobacter*) to pre-pollution levels. The abundance initially dropped in response to the petroleum pollutant

but slowly recovered in the amended soil with levels almost reaching those in the unpolluted control by the end of the study. This was not the case for *Rhizobium* and *P. aeruginosa* which both showed rapid proliferation in response to the exposure to the pollutant indicating their ability to utilise crude oil as an energy source. The abundance of *P. aeruginosa* in biochar-amended soil differed considerably from the unpolluted control but not from polluted control. *P. aeruginosa* cells seemed to be in a lag phase up until day 15 and subsequently and peaked on day 30. The response of *Azotobacter* was similar to that obtained with the nitrifiers. At the onset of the study, the counts obtained for *Azotobacter* in the polluted media were less than in the unpolluted control, however, with the application of biochar, there was a steady increase towards pre-pollution levels.

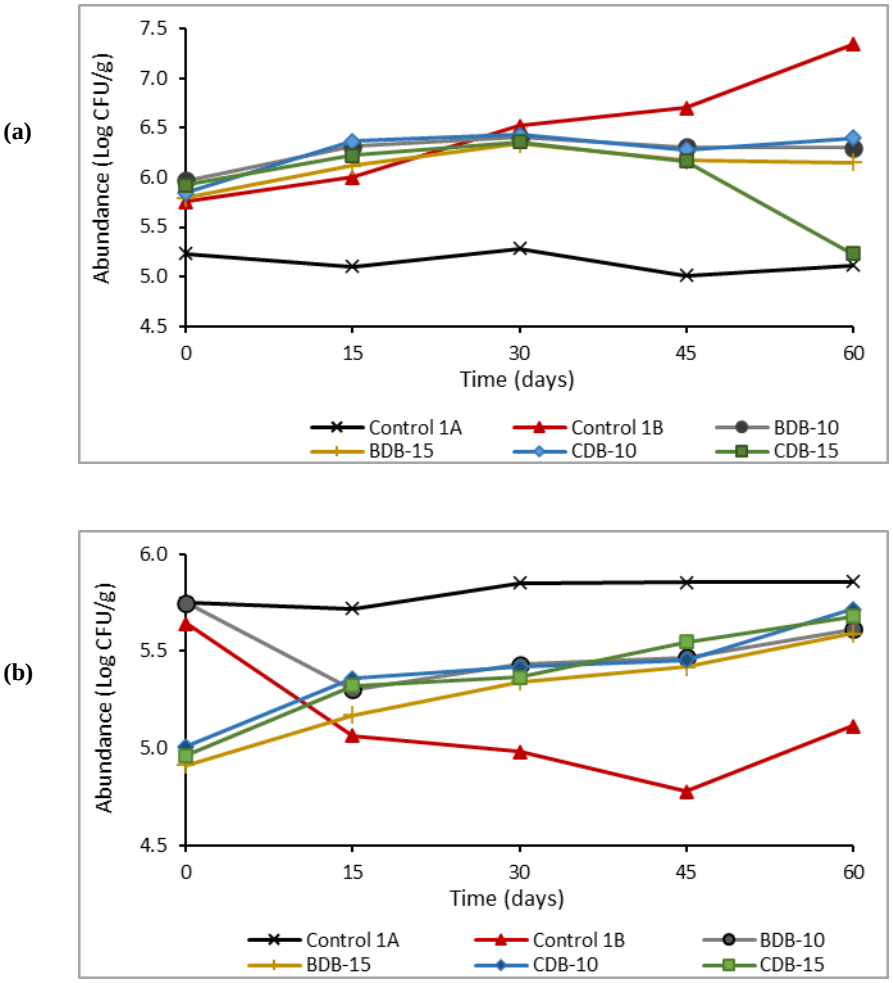
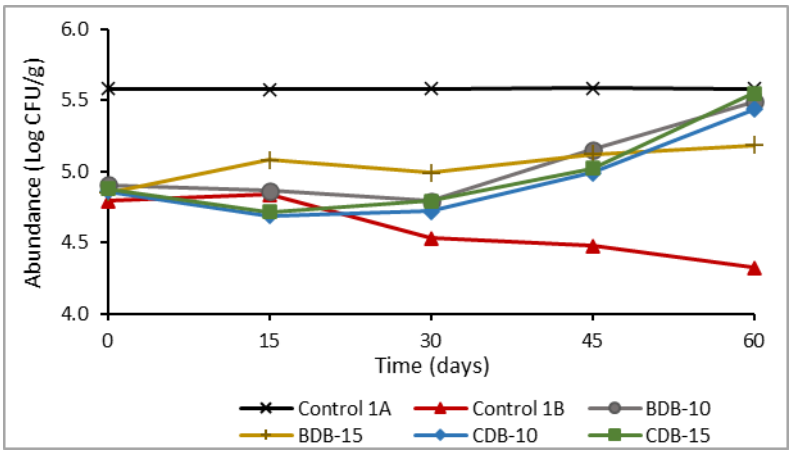


Fig.3. Variations in the mean abundance of nitrogen fixing bacteria, *Rhizobium* sp. (a) and *Azotobacter* sp. (b), during the study

BDB – bone derived biochar; CDB – corn cob derived biochar

The rise in abundance after an initial drop in the population of the bacteria may indicate a lag period during which the organism adapted to the newly introduced environmental stressor. This lag phase was not seen with *Rhizobium* (Fig. 3) or *P. aeruginosa* (Fig. 5). The variations seen in bacterial growth in the biochar-amended soil are likely due to facilitated natural attenuation of the hydrocarbon pollutant precipitated by the amendments; here, rapid proliferation preceded a decline in abundance and then a gradual return to initial unpolluted levels for all the nitrogen cyclers investigated.

(c)



(d)

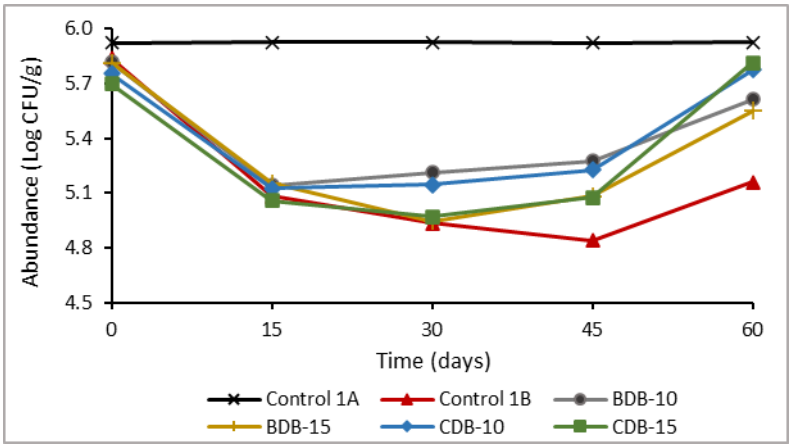


Fig.4. Variations in the mean abundance of nitrifying bacteria, *Nitrosomonas* sp. (c) and *Nitrobacter* sp. (d), during the study

BDB – bone derived biochar; CDB – corn cob derived biochar

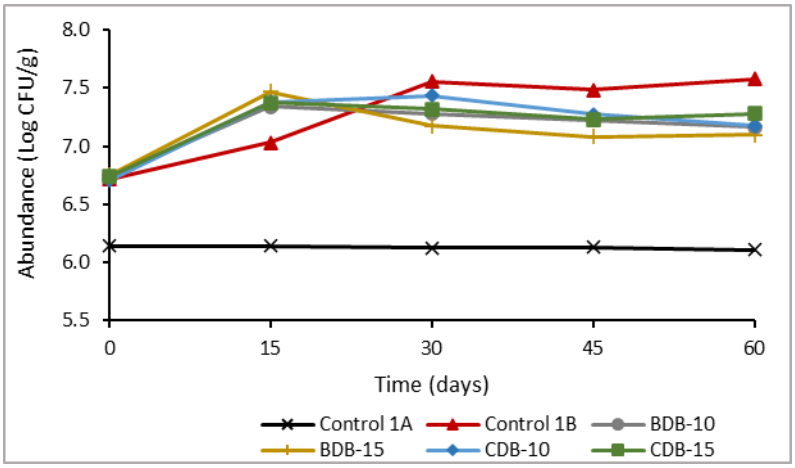


Fig.5. Variations in the mean abundance of *Pseudomonas aeruginosa* during the study

BDB – bone derived biochar; CDB – corn cob derived biochar

The increased concentration of nitrates in the soil samples does not tally with the observed nitrifier counts (*Nitrosomonas* and *Nitrobacter*); the latter decreased in the presence of petroleum and it would, thus, be expected that nitrate concentration would follow suit. This discrepancy is likely as a result of hyperactivity on the part of the genes responsible for ammonification and nitrate oxidation which would result in greater nitrate levels even when the nitrifying bacterial abundance has dropped. The reverse could be inferred for the nitrogen fixing bacteria; though the counts for *Rhizobium* increased relative to the nitrifiers, ammonium nitrogen levels in the amended soils did not vary much from the unpolluted control but differed markedly from the unamended (polluted) control. Under-expression of the relevant genes and the decrease in *Azotobacter* count could have further contributed to the observed discrepancy. Biochar clearly impacted soil ammonium nitrogen levels and the recovery of *Azotobacter* and the nitrifying bacteria positively.

Xu *et al.* [51] buttressed the observations in the current study when they reported that petroleum pollution led to diminished abundance of nitrifying functional genes and species richness in compromised soil and an eventual interruption of the nitrogen cycle. Several researchers confirm that nitrifying bacteria are more sensitive to hydrocarbon pollutants than other nitrogen cycling players especially the heterotrophic bacteria. Findings from the Deepwater Horizon spill detail an immediate drop in nitrifier abundance followed by a gradual rise as petroleum levels dropped. It was estimated that the abundance reached

pre-spill levels after about 60 days as also observed in the current study [52]. Van Dorst *et al.* [53] equally reported similar findings regarding the sensitivity of nitrifiers compared to other nitrogen cycling species. One study determined that nitrite oxidation was impacted by the presence of crude oil but cell lysis was not induced in *Nitrobacter*. Concentrations of 600 – 7500 mg/L resulted in a 50 % inhibition of the nitrification process [54]. *Nitrobacter* is considered more resistant to hydrocarbon pollution than *Nitrosomonas* [55].

Comparable to the present study, Zhoa *et al.* [56] confirmed increased abundance of denitrifying genes following exposure to petroleum in soil. They further found that potential denitrification rates increased alongside N_2O gas emission from the soil. Increases were 300 – 1000 times greater in soils spiked with 10% v/w of crude as in the current study. The result was the accumulation of N_2O in the soil. In addition, the denitrifying microorganisms displayed capacity for utilisation and degradation of the crude oil. They further reported that the abundance of the ammonium monooxygenase gene (*amoA*) responsible ammonification occurred in quantities significantly lower than other nitrogen cycling genes.

The activity of ammonia monooxygenase (AMO) enzyme initially increased during the study and then declined by the end of the study (Figure 6) but returned to levels close to but slightly higher than the pre-pollution state in the soils amended with biochar. The activity of AMO enzyme in the soil on day 60 for the biochar treatments were not significantly different from each other or from the day 0 unpolluted Control 1A at 95% confidence interval but were significantly different from the polluted Control 1B. The biochar amendment showed great potential for restoration of the pre-pollution AMO activity. Biochar is well-known to impact positively on enzymatic activity in soil ecosystems [20]. The use of biochar was found to enhance the activities of soil dehydrogenase and urease by 19 % and 44 % respectively in sandy loam soils [47,57]. The higher AMO enzyme activity may be considered an imbalance in the system and seems to result in the increased production of nitrates as evidenced in Figure 2. When the nitrate production occurs faster than the denitrifiers can handle, it could result in the accumulation of nitrates in the soil ecosystem.

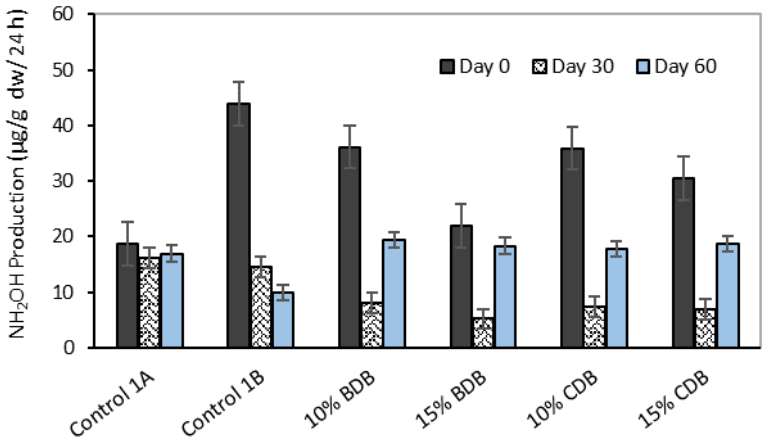


Fig.6. Effect of biochar amendments on ammonia monooxygenase (AMO) activity in crude oil polluted soils

Bars represent standard deviation; BDB – Bone derived biochar; CDB – Corn cob derived biochar

Elimination of Total Petroleum Hydrocarbons (TPH)

The CDB showed more effective elimination capacity than the BDB. The residual TPH levels at the end of the 15-week study were approximately 7730 mg/kg, 7266 mg/kg, 14108 mg/kg and 11397 mg/kg for 10% CDB, 15% CDB, 10% BDB and 15% BDB amendments respectively (Figure 7) representing reductions of 72.4% – 73.7% for CDB and 51.1% – 57.7 % for BDB.

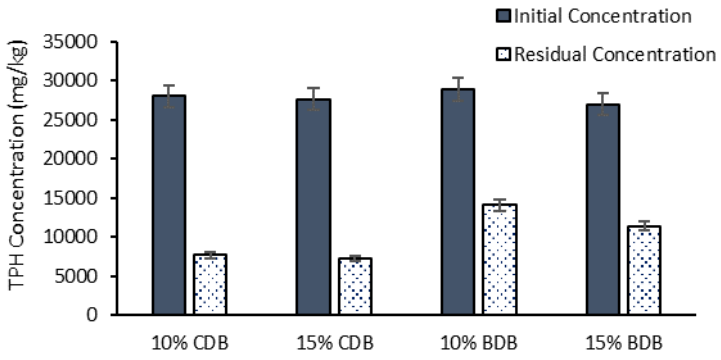


Fig.7. Initial and residual total petroleum hydrocarbon (TPH) levels in the biochar-amended crude oil contaminated soil samples during the study

Bars represent standard deviation; BDB – bone derived biochar; CDB – corn cob derived biochar

The pH of biochar alongside its CEC and structure play an important role in its remediation capacity [18]. Biochar from plant biomass has been noted by several researchers as having better adsorption capacity due to typically higher CEC and greater number and more uniform distribution of micropores in its structure [58].

Statistical Interactions between Groups

Statistical comparisons showed that there was a weak negative correlation between *Rhizobium*, *Nitrosomonas* and *Nitrobacter* counts and a medium negative correlation for *Azotobacter* counts. The r values were –0.18, –0.32, –0.27 and –0.49 for the four groups respectively. For *P. aeruginosa*, a weak positive correlation (r = 0.15) between bacterial counts and TPH concentration was found. The shared variance obtained was low at 2.25 %.

For all the nitrogen cycling bacterial groups studied except *P. aeruginosa*, the counts obtained in the amended soils for both types of biochar were significantly different from the controls (Controls 1A and 1B) at 95 % confidence interval. For *P. aeruginosa*, the counts were significantly different from the unpolluted control at 95 % confidence interval but were not significantly different from the polluted control. The microbial counts obtained for the CDB– and BDB–amended soils, generally, did not differ significantly from one another ($\rho \leq 0.05$). TPH removal levels, however, differ significantly between CDB and BDB at 95% confidence interval. The activity of ammonia monooxygenase enzyme in the soil on day 60 for all four biochar treatments were not significantly different from each other or the day 0 unpolluted control at 95% confidence interval but was significantly different from the polluted control.

4. CONCLUSION

The biochar used in this study exhibited potential for restoration of the nitrogen cycling ecosystem service in the soil as evidenced by the return of nitrogen cycling bacterial counts and soil ammonia monooxygenase activity to levels slightly higher than pre-pollution levels when compared to the control. Biochar had a better impact on restoration of nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) and *Azotobacter* with levels almost reaching those in the unpolluted control. This was not the case for *Rhizobium* and *P. aeruginosa* which both showed rapid proliferation despite the spike in petroleum in soil hence possibly indicating their ability to utilise the crude oil as an energy source. For nitrogen-fixing and nitrifying bacteria, the counts obtained in the amended soils for both biochar types were significantly different from the controls at 95% confidence interval. The biochar amendments restored soil ammonium nitrogen and nitrates to pre-pollution concentrations. The restorative impact on soil total

organic nitrogen concentration was negligible. The corn cob-derived biochar (CDB), generally, had a stronger restorative effect on soil ammonium nitrogen, nitrate and TON concentrations than the bone-derived biochar (BDB). Biochar amendment restored the activity of AMO enzyme in the soil by week 15. For all biochar treatment levels (week 15), AMO enzyme activity was not significantly different ($\rho \leq 0.05$) from each other or the unpolluted control but were significantly different from the polluted control. The CDB showed more effective total petroleum hydrocarbon (TPH) elimination capacity than the BDB. TPH reductions of 72.4 % – 73.7 % for CDB and 51.1% – 57.7 % for BDB were obtained in the amended soils. TPH elimination was greater at higher treatment levels of 15% w/w for both CDB and BDB. Further studies focusing on the abundance of the nitrogen cycling genes would provide useful data and contribute immensely to the research area. Biochar amendments exhibited great potential for restoration of nitrogen cycling while facilitating remediation of petroleum-polluted soils. It was concluded that the use of biochar for enhanced natural attenuation effectively supported an ecosystem services approach to bioremediation in petroleum compromised agricultural soil.

5. DECLARATION

The authors declare that no known conflicts of interest exist.

6. REFERENCES

[1] Griffiths B, Philippot L. Insights into the resistance and resilience of the soil microbial community. FEMS Microbiology Reviews 2012; 37: 112 – 119.

[2] MEA, Millennium Ecosystem Assessment. Ecosystems and Human Well-being: Current State and Trends. Findings of the Condition and Trends Working Group. Island Press, Washington, DC, USA; 2005.

[3] Baskent EZ. A Framework for Characterizing and Regulating Ecosystem Services in a Management Planning Context. Forests 2020; 11 (102): 1 – 20.

[4] Compton JE, Harrison JA, Dennis RL, Greaver TL, Hill BH, Jordan SJ, Walker H, Campbell HV. Ecosystem services altered by human changes in the nitrogen cycle: a new perspective for US decision making. Ecology Letters 2011; 14: 804 – 815.

[5] Urakawa H, Garcia J, Barreto P, Molina G, Barreto J. A sensitive crude oil bioassay indicates that oil spills potentially induce a change of major nitrifying prokaryotes from the Archaea to the Bacteria. Environmental Pollution 2012; 164: 42 – 45.

[6] USEPA, United States Environmental Protection Agency. Nitrification. U.S. Environmental Protection Agency, Office of Ground Water and Drinking Water, Standards and Risk Management Division, Washington, USA; 2002.

[7] Narayana B, Sunil K. A Spectrophotometric Method for the Determination of Nitrite and Nitrate. Eurasian Journal of Analytical Chemistry 2009; 4(2): 204 – 214.

[8] Halin S, Philippot L, Loffler E, Sanford RA, Jones CM. Genomics and Ecology of Novel N_2O-Reducing Microorganisms. Trends in Microbiology 2018; 26(1): 43 – 55.

[9] Robertson G, Groffman P. Nitrogen Transformations In: Eldor P., ed., Soil Microbiology and Biochemistry, 3rd Ed. Elsevier Academic Press, Amsterdam; 2007.

[10] Urakawa H, Rajan S, Feeney ME, Sobecky PA, Mortazavi B. Ecological response of nitrification to oil spills and its impact on the nitrogen cycle. Environmental Microbiology 2019; 21(1), 18–33.

[11] Kang YS, Park YJ, Jung J, Park W. Inhibitory effect of aged petroleum hydrocarbons on the survival of inoculated microorganism in a crude-oil-contaminated site. Journal of Microbiology and Biotechnology 2009; 19: 1672–1678.

[12] Burdige DJ. Estuarine and coastal sediments coupled biogeochemical cycling. In: McLusky D, Wolans E, eds. Treatise on Estuarine and Coastal Science, Academic Press, Waltham, MA; 2012.

[13] John RC, Ntino ES, Itah, AY. Impact of crude oil on soil nitrogen dynamics and uptake by legumes grown in wetland ultisol of the Niger Delta, Nigeria. Journal of Environmental Protection 2016; 7: 507 – 515.

[14] Kanter DR. Nitrogen pollution: a key building block for addressing climate change. Climate Change 2018; 147, 11 – 21.

[15] Bloem B, Breure A. Microbial Indicators. Chapter 2b, In: Markert B, Breure A, Zechmeister H, eds., Bioindicators and Biomonitors: Principles, Concepts and Applications. Volume 6, In: Nriagu J, ed., Trace Metals and other Contaminants in the Environment. Elsevier Science Limited, Oxford, UK; 2003.

[16] Urakawa H, Bernhard AE. Wetland management using microbial indicators. Ecological Engineering 2017; 108: 456–476.

[17] van den Belt M, Granek E, Gaill F, Halpern B, Thorndyke M, Bernal P. Assessment of Major Ecosystem Services from the Marine Environment (Other than Provisioning Services) Chapter 3. Scientific Understanding of Ecosystem Services Part III. United Nations; 2016.

[18] Guo M, Song W, Tian J. Biochar-Facilitated Soil Remediation: Mechanisms and Efficacy Variations. Frontiers in Environmental Science 2020; 8: 521512.

[19] Song L, Hou L, Zhang Y, Li Z, Wang W, Sun Q. Regular Biochar and Bacteria-Inoculated Biochar Alter the Composition of the Microbial Community in the Soil of a Chinese Fir Plantation. Forests 2020; 11: 951.

[20] Tomcyzk A, Sokoloswka Z, Boguta P. Biochar physicochemical properties: pyrolysis temperature and feedstock kind effects. Reviews in Environmental Science and Biotechnology 2020; 19:191–215.

[21] Noyce GL, Basiliko N, Fulthrope RR, Sackett TE, Thomas SC. Soil microbial responses over 2 years following biochar addition to north temperate forest. Biology and Fertility of Soils 2015; 51: 649 – 659.

[22] Mitchell PJ, Simpson AJ, Soong R, Schurman JS, Thomas SC, Simpson MJ. Biochar amendment and phosphorus fertilisation altered forest soil microbial community and native soil organic matter molecular composition. Biogeochemistry 2016; 130: 227 – 245.

[23] Ipppolito JA, Spokas KA, Novak JM, Lentz RD, Cantrell KB. Biochar elemental composition and factors influencing nutrient retention. In: Lehman J, Joseph S (eds.) Biochar for Environmental Management: Science, Technology and Implementation. 2nd Ed., Routledge, UK; 2015.

[24] Morris MC, McMurdie HF, Evans EH, Paretzkin B, Parker HS, Wong-Ng W, Gladhill DM. Standard X-ray Diffraction Powder Patterns. NBS Monograph 25 – Section 21. U. S. Department of Commerce, National Bureau of Standards, Pennsylvania, USA; 1985.

[25] Chen BL, Zhou D, Zhu L. Transitional adsorption and partition of nonpolar and polar aromatic contaminants by biochars of pine needles with different pyrolytic temperatures. Environmental Science and Technology 2008; 42: 5137 – 5143.

[26] Mustapha AA, Abdu N, Oyinola EY, Nuhu AA. Comparison of 3 different methods of CEC determination in Nigerian savannah soils. Journal of Applied Sciences 2020; 20(4): 159 – 165.

[27] Yuan JH, Xu RK, Zhang H. The forms of alkalis in the biochar produced from crop residues at different temperatures. Bioresource Technology 2011; 102: 3488 – 3497.

[28] Maynard DG, Katra YP. Nitrate and exchangeable ammonium nitrogen. Chapter 4 In: Carter MR., ed., Soil Sampling and Methods of analysis. Canadian Society of Science, Lewis Publishers, Boca Raton, Florida, USA; 1993.

[29] Keeney DR, Nelson DW. Nitrogen in organic form In: Page A., Ed., Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties. American Society of Agronomy, Inc., Madison, Wisconsin USA; 1982.

[30] Bremner JM. Nitrogen Total *In:* Sparks DL, Ed., Methods in Soil Analysis, Part 3, Chemical Methods. American Society of Agronomy Inc., Wisconsin, USA; 1996.

[31] Holt GT, Krieg RN, Sneath PHA, Staley TJ, Williams TS. Bergey's Manual of Determinative Bacteriology. 9th Ed. Williams and Wilkins, Baltimore, USA; 1994.

[32] Cheesbrough M. District Laboratory Practice in Tropical Countries, Part II. Cambridge University Press, London, UK; 2006.

[33] Vepsäläinen M. Poor enzyme recovery by extraction from soils. Soil Biology and Biochemistry 2001; 33: 1131 – 1135.

[34] Šnajdr J, Valaskova V, Merhautova V, Herinkova J, Cajthaml T, Baldrin P. Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. Soil Biology and Biochemistry 2008; 40: 2068 – 2075.

[35] Liu S, Vereecken H, Brüggemann N. A highly sensitive method for the determination of hydroxylamine in soils. Geoderma 2014; 232 – 234: 117 – 122.

[36] USEPA, United States Environmental Protection Agency. Method 3560 – Supercritical extraction for total recoverable petroleum hydrocarbons (TRPHs). USEPA, Washington DC, USA; 1996.

[37] Joseph S, Taylor P, Rezende F, Draper K, Cowie A. The Properties of Fresh and Aged Biochar. Armidale, NSW: Biochar for Sustainable Soils, Starfish Initiatives; 2019. Available at https://biochar.international/guides/properties-fresh-aged-biochar/, Accessed, 31st October, 2021.

[38] IBI, International Biochar Initiative. Standardized Product Definition and Product Testing Guidelines for Biochar that is used in Soil – Version 2.1. Westerville, Ohio, USA: International Biochar Initiative; 2016.

[39] EBF, European Biochar Foundation. European Biochar Certificate – Guidelines for a Sustainable Production of Biochar. European Biochar Foundation (EBF), Switzerland; 2012.

[40] Ronsse F, van Hecke S, Dickinson D, Prins W. Production and characterisation of slow pyrolysis biochar: influence of feedstock type and pyrolysis conditions. GCB Bioenergy 2013; 5: 104 – 115.

[41] Naeem MA, Khalid M, Arshad M, Ahmad R. Yield and nutrient composition of biochar produced from different feedstocks at varying pyrolytic temperatures. Pakistani Journal of Agricultural Science 2014; 51(1): 75 – 82.

[42] Domingues RR, Trugilho PF, Silva CA, de Melo ICN, Melo LCA, Magriotis ZM, Sanchez-Monedero MA. Properties of biochar derived from wood and high-nutrient biomasses with the aim of agronomic and environmental benefits. PLoS ONE 2017; 12(5): e0176884.

[43] Metcalf K. Biochar for Carbon Sequestration: Investigation and Outreach. Earth Science 2016; 32: 33 – 36.

[44] Janu R, Mrlik V, Ribitsch D, Hofman J, Sedlacek P, Bielska L, Soja G. Biochar surface functional groups as affected by biomass composition and pyrolysis temperature. Carbon Resources Conversion 2021; 4: 36 – 46.

[45] Wang S, Gao B, Zimmerman AR, Li Y, Ma L, Harris WG, Migliaccio KW. Physicochemical and sorptive properties of biochars derived from woody and herbaceous biomass. Chemosphere 2015; 134: 257 – 262.

[46] Yang F, Lee X, Wang B. Characterization of biochars produced from seven biomasses grown in three different climate zones. Chinese Journal of Geochemistry 2015; 34: 592–600.

[47] Mierzwa-Hersztek M, Gondek K, Baran A. Effect of poultry litter biochar on soil enzymatic activity, ecotoxicity and plant growth. Applied Soil Ecology 2016; 105:144–150.

[48] Wertz S, Leigh A, Grayston S. Effects of long-term fertilization of forest soils on potential nitrification and on the abundance and community structure of ammonia oxidizers and nitrite oxidizers. FEMS Microbiology Ecology 2012; 79: 142 – 154.

[49] Shetty R, Prakash NB. Effect of different biochars on acid soil and growth parameters of rice plants under aluminium toxicity. Scientific Reports 2020; 10: 12249.

[50] Bista P, Ghimire R, Machado S, Pritchett L. Biochar effects on soil properties and wheat biomass vary with fertility management. Agronomy 2019; 9(10): 623.

[51] Xu X, Liu W, Tian S, Wang W, Qi Q, Jiang P, Gao X, Li F, Li H, Yu H. Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: A perspective analysis. Frontiers in Microbiology 2018; 9: 2885.

[52] Huettel M, Overholt WA, Kostka JE, Hagan C, Kaba J, Wells WB, Dudley S. Degradation of Deepwater horizon oil buried in a Florida beach influenced by tidal pumping. Marine Pollution Bulletin 2018; 126: 488–500.

[53] Van Dorst J, Siciliano SD, Winsley T, Snape I, Ferrari BC. Bacterial targets as potential indicators of diesel fuel toxicity in subantarcctic soils. Applied and Environmental Microbiology, 2014, 80(13), 4021 – 4033.

[54] Okpokwasili GC, Odokuma LO. Tolerance of *Nitrobacter* to toxicity of some Nigerian crude oils. Bulletin of Environmental Contaminants and Toxicology 1993; 52: 388–395.

[55] Okpokwasili GC, Odokuma, LO. Tolerance of *Nitrobacter* to toxicity of hydrocarbon fuels. Journal of Petroleum Science and Engineering 1996; 16: 89-93.

[56] Zhao Y, Chen W, Wen D. The effect of crude oil on microbial nitrogen cycling in coastal sediments. Environment International 2020; 139: 105724.

[57] Ameloot N, De Neve S, Jegajeevagan K, Yildiz G, Buchan D, Funkuin YN, Prins W, Bouckaert L, Sleutel S. Short term CO_2 and N_2O emissions and microbial properties of biochar amended sandy loam soils. Soil Biology and Biochemistry 2013; 57: 401–410.

[58] Cao X, Harris W. Properties of dairy-manure-derived biochar pertinent to its potential use in remediation. Bioresource Technology 2010; 101 (14):5222–5228.