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Remediation by Enhanced Natural Attenuation (RENA): A Beneficial Strategy for Polyaromatic Hydrocarbon Degradation and Agrifood Production

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Abstract **–**The influence of crude oil remediated sites on agrifood production and the nutritional composition of plants is poorly understood. To address this knowledge gap, we assessed the efficiency of selected bioremediation strategies for the sustainable degradation of polyaromatic hydrocarbons, and their agricultural benefits. The crude oil we tested had a pristane/phytane ratio of 0.98 (within the 0.8 – 3.0 range of most crude oils) and higher concentrations of two of the USEPA priority pollutant polyaromatic hydrocarbons (PAHs) – phenanthrene and anthracene. The pH (6.6 ± 0.03) and moisture content (30.8 ± 1.0%) of the soil were within the acceptable level for oil degradation. Four treatments were prepared involving the planting and remediation-study controls. After 7 days, Tukey HSD post-hoc analysis showed a significant reduction in phenanthrene concentrations following RENA treatment compared to the remediation-study control ($p < 0.05$) whereas anthracene was below the limit of quantitation following RENA treatment. One-way ANOVA showed that although the concentration of major dietary minerals was lower in vegetable samples obtained from the other treatments compared to the planting control (with the exception of sodium and potassium levels) the differences were not statistically significant ($p > 0.05$). The Ca/P (> 1.0) and Na/K (< 0.60) ratios of the samples showed that they provide a good source of these minerals for bone formation and would not contribute to high blood pressure. Among the minor minerals analysed, copper, zinc and iron were below the FAO/WHO (2001) safe limit. Our findings suggest that RENA performs a dual function during the remediation of PAH contamination by favouring the growth of plants without reducing the mineral composition of the agricultural products, with the exception of chromium and zinc.

Keywords**:** Crude oil, Remediation by Enhanced Natural Attenuation (RENA), Polyaromatic Hydrocarbons (PAHs), Agrifood, Vegetables, Minerals

1. Introduction

The search for crude oil has expanded in many parts of the world with positive results in some members of the Organization of the Petroleum Exporting Countries (OPEC). Regardless of the numerous socio-economic gains from the discovery of crude oil, spills sometimes occur during production operations, or due to accidental discharges, leakages from corroded bunkers and transport vessels, and human errors. These spills contaminate the surrounding environment and negatively affect the ecosystem (Ajagbe et al., 2012; Khamehchiyan et al., 2007).

The quest for efficient and economical strategies to break down petroleum hydrocarbons at contaminated sites has led to the development of remediation methods, including bioremediation which involves the use of micro-organisms. Among the different bioremediation treatment approaches, natural attenuation, otherwise known as intrinsic or passive bioremediation, is considered when the other options - biostimulation and bioaugmentation - have been found to be too costly or unsuitable (King et al., 1998). Bioaugmentation, in which hydrocarbon-degrading microorganisms are introduced at the contaminated site to enhance the ability of the inherent microbial population to break down contaminants (Venosa and Zhu, 2003; Ogbonna et al., 2007), is often employed for the bioremediation of recalcitrant compounds (Atlas and Philip, 2005) which includes polyaromatic hydrocarbons (PAHs) (Eggen and Majcherczyk,

1998). However, this artificial introduction of microbial consortium in some cases may impede bioremediation effectiveness (Coulon et al., 2012).

On the other hand, Remediation by Enhanced Natural Attenuation (RENA), a land farming treatment technology (Wegwu et al., 2010; Onifade et al., 2007; Ebuehi et al., 2005) involving a form of natural bioremediation (Bento et al., 2005), has been suggested as a viable option in remediating crude oil-polluted agricultural lands (Wegwu et al., 2010) as it supports the provision of aerobic microbial processes required for the remediation of petroleum hydrocarbons (King et al., 1998).

In this paper, we compared the efficiency of these two aforementioned bioremediation strategies, i.e. the use of RENA to break down PAHs and thus promote agrifood production at crude oil remediation sites with bioaugmentation via the use of improved degradation abilities of *Pleurotus ostreatus* (Sukor et al., 2012; Zebulun et al., 2011; Bezalel et al., 1996) spent mushroom compost (Okparanma et al., 2011). The mesocosm study assesses the effect of these remediation methods on hydrocarbon degradation and the yield as well as the mineral composition of *Brassica juncea*, a member of the Brassicaceae family.

2. Materials and Methods

2. 1. Growing Medium and Chemicals

John Innes No. 2 soil-based compost (John Innes Manufacturers Association, Berkshire), spent *Pleurotus ostreatus* mushroom compost (Marlborough Mushrooms, UK) and *Brassica juncea* seeds (Herbiseed, England) were all purchased and stored appropriately. Reagents and HPLC-grade solvents (hexane, acetone, dichloromethane, deuterated EPA 525 PAH) were purchased from Sigma-Aldrich Co. Ltd (UK) and Fisher Scientific UK Ltd.

2. 2. Pot and Greenhouse Experimental Setup

The pot experiment was carried out using Pöpplemann TEKU 5-litre growing pots containing 2 kg soil-based compost, with four treatments in five replicates as follows:

Treatment A: 2 kg of soil-based compost (planting control);
Treatment B: 2 kg of soil-based compost + 0.5% w/w crude oil (remediation study control);
Treatment C: 2 kg of soil-based compost + 0.5% w/w crude oil + RENA treatment; and
Treatment D: 2 kg of soil-based compost + 0.5% w/w crude oil + bioaugmentation treatment.

The treatments were incubated for 42 days before planting *Brassica juncea* seeds. The crops were grown at the Cranfield University Research Glasshouse Facility, Cranfield, United Kingdom, where standardized John Innes Number 2 soil-based compost was used as the potting growing medium. The use of commercial potting compost is supported by OECDiLibrary (2006) guidelines for testing the effects of chemicals on plants, and Lawrence (1955) recommended the use of standardized John Innes compost to avoid large variations in plant growth and development which may arise from the improper making and handling of seed and potting composts.

2. 3. Initial Investigations, Spiking and the Incubation Process

In order to gain a useful knowledge of the distribution of the polyaromatic hydrocarbons (essentially the 16 USEPA priority pollutant PAHs), an initial investigation was carried out on the whole crude oil by gas chromatography – mass spectrometry (GC/MS). The physicochemical properties of the soil were also tested using appropriate standard methods.

The crude oil was thoroughly mixed with the growing medium using the modified method of Brinch et al. (2002) for spiking soil samples with organic compounds. The RENA treatment involved aeration via weekly tillage whereas the bioaugmentation treatment involved the introduction of spent *Pleurotus ostreatus* mushroom substrate to the growing medium (1:4 ratio) (Eggen, 1999) to enhance the bioremediation process.

Water was added at regular intervals to maintain the moisture content (Wu et al., 2013) and the pots were left to allow petroleum hydrocarbon degradation. We took 10-g soil samples at the start of the experiment [day (0) and day (7)] and other two points at the end [day (28) and day (42)], as well as after

harvesting to assess the level of degradation of the petroleum polyaromatic hydrocarbons over time. The soil samples were kept at -20^{o}C prior to analysis.

2. 4. Seed Planting and Sample Preparation

Ten *Brassica juncea* seeds were planted at 3 mm depth in each of the soil treatments after 42 days of incubation. The seeds were left to germinate in the greenhouse, and after 10 days of germination, thinning was carried out to remove weaker seedlings, leaving three seedlings in each pot. The plants were allowed to grow for 65 days while the environmental conditions were strictly monitored and the pots re-positioned periodically to minimize variations in plant growth. Collection trays were placed beneath each pot to retain leachate after watering and were re-applied immediately to the same pot. Plant growth parameters were recorded, and after harvesting the fresh above-ground plant parts were prepared for analysis. They were thoroughly washed in fresh running water to remove soil and surface particles, followed by snap-freezing, freeze drying and grinding into fine powder for analysis.

2. 5. Mineral Analysis

0.5g (dry mass) ± 0.1mg of each of the plant sample was digested in a microwave digestion liner using 5 ml of nitric acid (overnight at room temperature) followed by 5 ml of hydrogen peroxide solution for 2 h before the final digestion. An empty liner without the sample was used as the blank while the final digestion of all the samples with the blank was carried out in the Microwave Accelerated Reaction System (Model MARS®, CEM Corporation, USA). NIST 1515 apple leaves were prepared in the same manner as standard reference material. The filtrates were analysed by Inductively Coupled Plasma – Mass Spectrometry (Perkin-Elmer Elan 9000) whereas iron was analysed separately by Atomic Absorption Spectrometry (PerkinElmer AAnalyst 800).

2. 6. Hydrocarbon Analysis

The modified sequential ultrasonic solvent extraction technique of Risdon et al. (2008) was used for the extraction of hydrocarbons. Briefly, 5 ± 0.5 g of soil was dried with 5 g anhydrous sodium sulphate in a 50-ml centrifuge tube followed by the addition of 4 ml acetone and sonication for 2 min at 20°C. Acetone (6 ml) and hexane (10 ml) were added to the samples and sonicated for 10 min. The solvent and the soil were mixed, centrifuged for 5 min while the supernatant was passed through Sigma-Aldrich Supelco Discovery® DSC-Sci 3ml solvent phase extraction (SPE) tube. This was followed by a series of sequential extraction steps, while appropriate amounts of the sample extract and PAH internal standards were added to 2-ml Agilent GC glass vials and crimp capped with PTFE seals for GC-MS analysis.

USEPA priority PAHs (essentially phenanthrene and anthracene) in the crude oil and extracted soil samples were measured using an Agilent Technologies 6890N Network GC system coupled to an Agilent Technologies 5973 Network mass selective detector at 70 eV in positive ion mode. The column was a Phenomenes ZB-5HT (30 m × 0.25 mm internal diameter, film thickness 0.10 µm). Manual injection in splitless mode was used. The oven temperature was increased from 60^{o}C to 220^{o}C at 20^{o}C/min then to 310^{o}C at 6^{o}C/min using the full scan mode (*m/z* 50 – 500) for quantitative analysis of the target PAHs.

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were set at three and ten times the signal/noise ratio, respectively (Liguori et al., 2006; Rubert et al., 2013).

2. 7. Statistical Analysis

Where applicable, one-way analysis of variance (ANOVA) was used to compare the means of the values obtained using the IBM SPSS Statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

3. Results and Discussion

3. 1. Initial Findings

3. 1. 1. Crude Oil Characterisation

GC-MS analysis revealed the distribution of 10 of the USEPA priority PAHs in the crude oil with higher concentrations of phenanthrene and anthracene (Figure 1). The crude oil was also found to contain higher concentrations of the lighter alkane fractions at the instrument's highest oven operating temperature of 310^{o}C with a pristane/phytane (Pr/Ph) ratio of 0.98 (data not shown). This showed that the crude oil has a Pr/Ph ratio within the range of 0.8 - 3.0 of most crude oils (Peters et al., 2005) and also indicated that it was from an anoxic depositional environment which is characterised by Pr/Ph ratio <1 (El Nady and Harb, 2011).

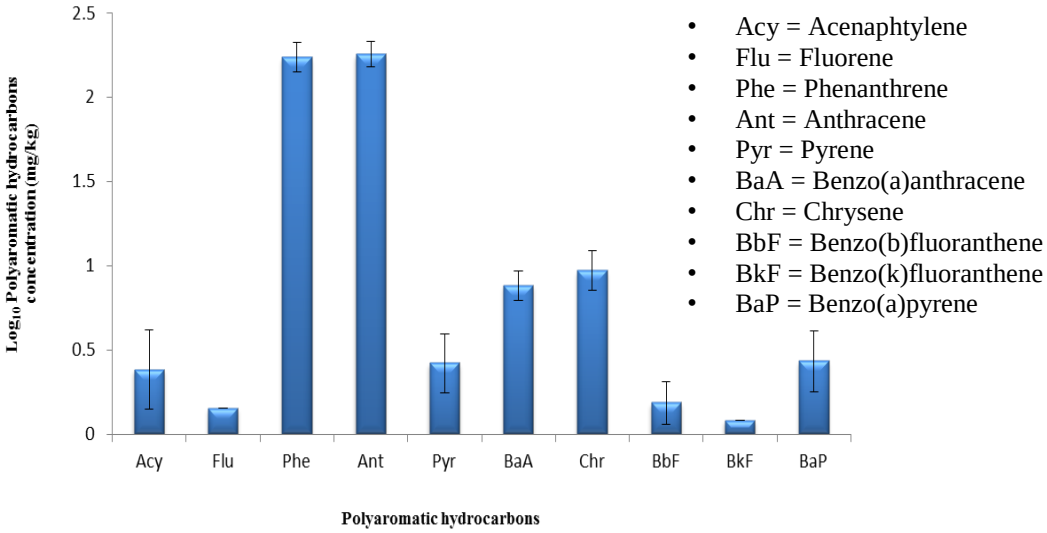

Fig. 1. Distribution of 10 USEPA pollutant priority PAHs in whole crude oil

3. 1. 2. Physicochemical Properties of the Growth Media

The physicochemical properties of the two substrates used for the experiment revealed that the pH and moisture content of the soil-based compost fell within the acceptable range for oil degradation as recommended by M. Vidali (2001) although the spent *Pleurotus ostreatus* mushroom compost had higher values of electrical conductivity and carbon/nitrogen ratio (Table 1). The spent mushroom compost also had a higher moisture content and water-holding capacity than the soil-based compost, indicating that it contained more organic matter, as confirmed in the higher total carbon content (Table 1).

According to Davidson et al. (2000), the bioaugmentation treatment involving the use of spent *Pleurotus ostreatus* mushroom compost would evolve more CO_2 due to the higher moisture content. Nonetheless, the water-holding capacity of the soil-based compost (within the range of 50–70%) would better support aerobic biodegradation of simple or complex organic material (Dibble and Bartha, 1979).

Although the two composts have C:N and C:P ratios higher than the acceptable theoretical values of 10:1 and 100:1, respectively, for a mixed microbial population in soil (Dibble and Bartha, 1979), these ratios are expected to be higher for petroleum biodegradation. The addition of spent *Pleurotus ostreatus* mushroom substrate with a high C:N ratio to the bioaugmentation treatment would reduce the nitrogen level in the soil, killing some of the microorganisms, which decompose to release nitrogen thus compensating for the loss in nitrogen level (USDA, 2011).

Table. 1. Physicochemical properties of compost.

Property	Method	Soil-based compost	Spent mushroom compost
pH	Sparks et al. (1996)	6.60 ± 0.03	4.75 ± 0.01
Moisture Content (%)	British Standard BS 7755: Section 3.1:1994 identical to ISO 11465:1993	30.80 ± 0.97	37.60 ± 0.24
Water-Holding Capacity (%)	Ahn et al. (2008)	58.07 ± 0.60	81.17 ± 0.54
Electrical Conductivity (S/m)	British Standard BS 7755: Section 3:4:1995 identical to ISO 11265:1994	0.128 ± 0.00	0.202 ± 0.00
Total Carbon (%)	British Standard BS 7755 Section 3.8:1995 identical to ISO 10694:1995	9.37 ± 0.37	47.53 ± 0.25
Total Organic Carbon (%)	British Standard BS 7755 Section 3.8:1995 identical to ISO 10694:1995	8.62 ± 0.16	44.95 ± 0.17
Total Nitrogen (%)	British Standard BS EN 13654-2:2001	0.31 ± 0.01	0.64 ± 0.01
Total Phosphorus (mg/kg)	British Standard BS 7755: Section 3.6:1995 identical to ISO 11263:1994	542.30 ± 15.81	712.05 ± 2.44
C/N Ratio		29.85 : 1.00	74.43 : 1.00
C/P Ratio		172.87 : 1.00	667.55 : 1.00

The electrical conductivity of the spent *Pleurotus ostreatus* mushroom compost also fell in the range within which the yield of sensitive crops could be restricted (Richards, 1954).

3. 2. Effect of Compost Treatments on Hydrocarbon Degradation

Four treatments (A, B, C and D) were used for the hydrocarbon degradation and plant response study, where treatment A was the control for the plant response and treatment B was the remediation-study control. At the start of the experiment, only phenanthrene and anthracene (Figure 2) were above the LOQ value of 0.02 mg/kg commonly used to assess PAHs in Nigerian sites with contaminated soils (Okparanma, 2013). The incubation period and consequent degradation study therefore focussed on these two PAHs, which are also USEPA priority compounds.

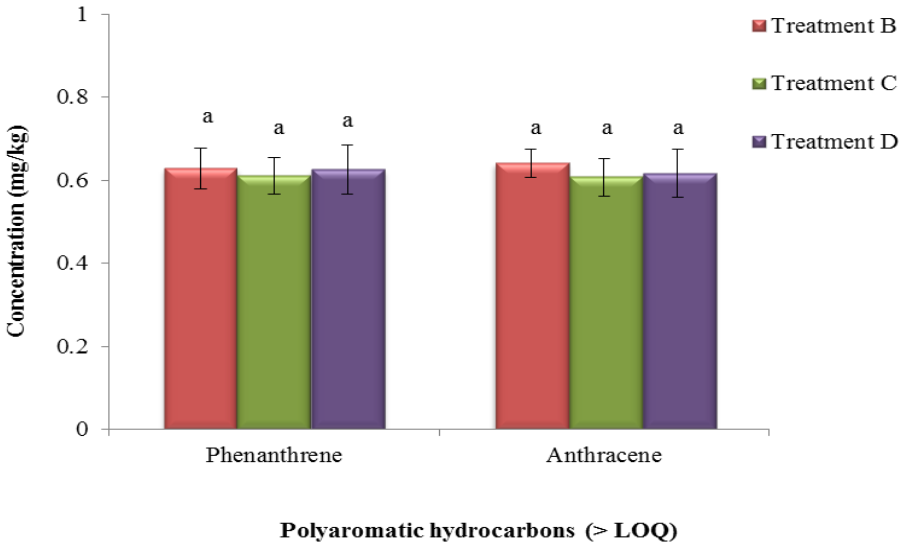

Fig. 2. Initial PAH distribution (> LOQ) in the pot treatments (Day 0)

There were no statistically significant differences in the concentration of these PAHs among the pot treatments at the start of the experiment, as shown in Figure 2, which indicates the homogenous mixing of the crude oil and substrates. However, after 42 days of incubation, the concentrations of the two priority PAHs fell below the LOD in the pot treatments where bioremediation strategies were applied (i.e. the RENA and bioaugmentation treatments) whereas phenanthrene concentration in treatment B remained above the LOD (Figures 3). The planting of *Brassica juncea* also supported the removal of phenanthrene from the soil.

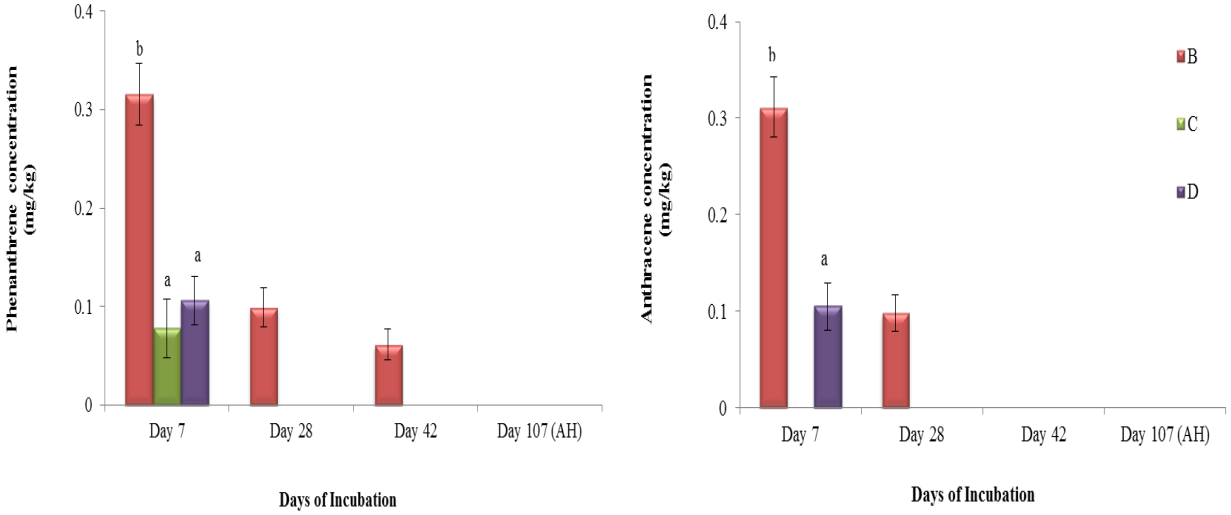

Fig. 3. Phenanthrene and anthracene degradation over time (AH: After Harvesting)

3. 3. Effect of Bioremediation Strategies on Seed Germination and the Nutritional Composition of Plants

3. 3. 1. Effect on Seed Germination

The effect of the selected bioremediation strategies on the germination of *Brassica juncea* seeds is shown in Figure 4. This experiment revealed that the lower percentage of seed germination in treatment D, involving bioaugmentation with spent *Pleurotus ostreatus* mushroom compost, was statistically significant compared to the other treatments. This may be as a result of the introduction of spent *Pleurotus ostreatus* mushroom compost (with unfavourable electrical conductivity) to the soil substrate.

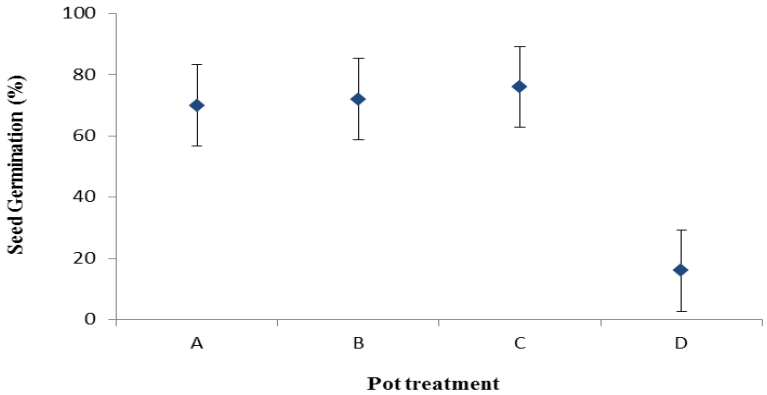

Fig. 4. Response of *Brassica juncea* to the different treatments

3. 3. 2. Effects on the Nutritional Composition of Plants

The nutritional composition of the plants was only determined in the plants under treatments A, B and C because the plants under treatment D were destroyed by pests. The effect of the three remaining treatments on the nutritional composition of *Brassica juncea*, i.e. the mineral distributions as found in the above-ground plants part of the plant after harvesting, was evaluated because vegetables are said to be good sources of minerals (Taura and Habibu, 2009) which can be classified as major and minor minerals (Nieman et al., 1992). The concentration of five of the major dietary minerals (Ca, P, Na, K and Mg) found in the vegetable samples after 65 days of growth is presented in Table 2.

Table. 2. The distribution of major dietary minerals (mg/kg) in each treatment.

Treatments	Distribution of Major Dietary Minerals (mg/kg)				
	Na	Mg	Ca	K	P
A (Clean soil, Planting control)	2766.6 ± 138.80[a]	5042.92 ± 138.80[a]	21,710.2 ± 2278.03[a]	64,696 ± 5363.73[a]	5712 ± 489.89[a]
B (Soil + Crude oil)	2949.93 ± 155.92[a]	4579.59 ± 83.53[a]	19,193.53 ± 1863.7[a]	65,226 ± 5642.18[a]	4798.67 ± 187.74[a]
C (Soil + Crude oil + RENA treatment)	2596.6 ± 300.22[a]	4739.59 ± 301.18[a]	20,073.53 ± 1008.32[a]	60,432.67 ± 551.04[a]	4965.33 ± 229.29[a]

All the values are the mean of three replicates ± SE.

Means followed by different letters are significantly different ($p < 0.05$) according to Tukey HSD post-hoc analysis.

A limited dataset is reported for treatment D because the plants were damaged by pests and we were not able to complete the mineral analysis for these plants.

There was no statistical difference in the levels of the major dietary minerals among the treatments ($p > 0.05$) but there was a gradual decrease in the concentration of metals, except sodium and potassium, compared to treatment A (Table 2). Based on the findings of Martínez-Ballesta et al. (2010), the concentrations of Na and P in the harvested samples are above the expected range of 22.8–940 mg/kg and 162–4370 mg/kg, respectively, whereas the high potassium content follows a similar trend to that observed in radish and lettuce leaves (McKeehen et al., 1996).

Nieman et al. (1992) have suggested that a food source is good if the Ca:P ratio >1 and poor if it is <0.5 (Aremu et al., 2006; Aremu et al., 2011; Olagbemide and Philip, 2014; Yusuf et al., 2007). The Ca:P ratios of the vegetable samples from the pot treatments were >2, so they are not only beneficial for bone formation, but they would also enhance the absorption of calcium in the small intestine (Aremu et al., 2006; Audu et al., 2013). Furthermore, the samples would not contribute to high blood pressure because the Na:K ratio was <0.6 (Nieman et al., 1992) as shown in Table 3.

Table. 3. Mineral ratios of the harvested *Brassica juncea* samples.

Treatments	Ca/P ratio	Na/K ratio
A (Clean soil, Planting control)	3.80	0.043
B (Soil + Crude oil)	3.99	0.045
C (Soil + Crude oil + RENA treatment)	4.04	0.043

One-Way ANOVA analysis of the minor minerals is shown in Table 4.

Table. 4. Distribution of minor dietary minerals (mg/kg) in each treatment.

Treatments	Distribution of Minor Dietary Minerals (mg/kg)						
	Cr	Mn	Co	Cu	Zn	Mo	Fe
A (Clean soil, Planting control)	3.95 ± 0.49[a]	107.66 ± 19.02[a]	0.17 ± 0.00[a]	1.85 ± 0.11[a]	42.78 ± 2.78[a]	0.53 ± 0.00[a]	159.35 ± 20.64[a]
B (Soil + Crude oil)	2.54 ± 0.25[a,b]	88.22 ± 15.26[a]	0.17 ± 0.04[a]	1.70 ± 0.35[a]	31.25 ± 1.56[b]	0.57 ± 0.16[a]	167.87 ± 19.91[a]
C (Soil + Crude oil + RENA treatment)	1.84 ± 0.38[b]	83.16 ± 4.42[a]	0.15 ± 0.03[a]	1.31 ± 0.25[a]	28.18 ± 0.69[b]	0.59 ± 0.11[a]	137.50 ± 18.99[a]

All the values are the mean of three replicates ± SE.

Means followed by different letters are significantly different ($p < 0.05$) according to Tukey HSD post-hoc analysis.

Among the seven minor minerals analysed, Cr, Fe, Mn and Co were higher than the expected concentration ranges of 4×10^{-4} – 0.06 mg/kg, 1.3 – 30.1 mg/kg, 0.1– 0.78 mg/kg and < 0.01 mg/kg respectively, reported by Martínez-Ballesta et al. (2010) (Table 4). The concentrations of copper and iron in the harvested samples did not exceed the FAO/WHO (2001) safe limits of 73 mg/kg and 425 mg/kg, respectively (Yahaya, 2013). Cu and Zn were also within the maximum permissible concentrations for human consumption of 50 mg/kg and 10–50 mg/kg, respectively (Samara et al., 1992).

Although zinc levels were below the WHO/FAO (2001) safe limit of 99.40 mg/kg (Yahaya, 2013; Aderinola and Kusemiju, 2012), it was the only mineral that was negatively affected by both the crude oil contamination and the RENA treatment (Table 4). Statistical analysis of zinc levels [$F(2, 6) = 26.28$, $p = 0.001$ and $\omega^2 = 0.85$] showed a statistically significant difference ($p < 0.05$) between treatments A and B [11.53, 95% CI (5.02 to 18.05) mg/kg] and between treatments A and C [14.60, 95% CI (8.08 to 21.12) mg/kg].

Finally, although the harvested vegetable samples have 'good' Ca/P ratios, high concentrations of major dietary minerals and some minor minerals within the recommended safe limits (Aderinola and Kusemiju, 2012), their chromium levels are above the WHO/FAO (1999) safe limit of 1.30 mg/kg. Although the chromium content of the RENA samples also exceeded this safe limit, the concentration was significantly lower than that of the planting control (Table 4).

4. Conclusion

The degradation of phenanthrene and anthracene over time observed in our study supports the finding that human health may not be at risk from these two priority pollutant PAHs at total petroleum hydrocarbon (TPH) crude oil: soil concentration below 10,000 mg/kg (McMillen et al., 2001) while the Ca:P and Na:K ratios in the harvested vegetable samples from all treatments suggest that they are within the recommended limit to maintain human health. RENA and bioaugmentation enhanced the degradation of PAHs at an initial TPH cut-off value of 5,000 mg/kg, but only RENA supported the optimum growth of *Brassica juncea* and had no negative effect on the nutritional composition of the harvested samples (major and minor dietary minerals) with the exception of zinc. RENA therefore performs the dual purpose of enhancing polyaromatic hydrocarbon degradation in soils and also supporting agrifood production. However, it will be necessary to analyse the level of PAHs bio-accumulated by harvested plants from RENA-treated sites in order to achieve a complete human health and safety assessment.

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