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Community level physiological profiling in a first generation phytoremediation experiment

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While biological methodologies have gained patronage in remediation of petroleum-impacted farmlands, only limited records are available on the functional ecosystem structure of such treated soils. Here, we conducted successfully a first generation soil heavy metal clean up in a 90d 4-factor phytoremediation pot experiment and studied the effect of such clean up on microbial communities. Influences with organic manure application were also evaluated. Microbial ecology of community was evaluated using BIOLOG. On analysis using multivariate factor, we observed correlation of consumption rates for polymer, carbohydrates, amines and amides correlates with metabolic patterns in the studied communities. Analysis of microbial diversity using Shannon H index, identified indigenous *Hevea brasilensis* cultivated soil populations with the highest diversity in polluted regimes and were more resistant, maintaining a steady growth after day 1 for the 9 days of incubation study. Lead removal was efficient using all four species studied with or without soil conditioner. *Vigna subterranea* may be a poor candidate for remediation of Cr contaminated soil following poor results obtained. Generally, legumes and indigenous plant species promoted distribution of communities more equitably among species. This study reveals the importance of plant-based bioremediation in microbial ecology and highlights the significance of soil conditioners and plants in microbial community behaviour.

Keywords: Crude oil pollution, Functional ecosystem, Heavy metals, *Hevea brasilensis*, Microbial community behaviour, Plant-soil interactions, Soil conditioners, Substrate consumption rate, *Vigna subterranea*

Given the heterogenous contaminants observed in constituents of Bonny light crude oil[1], and successes recorded with the biological methodologies at clean up of petroleum polluted soils, evaluating the functional ecosystem structure may be an edge in assessing the acclaimed site clean up performances, especially with fertilization[2-4]. Physiological profile characterization in a complex environment such a the petroleum impacted soil has been shown by Lladó and Baldrian[5] to explain the metabolic versatility of the entire bacterial communities in such ecosystem. Several researchers have reported adverse effects of crude oil pollution on soil[6], seed germination[7] and plant growth[8]. Heavy metals pose potential human health concerns when concentrations are high in soils and may be harmful to humans via ingestion of edible plants containing metals through normal uptake, splashed with contaminated soil or by accidental direct ingestion of soil, usually by children[9,10]. Similarly, breathing in dust from such soils may also pose a health risk. These metals include As, Cd, Cr, Cu, Pb, Ni, Se, and Zn. High levels of Pb in soil has been identified from paint and gasoline emissions while As-bearing pesticides and leaching from pressure treated lumber have been implicated in As pollution. Industrial activities or land-application of biosolids from wastewater treatment, and various by-products (animal/poultry wastes, fly ash, etc) that are land-applied due to their fertilizer value have been implicated[11] in some polluted soils.

Remediation of such polluted environments by cultivating suitable plants with symbiotic microbial communities yields promising results[10,12-14]. In this context, here, we studied the scope of four selected plants *viz*., *Vigna subterranea*, *Hevea brasiliensis*, *Cymbopogon citratus* and *Fimbristylis littoralis* for possible remediation of soils polluted with heavy metals such as chromium and lead. Bambara groundnut *Vigna subterranea* (L.) Verdc, an indigenous African legume, plays an important socioeconomic role in the semi-arid regions of Africa[15]. It is a drought resistant crop that can grow in

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marginal, low-input, environments[1]. *Hevea brasiliensis* is a commonly distributed species of tropical rainforest and also found growing in wet oil contaminated areas in the western part of the Niger Delta. It is the most economically important member of the genus Hevea. *Cymbopogon citratus* is a tall perennial tropical grass with good fibrous root system and vigorous deep root rhizomes that can check soil erosion and surface spreading of hydrocarbon contaminants. Distributed over western Niger Delta areas is also *Fimbristylis littoralis*, a weedy grass with a good fibrous root system. Considering these attributes, they were selected to evaluate their suitability for use in the phytoremediation of crude oil and associated contaminants polluted soils. Overall in this study, we tried to evaluate the suitability of Bambara plant, Rubber plant, Lemon grass and Fimbristylis for use in phytoremediation of chromium and lead polluted soils; the microbial functional diversity at such environments and the overall influence of soil conditioner applications.

Materials and Methods

Study site and experimental set - up

The surface (0-20 cm) soil used in this study was collected using a stainless steel hand auger in a randomized sampling quadrat design, from a fresh crude oil polluted agricultural soil in a Niger Delta community, Akala-olu in Ahoada Rivers state of Nigeria, following a field reconnaisance. This soil was reported to have caught fire soon after the oil spill and eye witnesses suspect sabotage as the cause of spill. Soil from a fallow-lying agricultural soil (with no history of pollution) from the Faculty of Agriculture, University of Port Harcourt served as control. Soil samples at 0 and 90 d were air-dried, gently crushed, sieved (2 mm), and used for basic characterization analysis using the method of Bradley *et al*.[16]. A phytoremediation pot experiment was designed using Bambara plant, Rubber plant, Lemon grass and Fimbristylis. Three replicates in 45 × 45 cm polyethylene pots, containing 6 kg soils per pot were watered and allowed to equilibrate for 2 weeks. Amount of characterized amendments for application, was calculated from the method of Akobundu[17], C = (R × A)/Q (where C = amount of amendment (animal dung), R, a constant = 2, A = weight of soil, and Q = product weight of substance as it is bagged). The pots were perforated for easy draining and aeration while growth indices were measured weekly for the duration of the experiment. Seedlings of Bambara and Rubber plants were raised on moist cotton lagged tray while those of Lemon grass and Fimbristylis were sourced directly from Botanical garden of the University of Port Harcourt Nigeria. Up to 12 seedlings were transplanted unto each pot and were thinned down to four after 14 d. Weeded plants were left on their various individual pots for duration of the experiment.

Sample preparation

At harvest, plant tissues were separated into root and shoot sections, carefully washed with deionised water and prepared for laboratory analysis. Both, shoots and roots, were oven dried at 65°C until constant weight and dry weight of biomass was measured. Homogenized soils were prepared following standard protocol, in ice chests coolers and transported to Institute of Agrophysics Lublin Poland for soil microbial assay. Agilent ICP-MS-AAS was used for elemental analysis following manufacturer's instruction.

Analytical methods

Physicochemical analyses

Total organic carbon in soil was determined by oxidation method described in Kalembasa & Jenkinson[18] while potentiometric pH was measured using portable Hannah pH meter. Kjeldahl method was employed for soil total nitrogen measurements while gravimetric method was used for moisture content determination. Total oil and grease content analysis was done using Horiba Oil content analyzer OCMA-350. Analyses were strictly conducted using scientifically accepted methodology and analar grade reagents.

Community level physiological profiling, (CLPP)

CLPP was done using BIOLOG Microstation™ Biotek Instruments USA. One gram soil was added to 99 mL sterile peptone water and calibrated using a peristaltic pump, and then autoclaved (Steril Clave 18 BHD Caminox 2009) at 121°C for 20 min alongside tips and tubes. Samples were shaken for 20 min at RT, followed by 30 min incubation at 4°C. Samples were shaken for 1 min and left to settle. The suspension of microorganisms (120 µL) were innoculated into labelled BIOLOG Ecoplates and first reading was taken thereafter as zero hour. There after, at 24 hourly intervals, up to 9 days the BIOLOG readings (27°C incubation) were taken. Shannon diversity indices were adopted for community diversity analyses.

Statistical analysis

The area under the 'absorbance versus time' curve was used for statistical testing to compare individual carbon source utilization. Also, a multivariate pattern analysis of community metabolism of the BIOLOG EcoPlates carbon sources was done. Analyses of variance and principal component analysis for triplicate data in this study were done using STATISTICA vs 10.

Results and Discussion

Community level physiological profiling (CLPP) revealed differences in the microbial community structure with increased heavy-metal contamination and conditioning (Fig. 1), as three major community cluster were identified. To account for differences in innocula densities, average well colour development (AWCD) was calculated. Garland[19] proposed an analysis point based on the time required to attain AWCD absorbance value such as 0.75. This point was identified on day 1 (Fig. 2), with conditioner influences. In relation to pollution, soil conditioner notably decreased the lag times and showed mixed effects for rate of colour development, maximum absorbance and overall community pattern. Less community degradation as indicated by decreased area under curve was observed for all cultivations, except with Bambara and Fimbristylis (Fig. 2).

Mutivariate factor analysis gives information on "organizing principle" of whatever is being measured with a number of observable measures (here Amines and amides, polymer, carbohydrates, carboxylic acids and amino acids consumption rates). Factor loadings indicate how each "hidden" factor is associated with the "observable" variables used in the analysis. Factor loadings of 0.91 and 0.78 (Fig. 3) indicate that percentage amines and amides and polymer substrates consumption can be used to describe hidden Factor 1; in other words, factor 1 has characteristics similar to the aforementioned consumption rates. Negative factor loading (–0.83) for carboxylic acid substrate

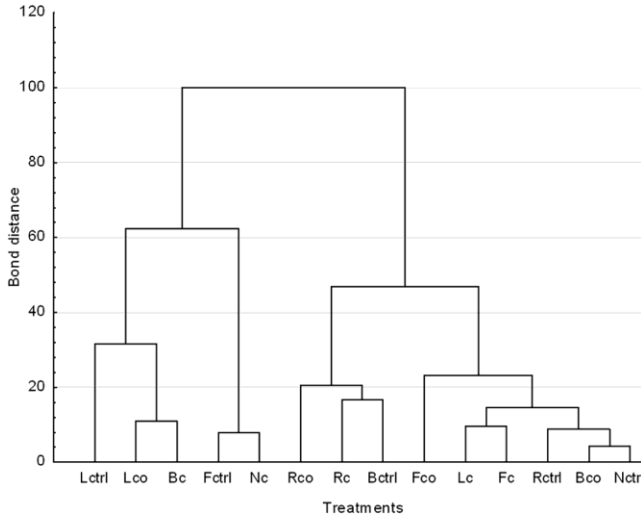

Fig. 1 — Tree cluster of community profiles in study soils. B, L, F, R, and N denotes Bambara, Lemon grass, Fimbristylis, Rubber, and unplanted regimes. Ctrl, co, and c denote control, crude oil polluted and organic manured soil and crude oil polluted and unamended soils.

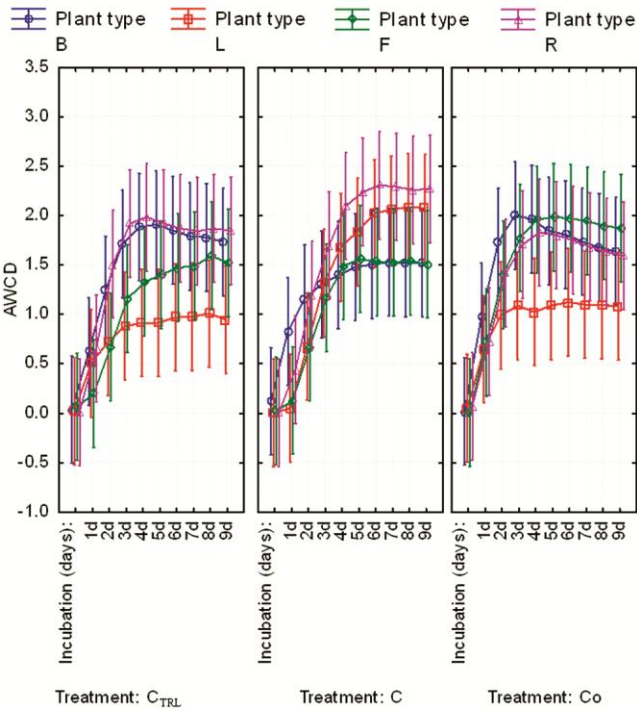

Fig. 2 — AWCD versus time variance with plant type and treatment. Vertical bars denote 0.95 confidence intervals

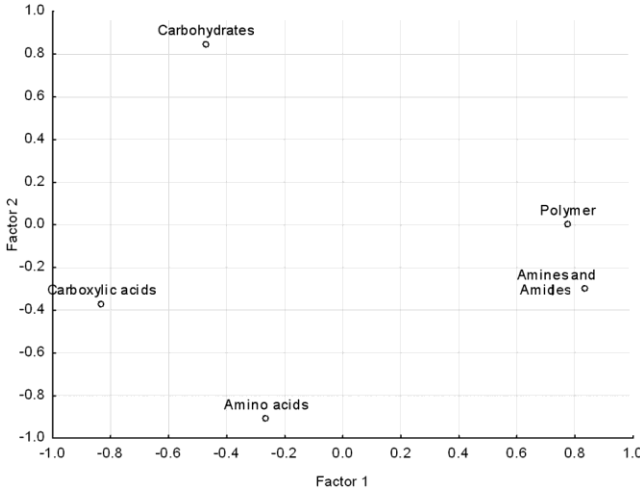

Fig. 3 — Multivariate principal component analysis of carbon source utilization

consumption rate, means that the hidden Factor 1 has the characteristics "opposite" of whatever consumption of this carbon source measures. Also, factor loading of 0.84 indicates that % carbohydrates consumption can be used to describe second hidden principle but factor loading of –0.91 implies that factor 2 has characteristics 'opposite' to whatever amino acids consuption rate measures. Amines and amides, polymer, and carbohydrates consumption rates were therefore selected to more directly compare with metabolic patterns and this agrees with observed pattern in Fig. 4.

Rate and utilization of different carbon substrates in BIOLOG wells after day 3 which gave relatively better growth pattern in comparison to control soil communities, were studied. Contamination with hydrocarbons and associated types increased amines and amides consumption, except in Bambara cultivated regimes and showed highest increment in lemon grass cultivated regimes. Conditioner (animal dung) application however, increased observed rate only in Bambara and Rubber cultivated communities (Fig. 4). Similarly, rate of polymer consumption, increased with pollution except for Rubber cultivated communities. Highest increment was recorded for Lemon grass cultivated communities. In relation to control, influence of conditioner was evident in increased consumption rate except for Lemon grass cultivated regimes. Consumption of carbohydrates in all polluted and unamended regimes, increased except for those cultivated with Lemon grass. Differences in pH (near neutral in amendments)[20] in the new environment (EcoPlates) as compared to their background pH (acidic) may have influenced observed community structure. Reduction in carbohydrate substrates however, was observed with communities for Bambara and Rubber cultivated regimes in amended variants. For Carboxylic acid carbon substrates, uniform reduction was observed with no significant change for Bambara cultivated unamended polluted regimes. This trend was reversed in conditioned variant except for Bambara cultivated communities where reduced consumption rate was observed. Consumption rate for amino acids sources decreased in polluted unamended regimes for Bambara and Fimbristylis cultivated soil communities. Conditioned polluted soil communities however, reduced amino acid consumption for all regimes. Such shifts were observed with sewage amendments by Guckert *et al*.[21]. Comparing within each plant treatments, only amines and amides and polymer consumption rates increased with conditioned polluted variants for Bambara cultivated soil communities. Amines and amides as well as amino acid consumption rates showed decreased pattern from those observed for unamended conterparts for Fimbristylis cultivated regimes. Starke *et al*.[22] posited that bacteria were majorly involved in the short term assimilation of plant-derived N and their depletion in hydrocarbon stress may have influenced observed consumption rate. For Lemon grass cultivated regimes, relief in decreasing consumption rate was seen in conditioned groups for carbohydrates and polymer carbon sources (Fig. 4).

Generally, there were observable shifts in carbon source utilization pattern with organic amendment for such mixed microbial communities and this agrees with the findings of Lladó and Baldrian[5] and Baldrian[23] that highlighted substrate dependent community shift in the original populations from the soil rhizosphere. Analysis of microbial diversity using Shannon H index, revealed highest diversity with native Rubber populations (Table 1) in polluted

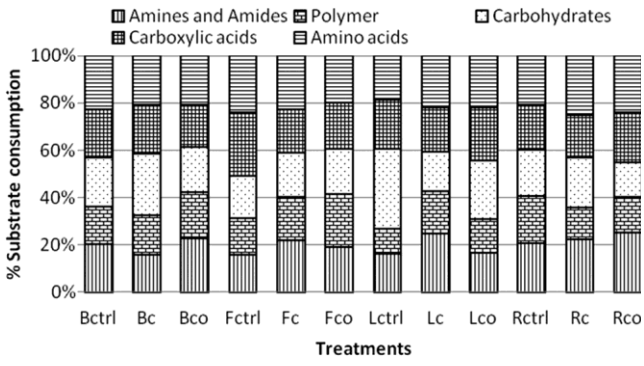

Fig. 4 — Carbon substrate consumption rate by soil communities

Table 1 — Microbial Diversity indices after day 1 of 37°C incubation. H, R, E and ENS denotes Shannon H inex, Richness, Evenness and Expected number of species

	H	R	E	ENS
Bctrl	2.84	24.00	0.89	17.12
Lctrl	2.60	74.00	0.60	13.46
Fctrl	2.80	23.00	0.89	16.44
Rctrl	3.18	40.00	0.86	23.93
Bc	2.70	30.00	0.79	14.88
Lc	2.20	163.00	0.43	9.03
Fc	2.40	30.00	0.71	11.02
Rc	2.73	37.00	0.76	15.40
Bco	2.80	28.00	0.84	16.44
Lco	2.50	38.00	0.69	12.18
Fco	2.50	25.00	0.78	12.18
Rco	3.14	34.00	0.89	23.06

regimes which were also more resistant and maintained a steady growth after day 1 for the 9 days of incubation study. Communities from legumes (Bambara) and native species (Fimbristylis and Rubber) cultivated soils gave relatively higher equitability values (Table 1). Expected no of species, ENS was defined by Tuomisto[24] as the number of equally abundant types needed for the average proportional abundance of the types to equal that observed in the dataset of interest. Rubber cultivated soil microbial communities, also gave the highest ENS while richness of species was highest in Lemon grass cultivated soil communities.

The pH range in all studied regimes was from 3.45 to 6.8, indicating acidic soils. The texture of study soil was clayey loam. Produced biomass as shown in Fig. 5 increased with contamination for Rubber and Fimbristylis plants and opposes the findings of Basumatary *et al*.[25] and Nwaichi *et al*.[3] that recorded decrease with contamination. These species however, are indigenous to study area and may have exhibited tolerance to posed toxicity. Lopez Martinez *et al*.[26] reported comparative advantage of such species over foreign or genetically modified species. Soil conditioning positively influenced biomass production but was significant only for Fimbristylis and Lemon grass. Initial Pb level (806.20 mg kg^{-1}), excceeding USEPA[27] set limit of 400 mg kg^{-1} in such soils were reduced by 87, 85, 86 and 86% using Fimbristylis, Bambara plants, Rubber plants and Lemon grass respectively (Fig. 6) in this phytoremediation study. Statistically (95% confidence level) significant differences in performance, with application of organic manure, were observed only in soil Pb removal as differences in decreased uptake capacities were not marked for all plants with conditioning and could be linked with organic matter transformation[22,28]. Similarly, increased reduction percentages were observed in soil Cr levels at 90 d with conditioner application from initial value of 30 mg kg^{-1}. Clean up potential increased from 50, 26, 47, and 52-60, 40, 65 and 63% with application of organic manure for Fimbristylis, Bambara, Rubber and Lemon grass respectively (Fig. 7A). Effect of amendments at Cr shoot and root uptake potentials were only significant with Lemon grass, although there was evidence of contaminant immobilization by conditioner used. There may have been influences due to induced cellular defense system in the root as reported by Jiang and Liu[29] and Kumar *et al*.[30]. USEPA Cr limit for unrestricted use soils is 11 mg kg^{-1}, based on human health risk. Although Fe levels were reduced signicantly from initial soil levels of 2064250 mg kg^{-1}, abundance of Fe in this area influenced observed performances

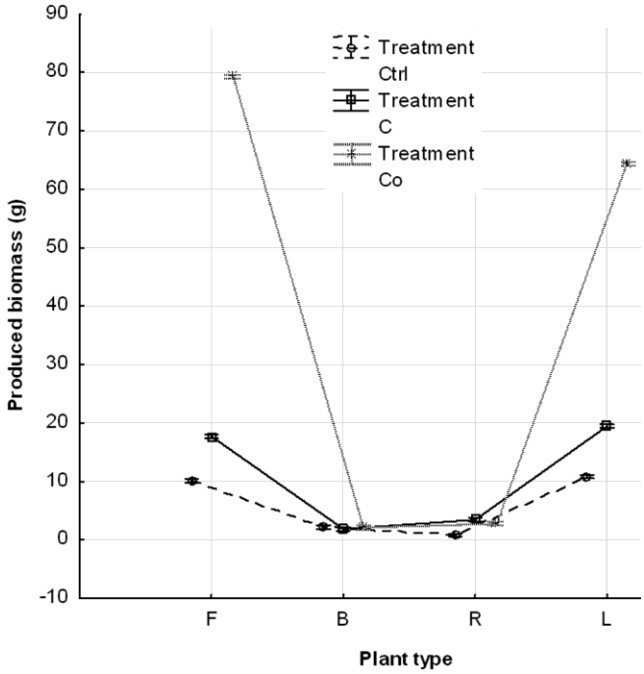

Fig. 5 — Variation of produced biomass (n=3) at 90 d

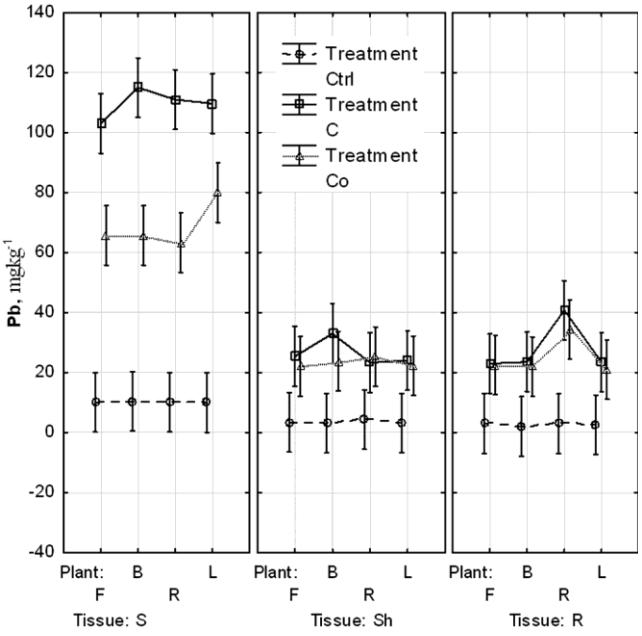

Fig. 6 — Variance of Pb levels (n = 3) among treatments, plant type and plant part/ soil. F, B, R, L, S, Sh and R denotes Fimbristyli, Bambara, Rubber and Lemon grass, Soil, Shoots and Roots, respectively.

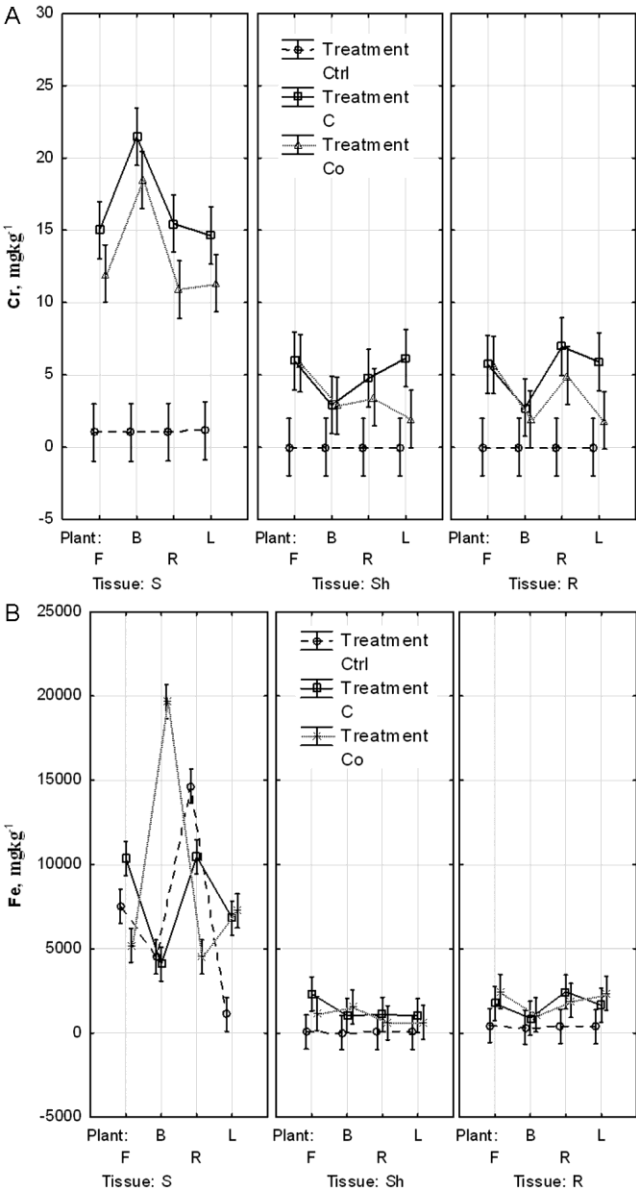

Fig. 7 — Variance of (A) Cr; and (B) Fe levels among treatments, plant type and plant part/soil

and did not show marked pattern as well in uptake potential (Fig. 7B). Statistical analysis of variance between treatments and distribution of contaminants (Fig. 8 A-C) showed consistent marked differences in contaminant fate only in soils. Comparatively, shoot and root tissue distribution showed no statistically significant difference with application of soil conditioner. It suggests that the transformation of study metals as low levels in these harvestable portions and are not reflective of failure given percentage removals obtained.

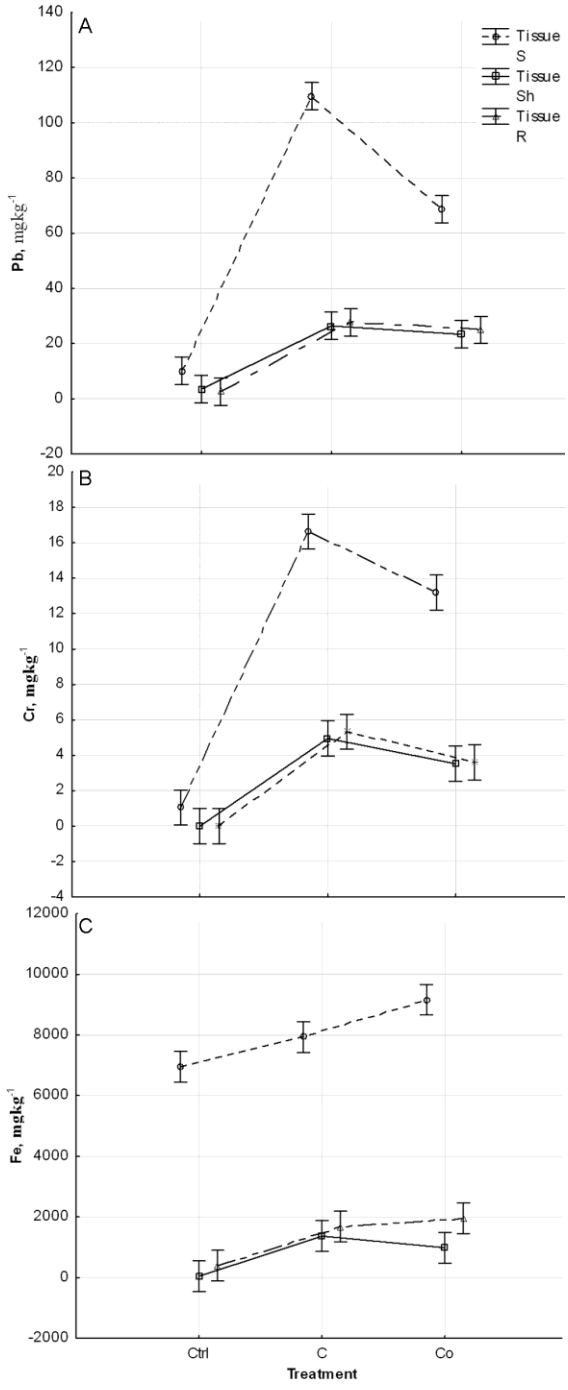

Fig. 8 — Variance of (A) Pb; (B) Cr; and (C) Fe levels between treatments and tissue

Conclusion

Results from this study indicate practical implications of phytoremediation in microbial ecology as technique preserved some communities while achieving good levels of contaminants removal. Influences with soil conditioners at phytoremediation

sites proved most useful within the soil matrix and improved the evenness of communities in a crude oil degraded soil diversity, and may be exploited. The comparative advantages of choice of plants for phytoremediation, therefore needs to consider the microbial ecology. Limitations in the study may include varied substrate concentration as those in the wells of the BIOLOG microplates may be much higher than those usually found in such parched environment.

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