Preliminary microbial studies on the impact of plants and South African fly ash on amelioration of crude oil polluted acid soils.

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ABSTRACT

Environmental changes as a result of pollution formed through energy production and use, affect soil pH, structure, fertility, and therefore fauna, flora and microbial communities. Soil pollution causes severe environmental disruption impacting agricultural practices and thus threatening food security, since affected soils become relatively sterile to all but resistant microbial life forms. Certain indigenous micro-organisms, are able to degrade pollutants in soil leading to *in situ* rehabilitation of polluted soils. Optimising their role in this process depends on understanding the diversity and function in these environments. Chemical changes taking place within the soil first impact microbial communities within the environment because they are the first to come into contact with these changes. Therefore, monitoring the microbial community shifts within target ecosystems can prove to be an effective means of determining the impact of pollution produced during industrial energy production and thus also the remediation of the environment during treatment thereof. Experiments were conducted in pots to assess the impact of pollution on soil microbial populations and on plant growth. Initial results, based on chemical tests, suggest the organic acids produced by soil inhabitants are able to degrade fly ash and release its liming potential. Using culture dependent and independent (denaturing gradient gel electrophoresis) microbiological techniques the soil communities of the trials have been mapped and shifts in microbial communities are evident as soils weather and begin to rehabilitate.

INTRODUCTION

Environmental changes due to various types of pollution, formed through energy production and use, affect soil structure, fertility, and therefore fauna and flora. Soil pollution causes environmental disruption impacting agricultural practices and thus threatening food security. Affected soils become relatively sterile to all but resistant microbial life forms. Certain indigenous micro-organisms, including bacteria and fungi, are able to degrade pollutants in soil leading to *in situ* rehabilitation of polluted soils. Optimising their role in this process depends on understanding the diversity and function in these environments.

Many organisms already present in an environment are capable of remediating pollution thus, the isolation and identification of microorganisms associated with soils

polluted during energy production and use is key. If land has been impacted by the energy industry, chemical changes taking place within the soil first impact the microbial communities within the environment because they are the first to come into contact with these changes. Therefore, monitoring the microbial community shifts within target ecosystems can prove to be an effective means of determining the impact of pollution produced during energy production and thus also the remediation of the environment during treatment thereof. After mapping microbial communities that can indicate when soil has reached an acceptable level of remediation, it is necessary to examine the ability of indigenous crops, and pasture grasses, to continue this bioremediation, thus providing rural communities not only with more arable land but also food security.

Fly ash is an alkaline product of fossil fuel power generation. It has a pH of approximately 11.5 when fresh and this value reduces over time (weathering) to stabilise at around 8.5. Owing to the process by which it is formed, fly ash is considered sterile. However, microbes are capable of adapting to extreme conditions such as those present within fly ash. The utilisation of fly ash for agriculture and rehabilitation is a well documented topic[17,19], fly ash has the potential to make positive contributions to agriculture and land reclamation as a liming agent. Fly ash can result in improved soil texture, water holding capacity, pH, soil fertility and plant productivity[1,17]. Most elements in fly ash occur as silicates, oxides, sulphates and alumino-silicates. When used in agriculture and mine reclamation, the application of fly ash benefits the soil by improving soil fertility[17]. Due to the presence of trace elements in the fly ash it has been applied to soils in order to correct micronutrient deficiencies of B, Mg, Mo, S and Zn[11,4,6,7,15]. Due to the long-term dissolution, the neutralizing capacity of fly ash is extended offering a liming potential that lasts longer and is less dramatic than agricultural liming[10]. This slower transmission of pH change allows for adaptation of the microbial communities and plants within the polluted soils. This is directly related to determining the possibility and extent of soil amelioration by microbes with the industrial by/waste-product fly ash.

This study represents the initial phase of an envisaged ongoing investigation to determine the impact on microbial diversity of fly ash added to agricultural soils and to polluted soils.

MATERIALS AND METHODS

Experimental layout

An experimental layout of the greenhouse pot trial was established using five (5) repeats to each treatment incorporated into 3L pots. Oil and ash mass added to the pots was corrected (table 1). The pot trial was laid out using an acidic soil with different levels of oil and ash (table 2). Before experiments began the soil being used was analysed for various parameters (table 3).

Table1: Application rate of oil and ash per treatment in 3L pots.

Low ash application				High ash application			
Concentration	Oil	Ash	Total	Concentration	Oil	Ash	Total
2ml kg^{-1}	6g	13g	19g	2ml kg^{-}	6g	26g	32g
5ml kg^{-1}	15g	13g	28g	5ml kg^{-1}	15g	26g	41g
20ml kg^{-1}	60g	13g	73g	20ml kg^{-1}	60g	26g	86g
40ml kg^{-1}	120g	13g	133g	40ml kg^{-1}	120g	26g	146g

Table 2: Shows the pot trial layout including each treatment. Thus 1 set of pots (green blocks) is 15 treatments and this is run across 5 replicates and at 3 plantings (no plant, Soya, Smuts finger) yielding a total of 225 pots.

Description	Oil Conc.	Ash Conc.	Acid Soil	Soya	Grass
controls	none	None (0 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		Low (50 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		High (100 ton/ha)	3 kg	1 plant/pot	1 plant/pot
Oil	2ml kg^{-1}	None (0 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		Low (50 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		High (100 ton/ha)	3 kg	1 plant/pot	1 plant/pot
	5ml kg^{-1}	None (0 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		Low (50 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		High (100 ton/ha)	3 kg	1 plant/pot	1 plant/pot
	20ml kg^{-1}	None (0 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		Low (50 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		High (100 ton/ha)	3 kg	1 plant/pot	1 plant/pot
	40ml kg^{-1}	None (0 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		Low (50 ton/ha)	3 kg	1 plant/pot	1 plant/pot
		High (100 ton/ha)	3 kg	1 plant/pot	1 plant/pot

Table 3: Soil properties as recorded at the beginning of the pot trail.

Parameter	Reading	Units
pH	6.6	
Total N	570	mg/kg
Total C	0.7	%
C/N Ratio	12.28	
Total P	220.7	mg/kg
P Bray	9	mg/kg
Total K	788.55	mg/kg
Total Ca	864.3	mg/kg
Total Mg	473	mg/kg
K	115	mg/kg
Ca	511	mg/kg
Mg	146	mg/kg
Na	5	mg/kg
Sand	84.4	%
Silt	3.8	%
Clay	11.3	%
Texture	Loamy sand	
CEC	7.49	cmol(+)/kg

Microbial culturing

Biological reproducibility was confirmed across replicates and bacterial counts were determined from composite soil samples. One gram of soil from each composite sample was placed in a tube containing 9 ml Ringers solution and 30 glass beads. The solution was shaken for 2 minutes in order to dislodge bacteria from soil particles. These solutions were used to make 10-fold serial dilutions and 0.1 ml of the diluents was plated onto half-strength nutrient agar plates. The plates were incubated at ambient temperature and colonies were counted after 48 hours.

DNA extraction

Total genomic DNA extraction from samples was performed using the ZR Soil Microbe DNA Kit™ (Zymo Research, Inqaba Biotec, Pretoria) is being maintained in stasis at –20°C at the University of Pretoria.

PCR

A portion of 16S bacterial gene of the rDNA was amplified, for DGGE, by means of PCR using the K and M primers below:

K: 5'ATT-ACC-GCG-GCT-GCT-GG3',[16]

M: 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG3' [5]

A reaction with no template DNA was included as a negative control for each PCR. Each PCR tube contained a total volume of 20 µl: 7 µl filter sterilised SABAX water, 10 µl of 2x PCR ready mix (Lucigen™ EconoTaq® Plus Green), 1 µl primer K (50 µM), 1 µl primer M (50 µM), 1 µl template DNA (27 ng/µ). Prokaryotic DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C, followed by 10 min at 72°C, and then held at 4°C. PCR product was analysed on a 1.5 % TAE agarose gel.

DGGE

PCR product was subjected to DGGE according to the method described by Muyzer *et al.*[13]. Seven microlitres (ca. 250 ng) of 16S PCR product was loaded per lane onto 45-55% denaturing gradient gels. A composite sample of PCR products from each replicate of 5 pots was loaded in order to determine reproducibility between PCR-DGGE analysis. Gels were run at 70V for 17hrs at a constant temperature of 60°C. Image analysis was performed using the Gel2K[14] programme and fingerprints were analysed in a cluster investigation using CLUST[14]. Dominant bands were compared and analysed for population diversity determination.

RESULTS AND DISCUSSION

The highest average colony count for treatments without plants was for ash applied at 50 tons/ha. This showed an even higher count than the average attained for the untreated control soil (table 4). This could possibly be due to an immediate liming effect as a result of the addition of fly ash causing an increase in bacterial species richness. Ash applied at 100 tons/ha shows a marked decrease in species richness possibly due to too drastic change in the environmental pH (fig. 1). The chemical and biological effects of soil amelioration with fly ash are as a result of increased mobility of calcium and hydroxide ions due to pH fluctuation[19].

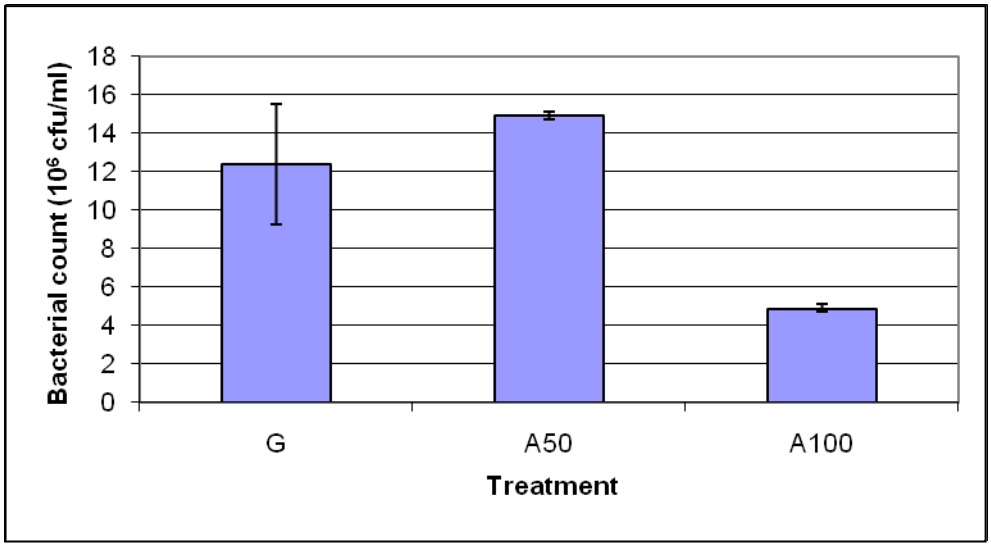

Figure 1. Bacterial counts of control and ash-treated soils.

Treatments with plants showed a overall higher average bacterial count. Roots improve physical and chemical properties within pollutant-stressed soil, by increasing contact between associated microbes and pollutants[2]. Soya treated with ash at 50 tons/ha showed the highest bacterial count (fig. 2) and this is to be expected as ash application at this rate is optimal for plant growth[18]. However, soya in general displayed the best growth conditions for bacteria in the control and ash ameliorated soils. Plant species involved in phytoremediation include various grass species and leguminous plants[9]. It is hypothesized that pots containing oil at various levels, which showed an increase in bacterial counts, did so because the oil provided an alternate carbon source. Bacteria degrade linear hydrocarbons via mono-terminal attack, forming a primary alcohol, an aldehyde and a monocarboxylic acid. Further biodegradation is via β-oxidation forming a two-carbon unit, shorter fatty acids, acetyl co-enzyme A and CO_2[3].

Table 4: Bacterial counts including repetitions and average count per treatment

Treatment	Plant	10^6 cfu/ml (rep 1)	10^6 cfu/ml (rep 2)	10^6 cfu/ml (rep 3)	Average
G (soil)	none	8.80	14.70	13.60	12.40
A50	none	15.10	15.00	14.70	14.9
A100	none	4.90	4.70	5.10	4.90
2ml	none	5.90	4.70	4.50	5.03
5ml	none	4.40	3.10	3.50	3.67
20ml	none	5.40	6.40	5.60	5.80
40ml	none	6.00	1.00	4.00	3.67
A50 + 2ml	none	0.00	4.00	2.00	2.00
A50 + 5ml	none	4.60	5.40	4.90	4.97
A50 + 20ml	none	4.50	4.50	4.70	4.57
A50+ 40ml	none	3.60	3.30	3.40	3.43
A100 + 2ml	none	5.10	3.40	4.80	4.43
A100 + 5ml	none	3.80	3.20	3.60	3.53
A100 + 20ml	none	2.60	1.60	2.10	2.10
A100 + 40ml	none	2.00	1.50	1.70	1.73
G (soil)	smuts	9.60	8.80	9.10	9.17
A50	smuts	29.20	26.70	25.90	27.30
A100	smuts	85.00	79.00	82.00	82.00
2ml	smuts	7.40	8.10	7.90	7.80
5ml	smuts	22.80	20.80	21.00	21.50
20ml	smuts	20.00	22.20	22.00	21.40
40ml	smuts	5.90	4.00	5.30	5.07
A50 + 2ml	smuts	22.40	22.00	22.70	22.40
A50 + 5ml	smuts	8.40	13.40	9.10	10.30
A50 + 20ml	smuts	23.30	25.70	24.10	24.40
A50+ 40ml	smuts	5.10	4.30	4.80	4.73
A100 + 2ml	smuts	18.30	13.30	14.40	15.30
A100 + 5ml	smuts	78.00	99.00	84.00	87.00
A100 + 20ml	smuts	9.40	14.20	11.40	11.70
A100 + 40ml	smuts	5.80	4.10	4.90	4.93
G (soil)	soya	106.00	91.00	99.00	98.70
A50	soya	172.00	16.90	154	165
A100	soya	113	113	119	115
2ml	soya	5.00	4.10	4.70	4.60
5ml	soya	21.60	25.60	23.40	23.50
20ml	soya	27.00	23.50	25.10	25.20
40ml	soya	3.10	0.8	3.60	2.50
A50 + 2ml	soya	37.00	40.00	39.00	38.70
A50 + 5ml	soya	35.00	33.00	30.00	32.70
A50 + 20ml	soya	14.40	21.00	16.40	17.30
A50+ 40ml	soya	5.50	12.40	7.10	8.33
A100 + 2ml	soya	20.10	20.30	21.00	20.50
A100 + 5ml	soya	38.00	36.00	33.00	35.70
A100 + 20ml	soya	5.60	6.20	5.80	5.87
A100 + 40ml	soya	4.30	10.70	6.40	7.13

Average bacterial plate counts across treatments increased progressively from 5.15 ($x10^6$ cfu/ml) for pots containing no plants, 23.66 ($x10^6$ cfu/ml) for pots containing smuts finger and 40.05 ($x10^6$ cfu/ml) for pots containing soya. It is evident from this that the presence of plants dramatically increases bacterial numbers and that different crops stimulate this growth at different rates. This is supported by studies which have shown that root length, surface area, volume and diameter play a role in the rehabilitative effect of plants in crude oil-polluted soil[12].

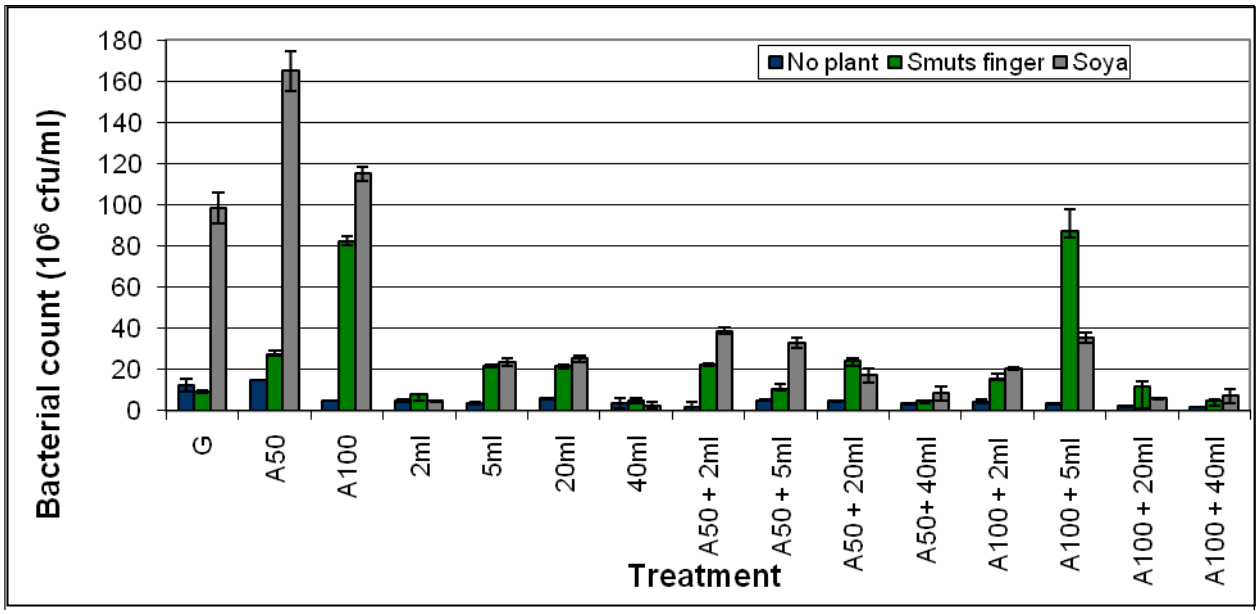

Figure 2: Bacterial counts of control, ash and oil-treated soil with and without plants

Initial bacterial culture based plating techniques indicate that ash at the application rate of 50ton/ha is the most supportive of microbial growth in the absence of plants (fig. 2). However, bacterial counts (in the absence of plants) decrease with increasing oil concentration. There is also initial evidence that microbes are growing better in soils polluted up to 20ml/kg of oil. Pots containing plants showed a higher overall bacterial count, this is to be expected due to the nutritional advantage to be gained from association with plant roots and exudates.

DNA was successfully extracted from all samples collected (fig. 3). No evidence of RNA or protein contamination that could inhibit further application of DNA is visible either below the lanes or in the wells of the gel, respectively. It is important to note that although some of the DNA appears to be of low concentration, this is still application ready DNA and will still yield a PCR product for further analysis.

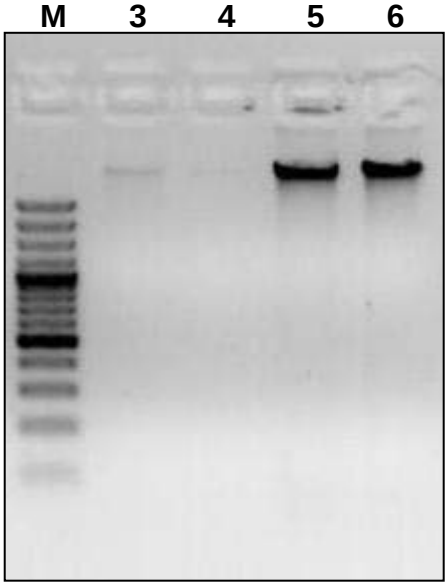

Figure 3: 1.5% TAE agarose gel showing high-quality, clean genomic DNA extracted from soil samples by means of the BIO101 Fast DNA Spin Kit for soil.

PCR of prokaryotes was successful yielding a ca. 510bp PCR product on a 1.5% TAE agarose gel (fig. 4). The negative control lane (first in row) shows that there was no contamination of the reaction and that PCR product is thus a true indication of the microbial population being targeted.

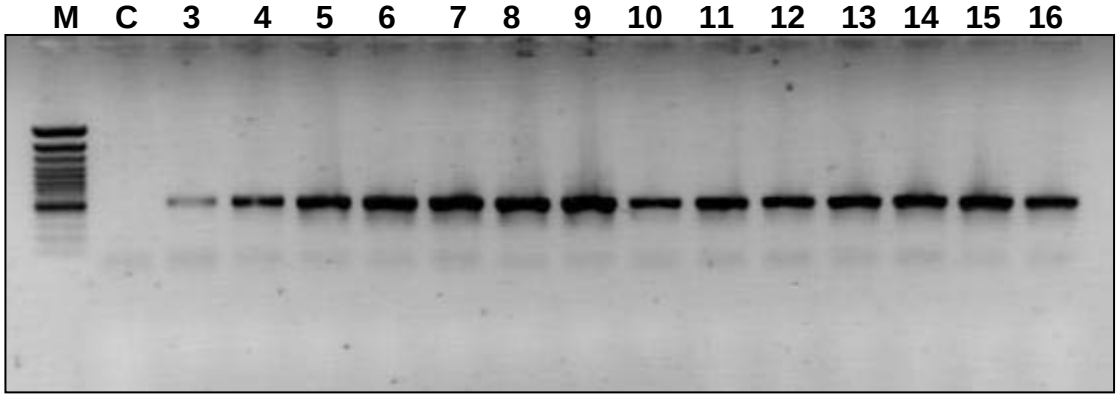

Figure 4: 1.5% TAE Agarose gel, showing 5µl of PCR product from each of the 16S bacterial gene amplifications.

DGGE yielded gels showing clear multiple banding, forming a fingerprint in each lane (Fig. 5). These gel images were loaded into Gel2K[14] and a graphical image of the gel was produced for further species diversity bioinformatics analysis. Dominant species per lane are indicated as dark prominent bands across the lane.

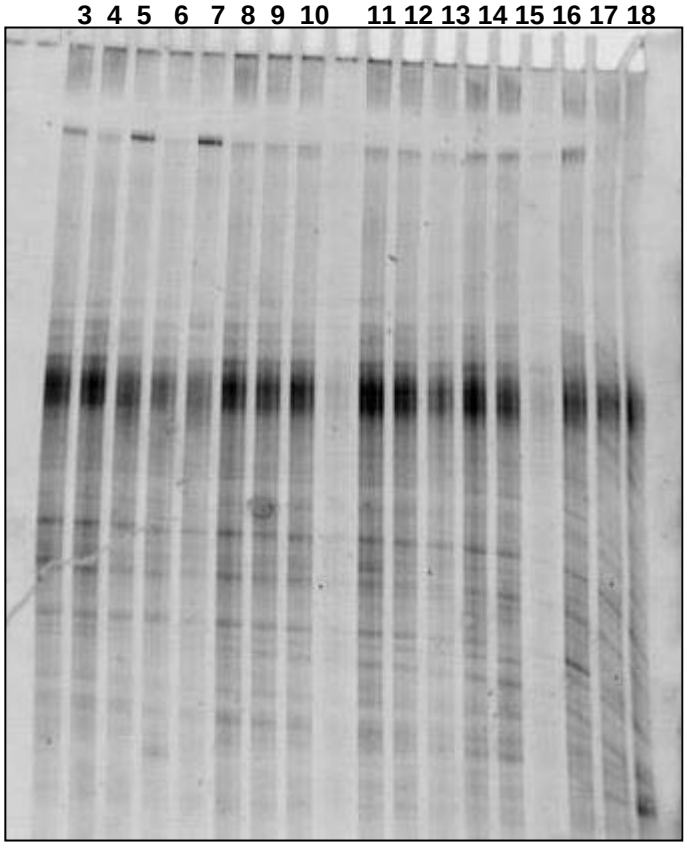

Figure 3: DGGE gel showing species diversity of bacteria from soil samples, run at 45-55% denaturants. PCR product is separated according to base-pair sequence differences to determine community richness and diversity of microorganisms based on these fingerprints.

Fingerprints were analysed independently and the focus was initially on the control and fly ash treated soils (fig. 4 & 5). Samples grouped according to ash amelioration level with no ash in clade I, 50 ton/ha in clade II and 100 ton/ha in clade III. Sample 2 (weathered ash) was added for resolution to indicate that over time ash does build up a microbial population (fig. 5). The number of dominant bands for each treatment was equivalent for soil without ash and with ash applied at 50 ton/ha. However, the soil treated with 100 ton/ha showed a slight drop in microbial diversity as seen with the cultured species richness in figure 1 above. Several bacterial species persisted between treatments while others disappeared from the diversity profiles. In this case, soil disruption caused by the addition of fly ash impacted biodiversity and, as shown in the study of Kozdrój and Van Elsas[8], chemical changes selected for microbial species better adapted to survive in the changed environment.

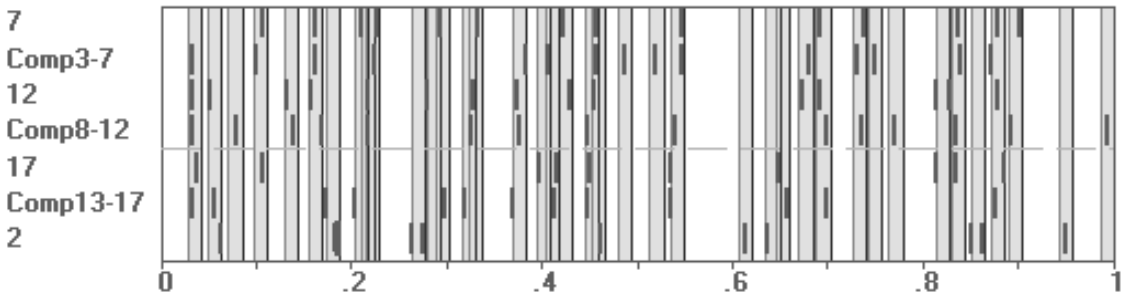

Figure 4: A graphical diagram of the banding pattern of PCR samples across the different single (7, 12, 17), composite (Comp3-7, Comp8-12, Comp13-17) and control ash (2) samples displayed.

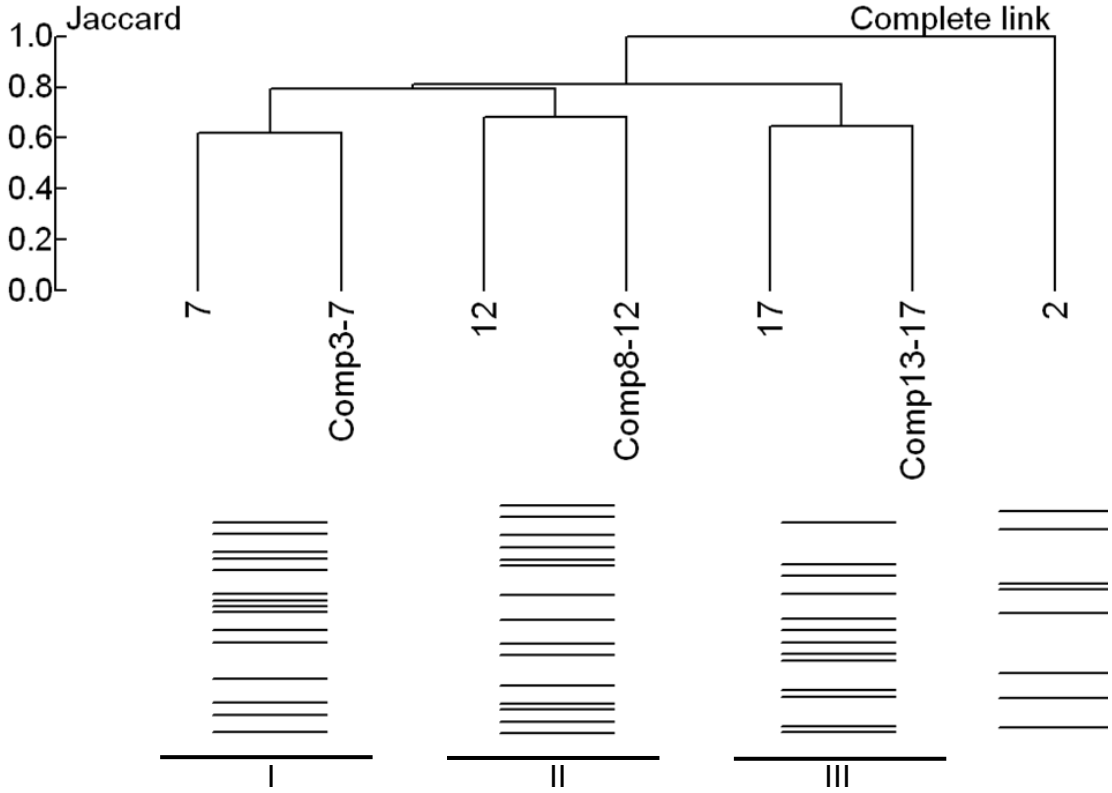

Figure 5: Cluster analysis of single (7, 12, 17), composite (Comp3-7, Comp8-12, Comp13-17) and control ash (2) samples using a Jaccard complete link setting.

Fingerprints were analysed independently and the focus shifted to all the fly ash and oil treated soils (fig. 6 & 7). The highest diversity is in clade I (fig. 7) where 5ml/kg of oil was added and 100 ton/ha of ash. However, the trend across treatments shows an erratic population reaction to oil pollution and ash amelioration. This is possibly due to these samples being taken soon after blending and thus a shorter time frame was available for bacteria to respond and/or adapt to the changed environmental conditions.

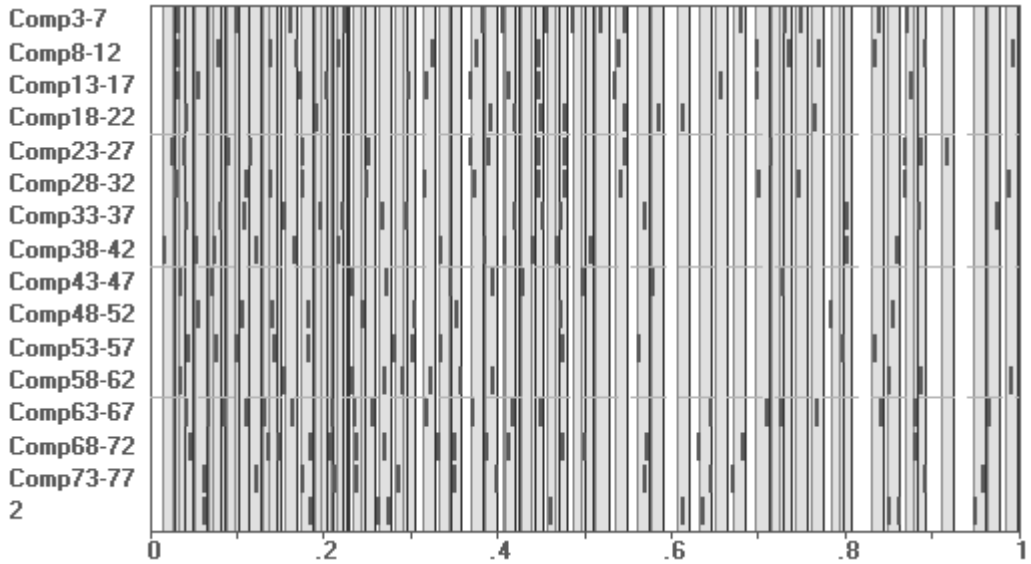

Figure 6: A graphical diagram of the banding pattern of composite PCR samples across the oil and ash treatments.

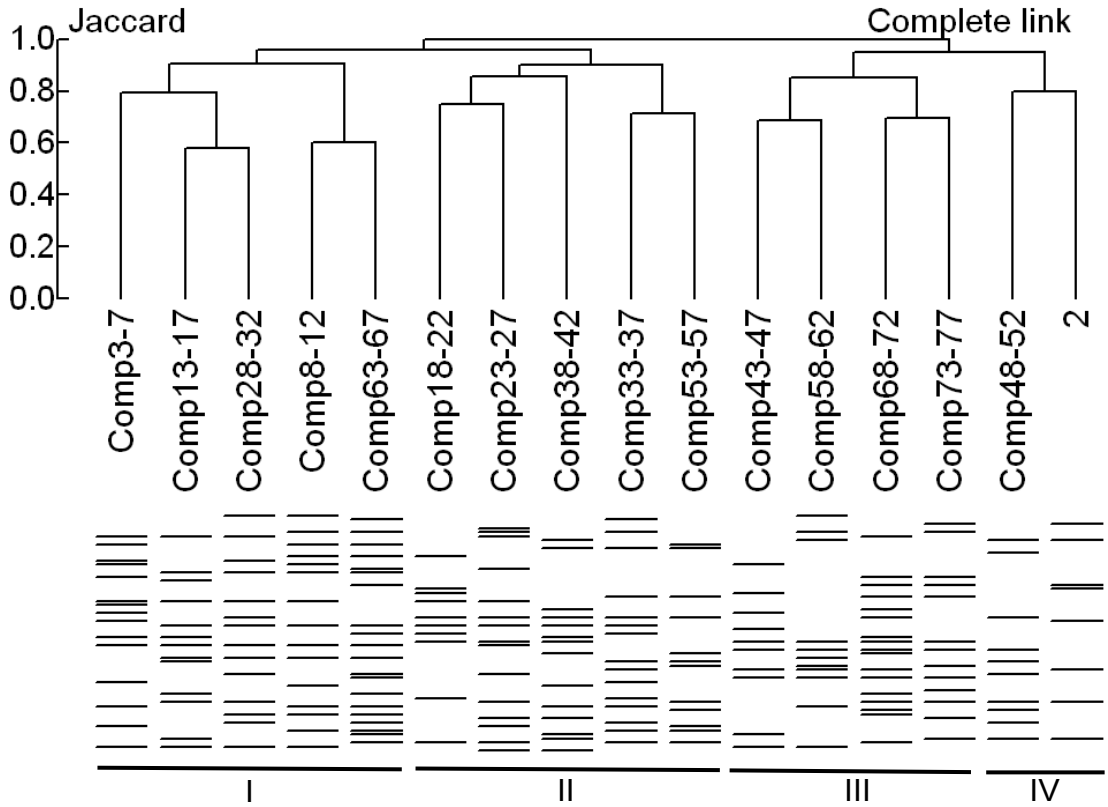

Figure 7: Cluster analysis of composite fly ash and oil treated samples and a control weathered ash (2) using a Jaccard complete link setting.

This study constitutes the first phase of an ongoing project targeting the remediation of oil polluted soils using fly ash as an ameliorant. Soils are expected to respond differently based on oil, ash and plant presence and/or type. The results presented here show that plants affect bacterial richness; this was shown by an increase the bacterial count during plating. Addition of ash is favourable depending on the application level, plant presence and oil pollutant level. Oil applied in the medium range during this study showed an increase in bacterial richness as it provided an alternate carbon source. Both the bacterial diversity and richness decreased when ash was applied at 100ton/ha in the absence of plants. There are several follow up phases to this study during which the longer term effect of the ash and the oil on the bacterial diversity and richness as well as the plants will be determined.

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