# Ash Microbiology: A Molecular Study

**Kelley A. Reynolds[1] and A. Karen J. Surridge[2]**

**[1]Eskom ERID, Private Bag 40175, Cleveland, 2022, Johannesburg, South Africa.**
**[2]MicroSci Consultants, PO Box 1387, Wapadrand, 0050, Pretoria, South Africa.**

**Keywords**: Ash microbiology, denaturing gradient gel electrophoresis, DNA, Polymerase Chain Reaction

## INTRODUCTION

Fly ash is a by-product of the fossil fuelled power industry. This product is a spherical aluminosilicate with trace level inclusions of non-organic elements which were present in the parent coal. It is highly alkaline with an initial pH of approximately 12 and high buffering capacity. Due to the very high temperature (<1700°C) process by which fly ash is formed, it must be considered to be sterile at the point of generation.

The coal ash from the power station boilers is collected through electrostatic precipitators and transported to an ash handling facility. In each case waste water is utilised as a wetting agent that, in downstream handling, may allow elements to be leached from the ash. The intrinsic nature of leachate has been attributed to chemical activities within the dump (Petrik *et al.*, 2001). These activities may include exothermic reactions of moisture with free lime, redox reactions between relevant oxidation states due to the pH change or ion solubilisation.

The test site is a dry ashing facility; i.e. ash is only conditioned to 10%, not slurried. The ash dump covers an area 1.8km long, 1km wide and 45m high. This site has two ash conveyors in the case of a breakdown. The second conveyor is routed along the side of the ash dump for dumping during emergencies. This stack is, however only operated on one level and only has one toe and drain. This ash is not subjected to rehabilitation as the remaining dump and is subsequently often over irrigated.

The ash is conditioned, post precipitators, to 10% moisture with waste water, to limit billowing (wind dispersion). This waste water consists of reverse osmosis (RO) brine, demineralisation train regeneration chemicals, cooling water blow down, final sewage effluent, station drains effluent and recovered mine water. Conditioned ash is conveyed from the power station to the ash dump and is stacked in large heaps, which are levelled and compacted by a grader to form the base level or front stack. Once the front stack has been formed, approximately 40m wide and 20m high, the stacker returns to the back stack or upper level and fills the next 20-25m. For this reason, the growing dump has two faces/stacks with a top and a toe each. Waste water is additionally used for dust suppression on the dump. A sprinkler system is networked over any un-rehabilitated areas with constant irrigation, to minimise particle distribution. This water percolates into the ash and either moves into the ground-water or to the toe drains. Toe drains run at the base of the lower stack, around the border of the dump collecting any run-off. Ash dumps are occasionally

used as rubbish dumps and debris (tyres, conveyor belting, tin cans and grease) can often be found at the base of the front stack.

Although the chemistry of maturing fly ash, on ash storage facilities, is well known, little or no information is available on its microbiology. It is accepted that some extremophilic microorganisms are capable of adapting to harsh environments (e.g. pH 10-11; low organic nutrients) such as those present within maturing fly ash. It is thus reasonable to hypothesise that leaching products may be generated, at least in part, by microbial action. In order to explain ash microbiology results, it is essential to understand the operation of the tested ashing system. This paucity of information justifies a molecular study of the microbiological populations of stored, weathered ash and of changes in the populations from fresh to matured ash.

Molecular methods used in ecological studies usually involve the separation of PCR product/amplicons on the basis of DNA nucleotide sequence differences, most often the 16S rRNA gene. Denaturing gradient gel electrophoresis (DGGE) is a most appropriate molecular method for monitoring microbial community ecology. It relies on variation in genetic sequence of a specific amplified region to differentiate between species within microbial communities (Banks and Alleman, 2002; Koizumi *et al.*, 2002). PCR product is electrophoresed through a polyacrylamide gel containing a linear DNA-denaturing gradient. The resulting band pattern on the gel forms a genetic fingerprint of the entire community being examined (Gillan, 2004). Most commonly, 16S rRNA genes are used to give an overall indication of the species composition of a sample since they can easily be compared on gene databases. Partial sequence of this gene has been analysed from environments as complex as soil (Throbäck *et al.*, 2004). Thus, global gene databases can yield invaluable information with regard to microbial organisms within communities that can be used as indicator organisms.

In this study the presence of microorganisms in fly ash was confirmed and DGGE was employed to map microbial community diversity present in fly ash and leachate. Two genes were targeted to give an overall impression of the prokaryotic (bacteria) and eukaryotic (Fungi/Plantae/Animalia) populations. Tentative identification of the prokaryotes was determined by DNA sequencing of DGGE fragments.

## MATERIALS AND METHODS

### Ash dump sampling

Samples were collected from a dry ashing test site in Mpumalanga, South Africa (S 26.77830; E 29.41525). Ash samples (5g) were taken from areas at various stages of weathering, ranging from fresh ash off the conveyor belts to ash which is approximately 10 years old. The chemical composition of the test fly ash and the wetting brine is shown in tables 1 and 2, respectively. It was sampled at a depth of approximately 10cm to ensure that samples were not contaminated by airborne organisms. Two samples of approximately 5ml of the ash leachate, from the dump toe drains were also collected for analysis. Details of all samples are shown in table 3. The samples were collected in sterile Whirl-pak™ bags. The samples were transported to the laboratory in a cooler box and stored at 4°C until DNA extraction could be completed.

Table 1: Chemical composition of test ash

| Element | Value % |
|---|---|
| Aluminium (as $Al_2O_3$) | 25.5 |
| Calcium (as CaO) | 5.5 |
| Carbon (as C) | 2.0 |
| Iron (as $Fe_2O_3$) | 4.0 |
| Magnesium (as MgO) | 1.9 |
| Manganese (as MnO) | 0.05 |
| Phosphorous (as $P_2O_5$) | 0.39 |
| Potassium (as $K_2O$) | 0.6 |
| Silicon (as $SiO_2$) | 54.3 |
| Sodium (as $Na_2O$) | <0.01 |
| Sulphur (as $SO_3$) | 3.7 |
| Titanium (as $TiO_2$) | 1.4 |

Table 2: Chemical composition of test site brine

| Component | Unit | Value |
|---|---|---|
| Alkalinity P | mg/l | BD |
| Aluminium (as Al) | mg/l | <0.06 |
| Ammonia (as N) | mg/l | 2.82 |
| Arsenic (as As) | mg/l | BD |
| Barium (as Ba) | mg/l | 2.52 |
| Bicarbonate (as $HCO_3$) | mg/l | 107 |
| Bicarbonates (as $CaCO_3$) | mg/l | 88 |
| Cadmium (as Cd) | mg/l | <0.005 |
| Calcium (as Ca) | mg/l | 372.8 |
| Chloride | mg/l | 4300 |
| Conductivity (at 25°C) | µS/cm | 28300 |
| Copper | mg/l | 0.052 |
| Fluoride | mg/l | 10.45 |
| Iron | mg/l | 1.30 |
| Lead | mg/l | <0.02 |
| Magnesium | mg/l | 362.80 |
| Mercury | mg/l | <0.0007 |
| Nitrate | mg/l | 7.5 |
| pH | pH @ 20°C | 6.5 |
| Potassium | mg/l | 91.94 |
| Selenium | mg/l | 0.0065 |
| Sodium | mg/l | 6720 |
| Strontium | mg/l | 1.087 |
| Sulphate | mg/l | 10700 |
| Suspended Solids | mg/l | 190 |
| TDS @180°C | mg/l | 23000 |
| TOC (as C) | mg/l | 11.5 |
| Total alkalinity (as $CaCO_3$) | mg/l | 88 |
| Total hardness (as $CaCO_3$) | mg/l | 2425 |
| Total Silica (as $SiO_2$) | mg/l | 26.1 |
| Zinc | mg/l | 0.030 |

Table 4: Fly ash and leachate samples collected at the test ash dump.

| Sample number | Sample description | Approximate age | Collection point |
|---|---|---|---|
| 1 | Ash | One year | Top of back stack |
| 2 | Ash | Five years | Toe of the dump |
| 3 | Ash | Three hours | Conveyer dump |
| 4 | Ash | One hour | Ash stacks |
| 5 | Water | Unknown | Toe seep of emergency dump |
| 6 | Ash | One year | Toe of back stack |
| 7 | Ash | Unknown | Toe ash emergency dump |

**Ash Core sampling**

Ash dump core samples were collected from five different points at the test site, selected from a resistivity graph over the dump (Fig. 1). Samples of approximately 50g were taken in sterile containers from the inside of the core at 1m intervals. Core 79 is the oldest ash (approx 20-25 years) and core 83 being the youngest (approx 2-5 years) (Table 4). For the purpose of analysis, samples from the surface the ash/soil interface, the 5m level, each subsequent 5m level and the base sample were analysed for each core.

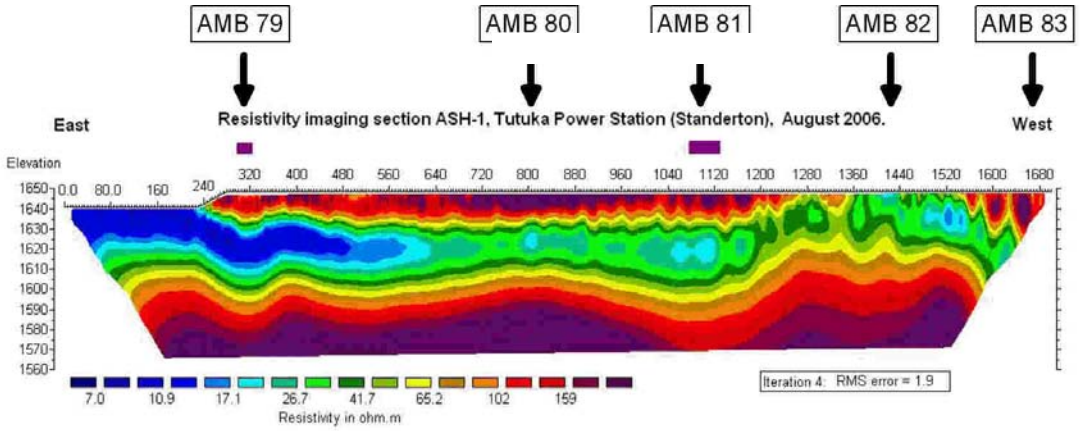


Figure 1: Resistivity graphs of test site ash handling facility, indicating the position of the core holes from old to new ash.

Table 4**:** Fly ash core samples collected in January 2007 at the test site ash dump.

| Sample no. | Sample description |
|---|---|
| 1 | Core B79 0.75m |
| 2 | Core B79 5m |
| 3 | Core B79 10m |
| 4 | Core B79 12m water |
| 5 | Core B79 15m |
| 6 | Core B80 1m |
| 7 | Core B80 5m |
| 8 | Core B80 10m |
| 9 | Core B80 15m |
| 10 | Core B80 20m |
| 11 | Core B80 25m |
| 12 | Core B81 1m |
| 13 | Core B81 5m |
| 14 | Core B81 10m |
| 15 | Core B81 13m |
| 16 | Core B82 1m |
| 17 | Core B82 5m |
| 18 | Core B82 10m |
| 19 | Core B82 15m |
| 20 | Core B82 20m |
| 21 | Core B82 25m |
| 22 | Core B82 28m |
| 23 | Core B83 1m |
| 24 | Core B83 5m |
| 25 | Core B83 10m |
| 26 | Core B83 15m |
| 27 | Core B83 20m |
| 28 | Core B83 25m |
| 29 | Core B83 30m |
| 30 | Core B83 31.5m |

**DNA extraction**

Total DNA was extracted using the BIO101 Fast DNA Spin kit (Soil) (Qbiogene Molecular Biology products, Pretoria, South Africa). Half a gram (0.5 g) of sample was utilised and extracted DNA was stored at -20ºC at the University of Pretoria, South Africa.

**16S PCR**

A portion of the bacterial 16S rRNA gene was amplified by means of PCR using K and M primers:

K (PRUN518R) : 5'ATT-ACC-GCG-GCT-GCT-GG3' (Siciliano *et al.* 2003)

M (pA8f-GC) : 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG3' (Fjellbirkeland *et al.* 2001)

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: 10.8 µl sterile SABAX water, 2.5 µl PCR buffer (10x), 2 µl $MgCl_2$ (25mM), 2 µl dNTPs (2.5 µM), 1 µl primer K (50 µM), 1 µl primer M (50 µM), 1 µl template DNA (27 ng/µl), 0.2 µl *Taq* polymerase (5 U/µl).

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.

**ITS PCR**

A portion of the eukaryotic internal transcribed spacer (ITS) gene sequence was subjected to PCR using the primer set ITS3 and ITS4:

ITS3 (34-53 (5S)) : 5'CAT CGA GAA GTT CGA GAA GG3'
ITS4 (57-38 (25S)) : 5'TAC TTG AAG GAA CCC TTA CC3'
(White *et al*. 1998)

Reactions were carried out essentially as described above, but with the use of the ITS3/4 primer set and the following thermal cycler programme: 1 min at 92°C, 35 cycles of 1 min at 92°C, 1 min at 50°C and 1 min at 72°C, followed by 5 min at 72°C, and then held at 4°C.

**DGGE**

PCR products were subjected to DGGE according to the method described by Muyzer *et al.* (1993) and briefly described above. Ten microlitres (ca. 250 ng) of each PCR product was loaded per lane onto a urea/formamide denaturing gradient gel. Gels were run at 70V for 17hrs at a constant temperature of 60°C. Image analysis was performed using the Gel2K programme (Norland 2004). The fingerprint comparisons were analysed using CLUST (Norland 2004). Dominant bands were compared and analysed to determine population diversity. Several comparison options are available but Jaccard and Simple indices (matchings) were used in this study. DGGE gels were viewed under blue light and bands were excised using a sterile scalpal blade. Gel fragments were placed in 30 µl of sterile DNAse free water and left for 48 hours before being re-amplified using the PCR protocol described above.

**Sequencing**

Each excised band was sequenced in an Eppendorf tube containing 1µl clean PCR product, 2µl "Big Dye" (Roche) sequence mix, 0.32µl primer K (without a GC clamp) and 1.68µl filter-sterilised deionised water. The PCR product was cleaned by adding 15µl sterile water, transferring the entire volume to a 0.5ml Eppendorf sequencing tube, adding 2µl of 3M sodium acetate and 50µl 95 % ethanol, and allowing it to stand on ice for 10min. The tubes were then centrifuged at 10 000 rpm for 30 min. The ethanol solution was removed, the pellet rinsed in 150µl 70 % ethanol, and the tubes again centrifuged for 5 min at 10 000 rpm. The ethanol was aspirated and the pellet dried under vacuum for approximately 10 min. Tubes were transferred on ice to the sequencer. DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK). Partial sequences of the bacterial 16S rRNA gene were obtained using the K primer (above).

Each sequence was subjected to BLAST analysis on the GenBank database. Matching hits, with e-values closest to 0.0 indicating a statistically plausible match, were selected for alignment. In all cases, sequences of several species were included in the alignments. Sequences were aligned with Clustal X (Thompson *et al.* 1994) and inserted gaps were treated as missing data. Missing, ambiguous and

uninformative characters were excluded from the data set before analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford 2000). Heuristic and bootstrap searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping, MULPAR-effective and MaxTrees set to auto-increase. Phylogenetic signals in the data sets were assessed by evaluating tree length distributions over 100 randomly generated trees.

## RESULTS AND DISCUSSION

DNA was successfully extracted from all samples collected (Fig. 2). No evidence of RNA or protein contamination that could inhibit further applications of DNA was visible either in the lanes or in the wells of the gel, respectively. It is important to note that although some of the DNA appeared to be of low concentration, both the concentrations and DNA purity was considered to be suitable for further analysis.

PCR amplification of prokaryote and eukaryote partial 16S and ITS2 sequences was successful, yielding ca. 500bp and ca. 300bp PCR products on 1.5% TAE agarose gels, respectively (Figs. 3A and B). Negative control lanes (first in each row) indicated an absence of background contamination.

DGGE gels, using bacterial primers pA8f-GC and PRUN518r, and eukaryotic primers ITS3-GC and ITS4, showing clear multiple banding, forming a fingerprint in each lane (Figs. 4 and 5). Bands travelled the full length of the gel, indicating that an appropriate denaturing gradient had been used.

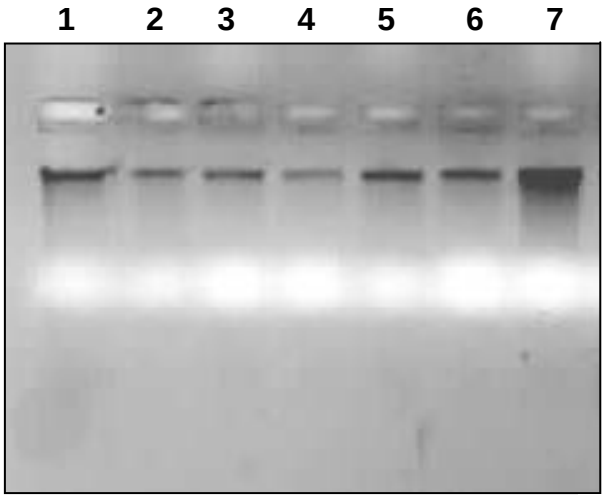


Figure 2: 1.5% TAE agarose gel showing high-quality, clean genomic DNA extracted from fly ash samples (Table 1) by means of the BIO101 Fast DNA Spin Kit for soil. (M = 100bp marker, 1A-6B = DNA from samples).

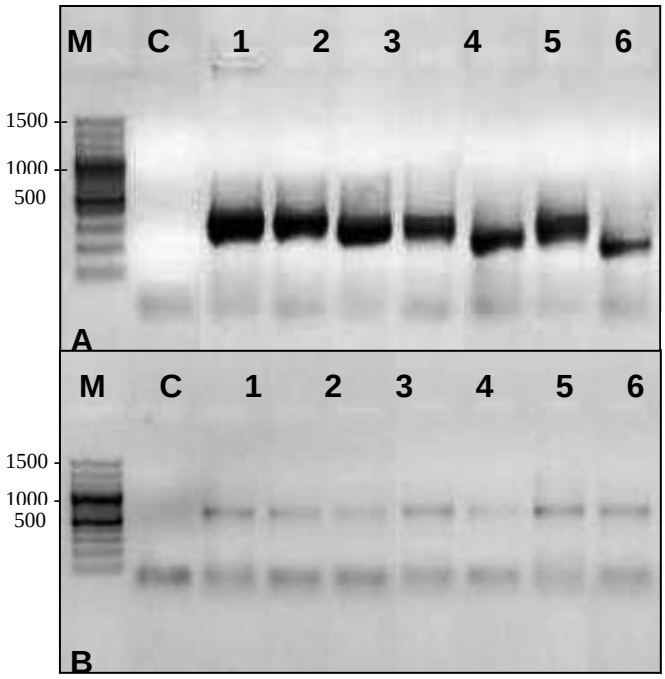


Figure 3: 1.5% TAE agarose gel, showing the PCR products from the 16S (A) and internal transcribed spacer sequence (B) PCR amplifications.

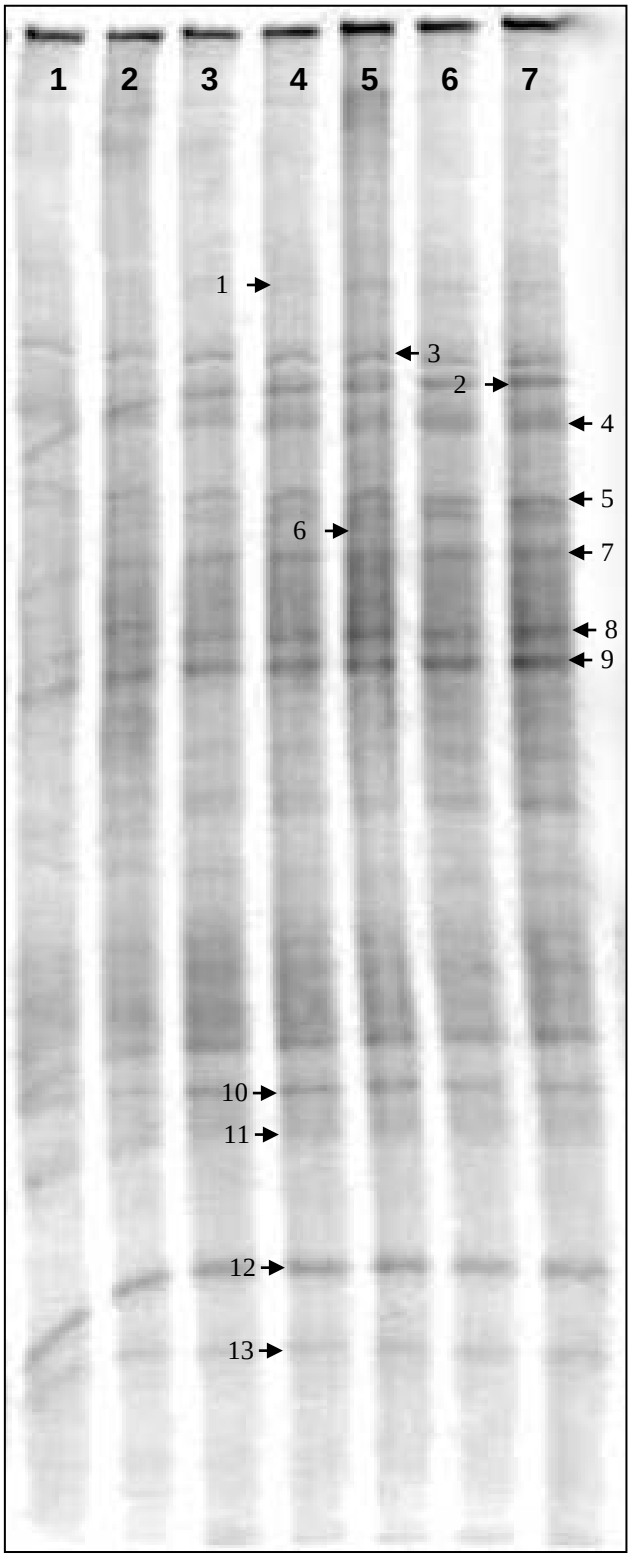


Figure 4: DGGE gel depicting the banding pattern of the 16S rRNA gene PCR amplicons, separated with a 40-60%.urea and form amide gradient.

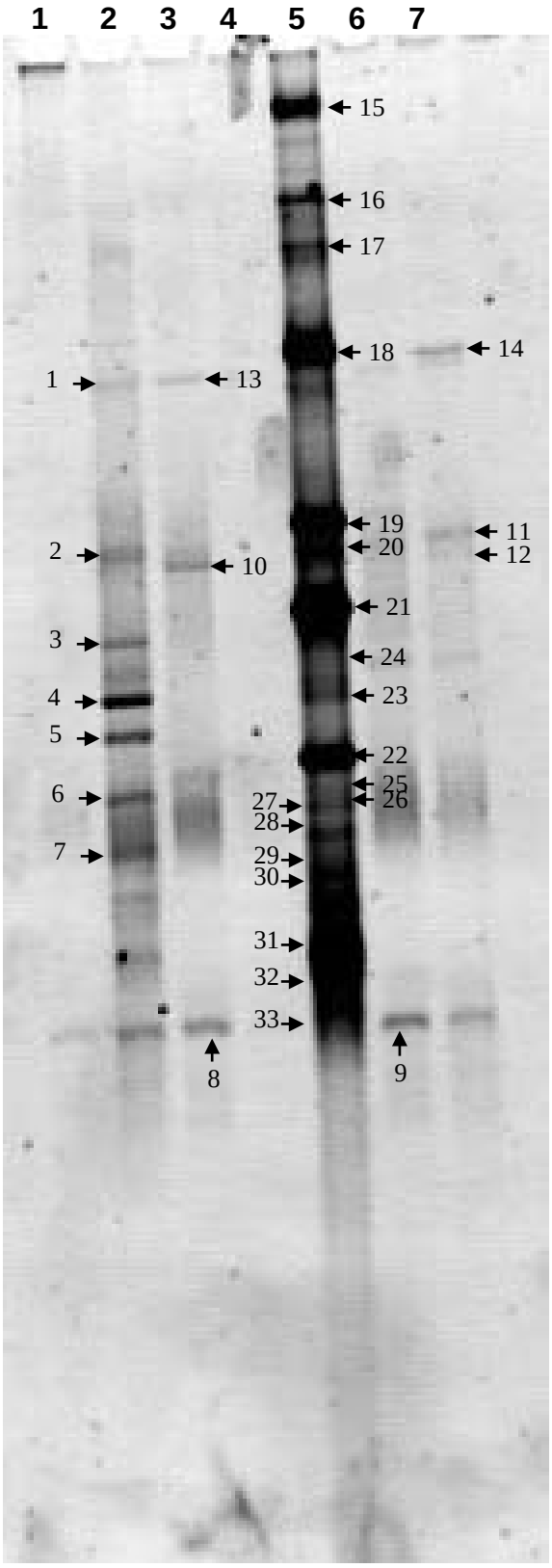


Figure 5: DGGE gel depicting the banding pattern of the ITS gene PCR amplicons, separated with a 45-55%.urea and formamide gradient.

On average, more DGGE bands were found for prokaryotes than for eukaryotes (Fig. 6), suggesting that prokaryotic diversity was higher than that of eukaryotes. Species richness may also be higher within the prokaryotes; i.e., more dark/dominant PCR bands were observed within the 16S gene gel lanes than in eukaryotic lanes.

In a comparison of the numbers of prokaryotic species between samples, it is evident that samples 2 and 5 (toe salts and toe seep emergency dump) have significantly lower species diversity than ash and leachate samples (Fig. 6). Samples 4 and 6 (New stack and toe of back stack) fall within the midrange of species diversity and samples 1, 3 and 7 (top of back stack, conveyor dump and toes of new emergency stack) show the highest apparent diversity of all the samples.

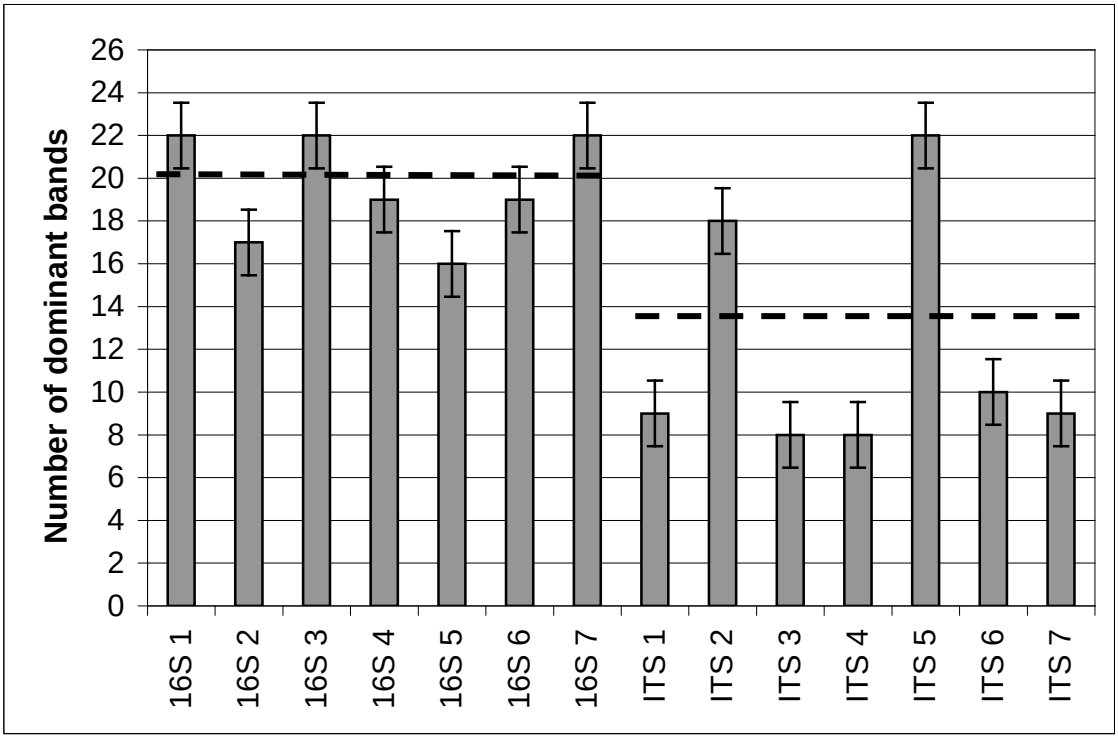


Figure 6: Number of dominant phylotypes per fly ash and leachate sample as indicated by DGGE of 16S rDNA (16S) and partial internal transcribed spacer sequence (ITS) PCR products. Note that the mean number of dominant species is 18.6 for prokaryotes and 12.0 for eukaryotes (dotted lines).

A eukaryotic species diversity comparison between samples, indicate that samples 1, 3, 4, 6 and 7 (top of back stack, conveyor dump, new stack, toe of back stack and toe of new emergency stack leachate) have less diversity than the average eukaryotic diversity of the ash and leachate samples (Fig. 6). Samples 2 and 5 show the highest eukaryotic diversity. However, it is noted that these samples exhibit the lowest species diversity in the prokaryotic diversity profile. This may be due to the fact that both samples are at the base of the ash (2 at the toe and 5 in the toe drains) the environment may be harsher in a chemical or moisture sense due to the presence of the toe seep leachate

Using the DGGE gel banding patterns, graphical cluster representations were drawn using Gel2K (Norland 2004) (Figs. 7 and 8 for prokaryotes and eukaryotes, respectively). This analysis estimates band peak intensity along each lane. The programme CLUST (Norland 2004) groups the profiles of the species in each sample according to similarity in population composition/diversity. Thus, samples from similar environments would be expected to display similar populations and group together in the CLUST dendogram. Dominant species per lane are indicated as darker, more prominent bands within the lane.

Prokaryotic populations of fly ash and leachate samples were separated into three distinct clades (Fig. 7). Clade I contained samples 1 and 2 (top of back stack and toe of dump), clade II contained samples 3 and 6 (conveyor dump and toe of back stack) and clade III contained samples 4, 5 and 7 (stacks new, toe seep of new stack and toe leachate of new emergency dump). However, while samples that group together, under Jaccard, complete link statistical, comparison, do so as a result of similar species and diversity level, not all of the same species are necessarily present in each sample. This means that the samples may contain similar species but not necessarily display the same extent of diversity.

Ash samples 1 and 2, one and five year old ash respectively, group in clade I. This clade is separate from clades II and III and indicates the oldest ash samples. The fact that these samples cluster separately from the other ash, indicates that there may be a change in bacterial populations over time, perhaps as an effect of ash weathering.

Ash samples 3 and 6 group together in clade II. These samples are from the conveyor belt (approx 1 hour old) and the toe of the back stack respectively. These areas are regions of high aeration and agitation, which may lead to the similar species detected in the samples.

Samples 5 (water), 7 and 4 (Clade III) group together, this indicates that the toe seep water (5) and toe of the ash dump (7) are similar in bacterial populations but that the hour old ash (4) groups more closely with the emergency toe dump (7). This maybe misleading as sample 4 is fresher than sample 7 (further investigation to this phenomenon showed that ash had been dumped on the emergency stack the previous day and was thus less than 24 hours old). In addition water being used to irrigate the fresh ash percolates through the ash and discharges at the toe seep.

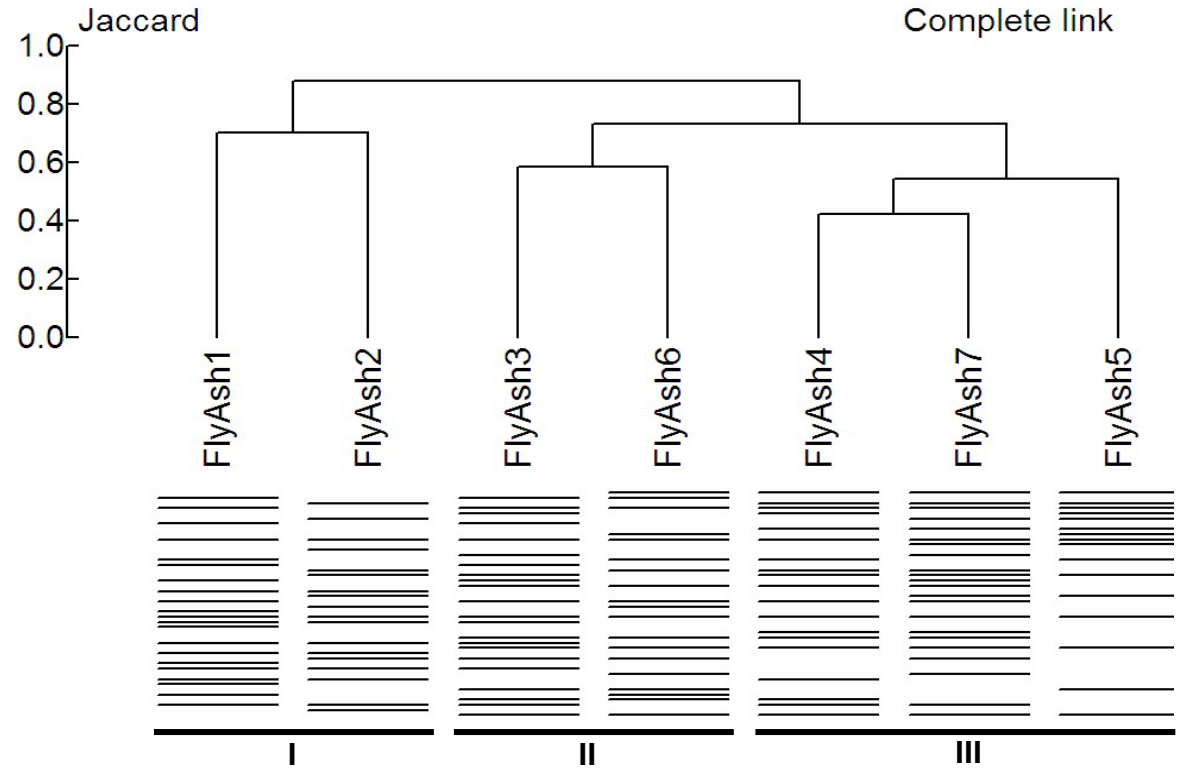


Figure 7: Prokaryotic Cluster analysis of the gel banding pattern in Fig. 4, using a Jaccard, complete link statistical setting to separate populations on the basis of species sequence differences.

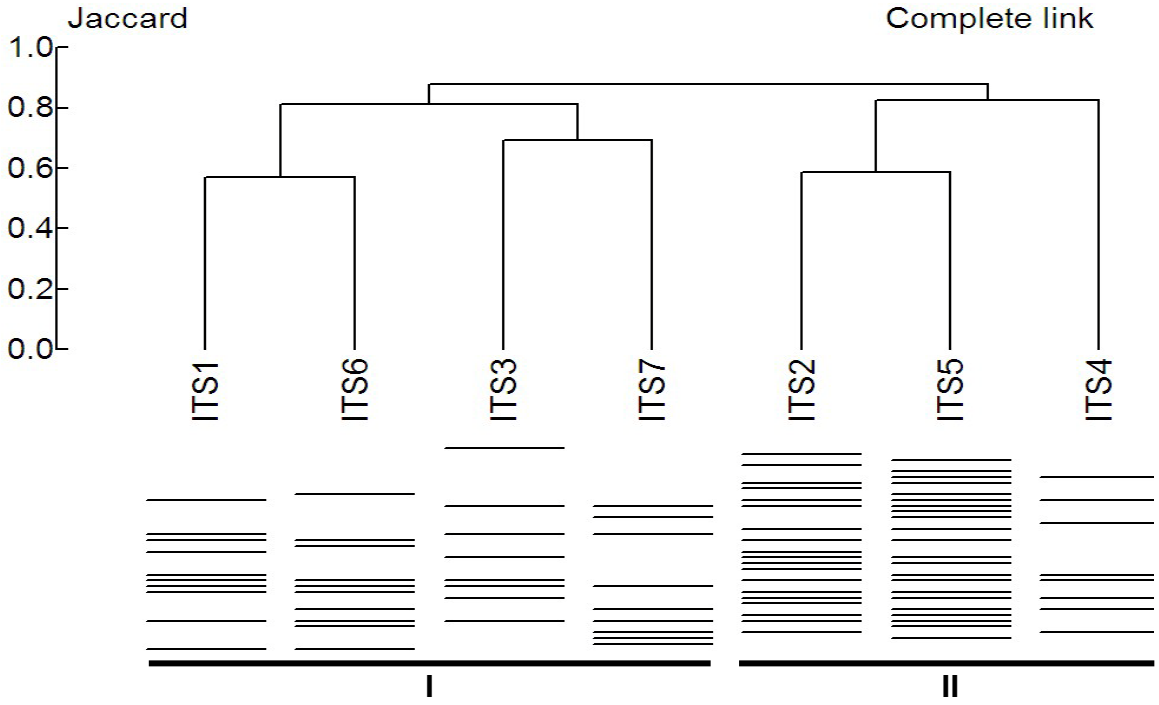


Figure 8: Eukaryotic Cluster analysis of the gel banding pattern in Fig. 5, using a Jaccard matching, complete link setting to separate populations on the basis of species sequence differences.

Eukaryotic populations of fly ash and leachate samples split into two distinct clades under a Jaccard, complete link setting (Fig. 8). Clade I contained samples 1, 3, 6 and 7 (top of back stack, conveyor dump, toe of back stack and toe leachate of new emergency dump), clade II contained samples 2 and 5 (Toe salts and toe seep of new stack) and sample 4 fell basal to clade II.

Within clade I samples 1 and 6 group together apart from 3 and 7. Samples 1 and 6, from the top and toe of the back stack are both approximately 1 year old. These ashes are continually exposed to the environment. Samples 3 and 7 are one hour old ash and ash from the base of the emergency dump toe, indicating ash less than 24 hours old.

Samples 2, 4 and 5 are fresh ash, ash at the toe seep and toe seep water respectively. These samples have all been impacted by water; the fresh ash (4) was conditioned to 10% moisture with the ash water brine, prior to being transported to the ash dump. The water (5) has percolated through the ash from the irrigation on the top and sides of the dump and impacts on the surrounding ash (2), potentially 'homogenising' the microbial populations.

**Sequencing**

Prokaryotic sequences obtained from extraction and re-amplification of DGGE bands, and their BLAST results, were rooted with *Thermotoga maritima* as a monophyletic outgroup. Bootstrap analyses were conducted to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated. This resulted in the DGGE bands falling within four distinct clades (Fig. 9), where *Hydrocarboniphaga, Pseudomonas, Acinetobacter and Bacillus* form clades I to IV respectively.

Phylogenetic trees generated from the partial 16S sequences obtained from fly ash and leachate, (Figure 9). DGGE bands 1, 2 and 4 appear to be of the genus *Pseudomonas* as they cluster directly within clade II, whereas DGGE bands 6, 7 and 8 are closely related to the *Pseudomonas* genus but group separately in a subclade within clade II. DGGE bands 2, 4 and 7 show closest similarity to *Pseudomonas* species commonly isolated from unpolluted soil as do DGGE bands 5, 12 and 13 that fall into Clade IV clustering with known soil *Bacillus* species.

Half of the DGGE band sequences grouping with the genus *Pseudomonas* have been isolated from activated sludge samples and BTEX (benzene, toluene, ethybenzene and xylene) contaminated industrial sites (DGGE bands 1, 6 and 8). This is understandable as the ash has been conditioned with station drain and blow down water, which contains grease and hydrocarbon waste from the station surfaces.

Clade I comprises *Hydrocarboniphaga effuse* sequences grouping closely with DGGE bands 9 and 11, supported by a 100% bootstrap value. Species of the *Hydrocarboniphaga* genus are known to degrade alkanes and aromatic hydrocarbons *in situ* (Palleroni *et al.* 2004).

DGGE band 3 falls into Clade III grouping with *Acinetobacter calcoaceticus.* This bacteria is known to be isolated from soil and is capable of degrading aliphatic polyesters (Poly B-hydroxyalkanoate) used in biodegradable plastics (Suyama *et al.,* 1998*).* At the time of publishing the results for the ITS sequencing were not yet finalised.

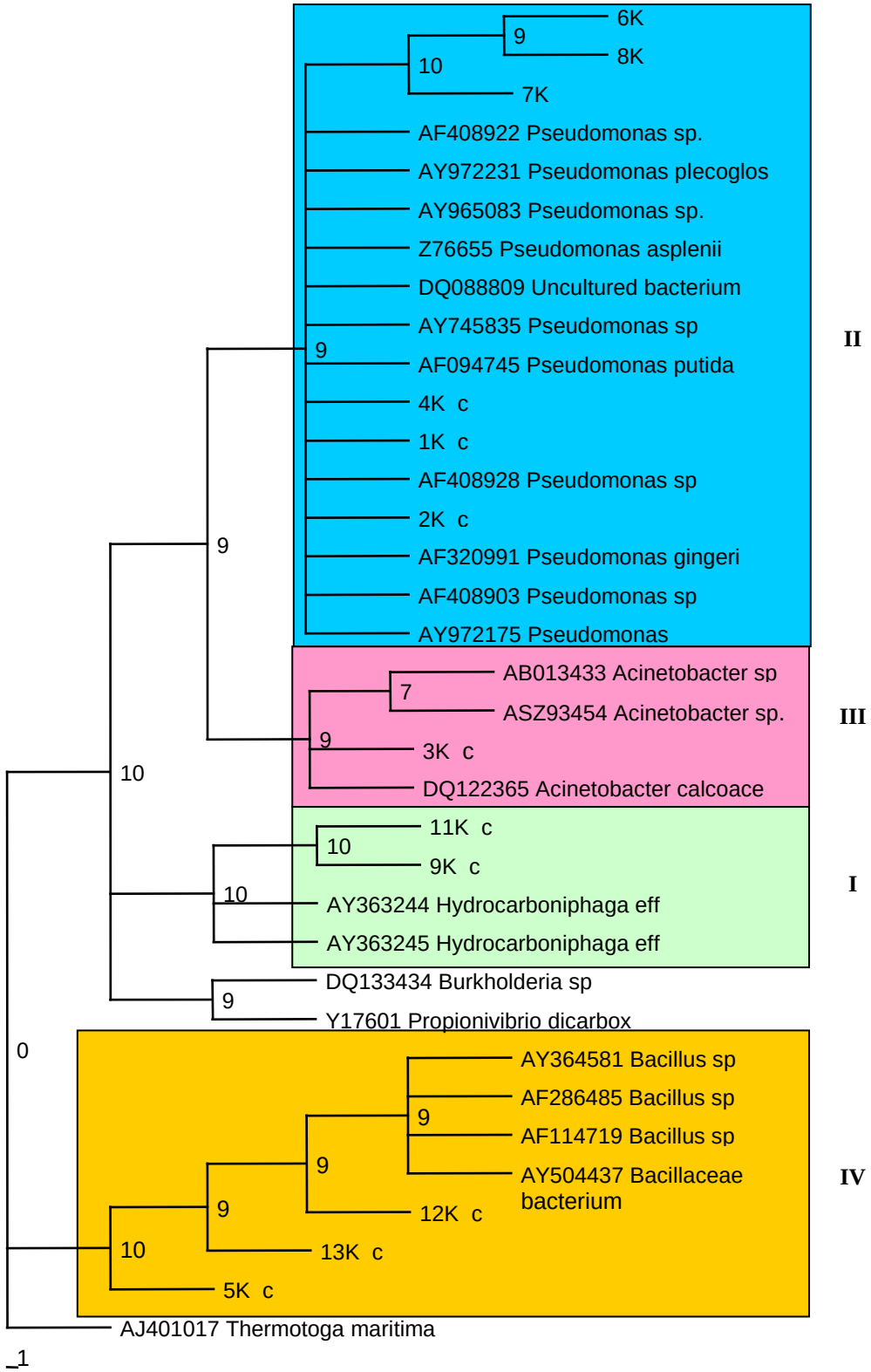


Figure 9: Phylogeny of the 16S bacterial gene of the rDNA, showing sequences from fly ash and leachate (bootstrap support values are indicated on branches). Parsimony informative characters: 164, CI: 0.7050, RI: 0.8839, Number of trees: 1, Tree length: 238.15264, *g1*: -0.915477.

**Ash Core microbiology**

DNA was successfully extracted from all samples collected (Figure 10).

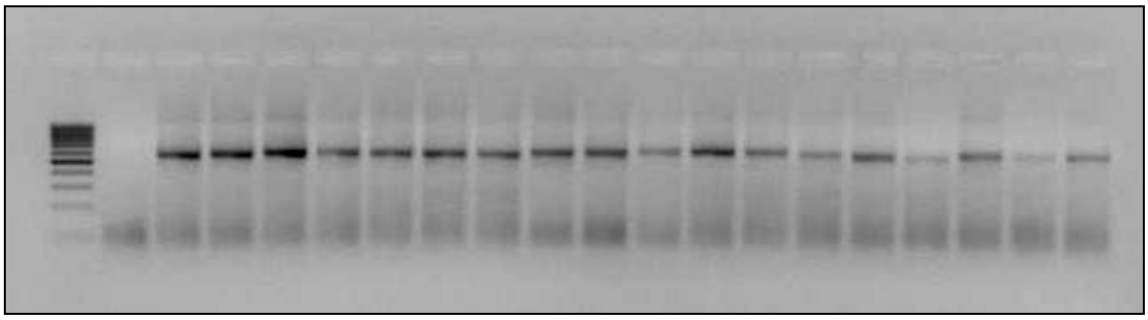

Figure 10.**:** 1.5% TAE Agarose gel, showing the PCR products from the 16S bacterial gene amplifications (ITS not shown).

DGGE gels of PCR products showed a clear banding pattern (Figures 11 A and B). In general, core samples yielded more prokaryotic amplification bands than eukaryote (Figures 12), suggesting that prokaryotic diversity was higher than that of the eukaryotes. Species richness was also more evident within the prokaryotes. Graphical cluster representations of the band patterns were drawn using Gel2K (Norland 2004) (Figures 13 and 14).

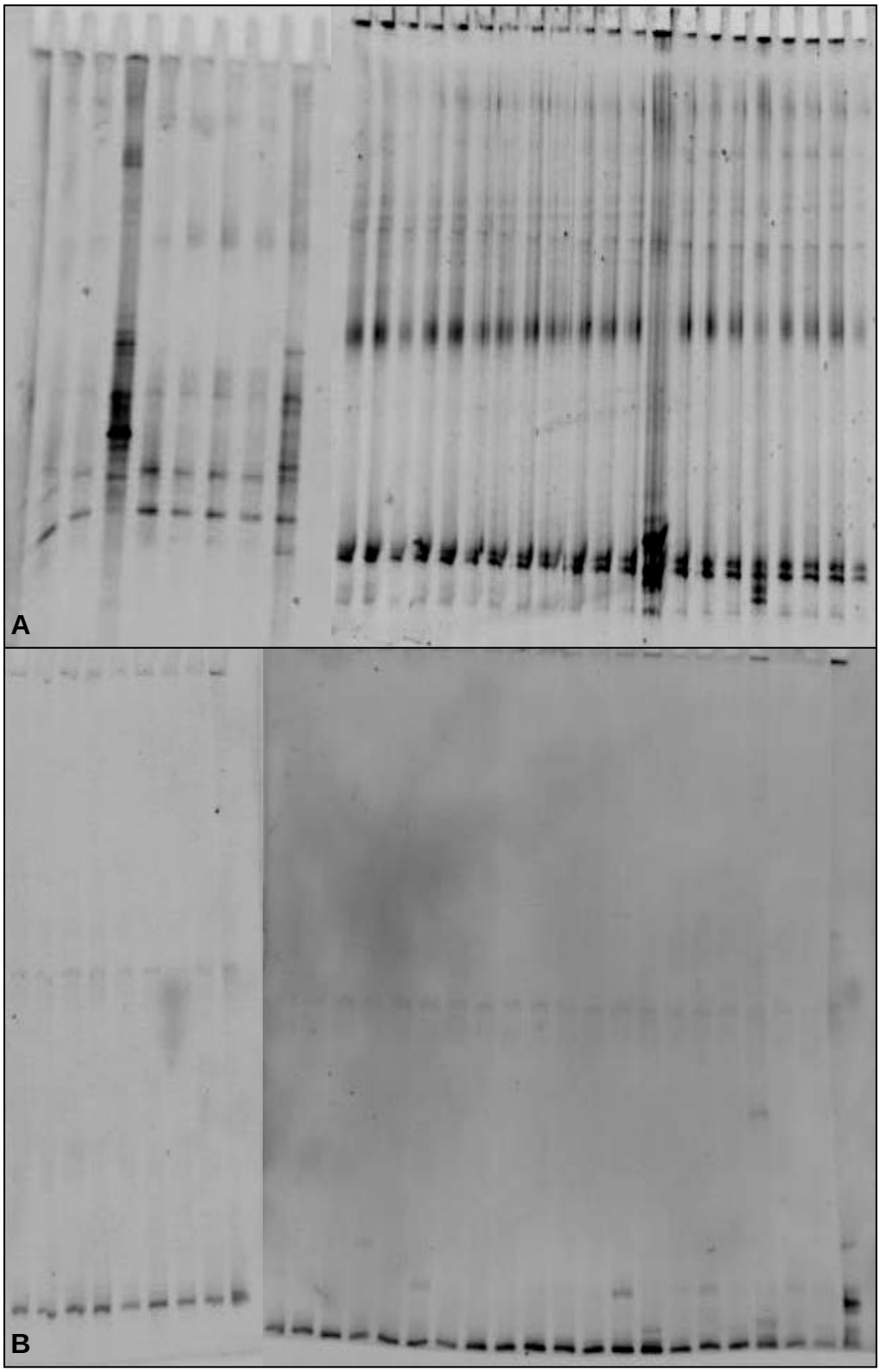


Figure 11**:** Denaturing gradient gel electrophoresis gel showing species diversity: (A). Ash core prokaryotic/bacterial and (B). Ash core eukaryotic species.

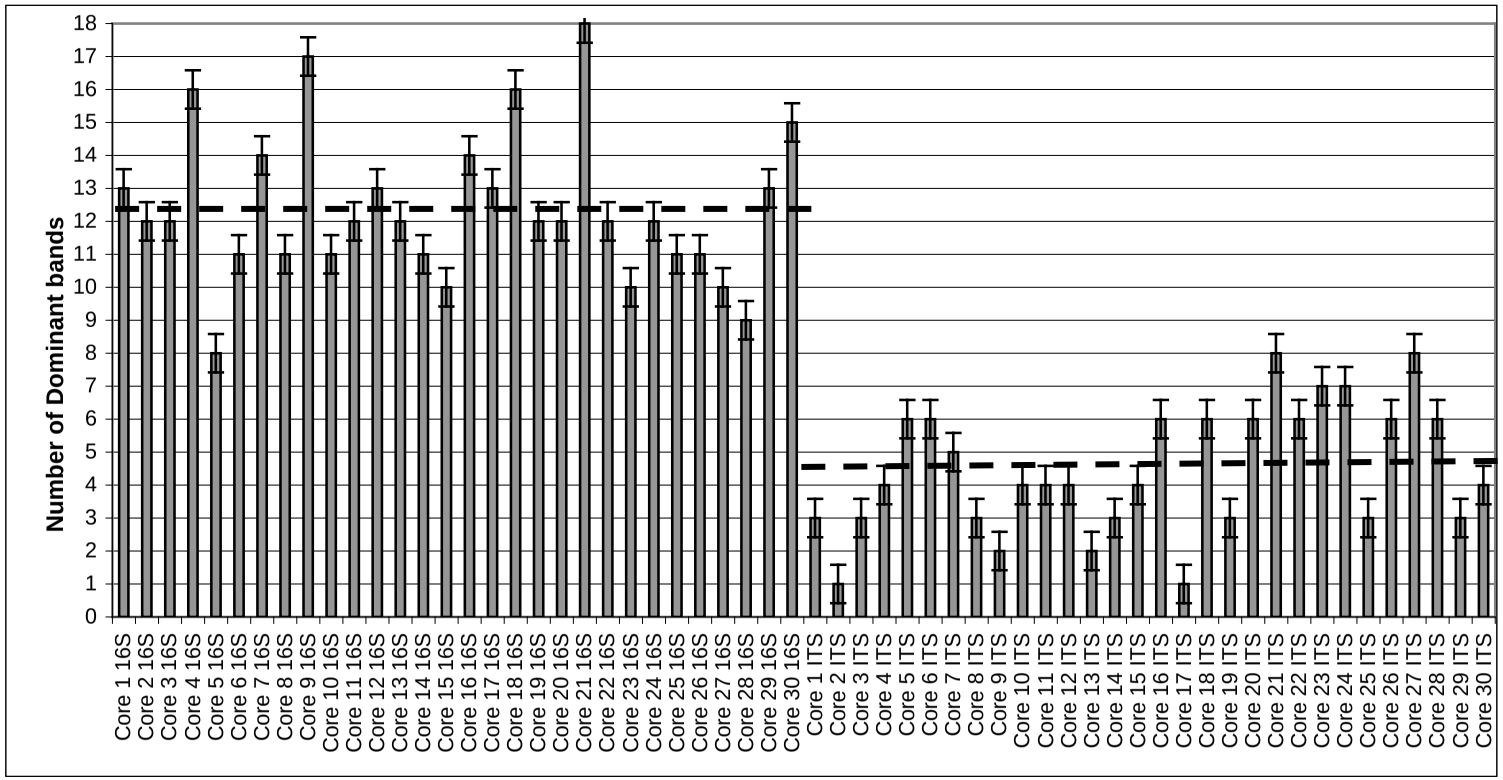


Figure 12**:** Number of dominant species per core sample as indicated by DGGE of 16S rDNA (16S) and partial internal transcribed spacer sequence (ITS) PCR products. Note that the mean number of dominant species is 12.4 for eukaryotes and 4.5 for prokaryotes (dotted lines).

Core B79, samples 1-4 group together in clade V of both of the dendrograms in figure 13. Additionally, sample 5 (core B79) groups with samples 26 and 27 (core B83). Core B83 is the newest core and shows lower diversity, sample 5 was taken at 15m into core B79 so diversity is expected to be low this may explain why these samples group together. These groupings can be identified throughout the dendrograms and will imply similar species diversity. It is important to note that within the Jaccard analysis, clades parallel the core site and thus share similar species viz. clades I and II form one large clade and contain mainly core B83; clade III contains samples from cores B80, B81 and B82 that tend to fall closer to one another within the clade; clade IV contains samples from core B82. This observation is understandable because cores are numbered according to age where B79 is the oldest and B83 the newest. Eukaryotic analysis does not show this distinct "core diversity differentiation" observed within the prokaryotes (Fig. 14). The simple dendrogram shows that most samples group within clade II. However the Jaccard dendrogram does not indicate a high level of diversity between samples, this is evident from the long branches extending from the base line. Taking into account the results of the DGGE gels themselves it seems that eukaryotic diversity is very low and thus should not be used as an indicator to imply similarities within this environment.

## CONCLUSIONS

Measuring reservoirs of environmental microbial diversity, such as in fly ash, is challenging and it is widely accepted that direct culturing of the organisms is unreliable (McCaig *et al.*, 1999). Consequently, DNA-dependent analyses provide the most comprehensive option (Muyzer, 1999). Currently, diversity is estimated by targeting conserved genes (e.g. 16S rRNA genes) that occur ubiquitously in the organisms being screened (Yeates *et al.*, 1998). DGGE is an appropriate method to employ in diversity studies, as it facilitates fingerprinting of populations at species level, and can allow specific species to be targeted during diversity estimation (Heuer and Smalla, 1997). Prokaryotic and eukaryotic species diversity was analysed in parallel in this study.

Sequence data indicated that the predominant prokaryotic genus within the samples was *Pseudomonas*. Six of the sequences group with the *Pseudomonas* genus. The *Pseudomonas* species with which the sequences group have been isolated from activated sludge and from BTEX polluted environments (Shim *et al.*, 2005). This is feasible due to the conditioning waste water incorporating the station drains effluent, which contains grease, oil and chemical solvents. This hypothesis is strengthened by the close grouping of some sequences with *Hydrocarboniphaga* which are also well researched aromatic and aliphatic hydrocarbon degraders, often isolated from polluted oil spill sites (Palleroni *et al.*, 2004). The link to *Acinetobacter* indicates an ability to further degrade hydrocarbons and is also one of the main infectants in common human infections, supporting the sewage effluent theory (Suyama *et al.,* 1998*).*

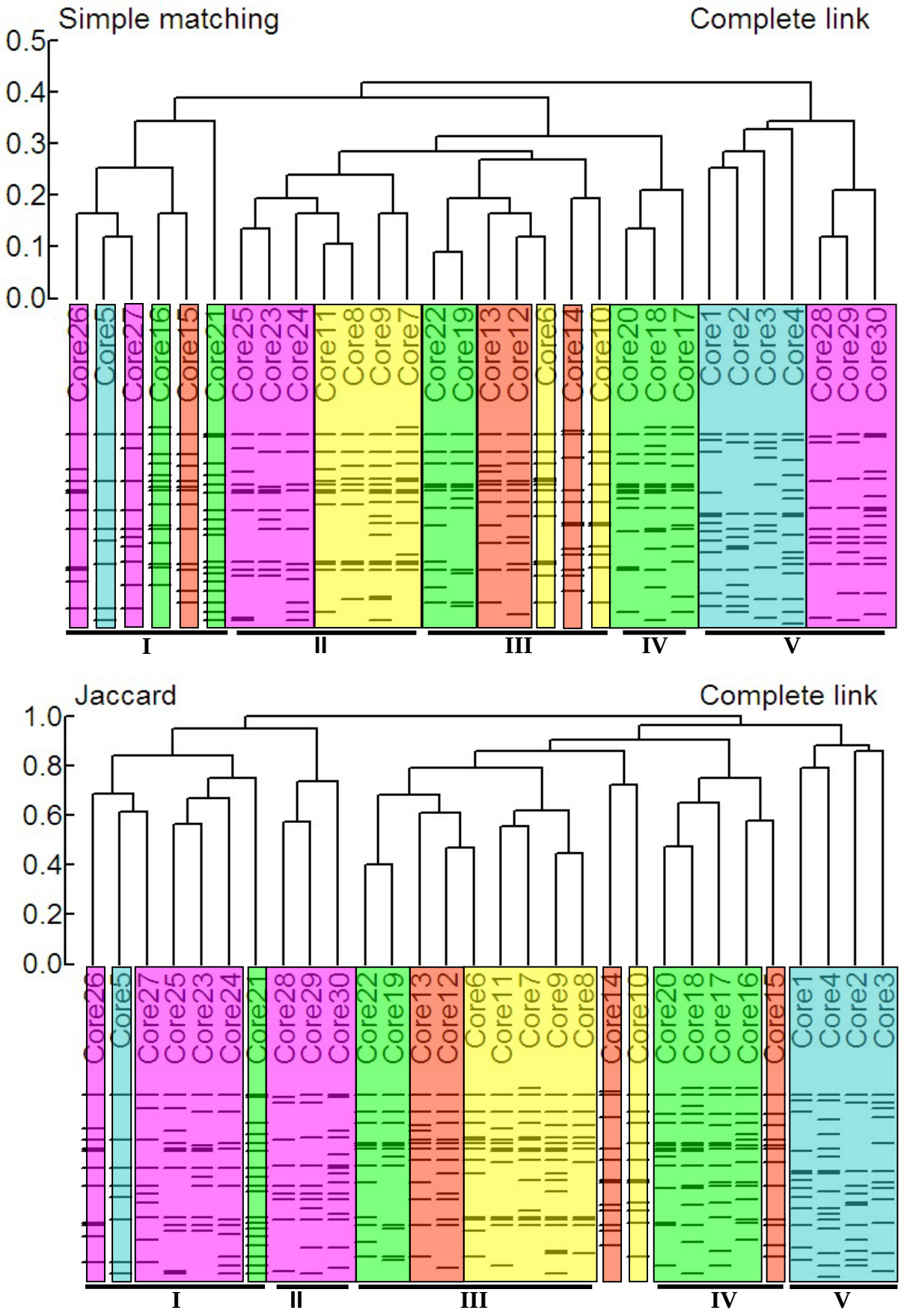


Figure 13: Cluster analysis of the banding pattern, using both a simple matching and a Jaccard setting with a complete link to separate prokaryotic core populations from cores 79, 80, 81, 82 and 83 on the basis of species sequence differences

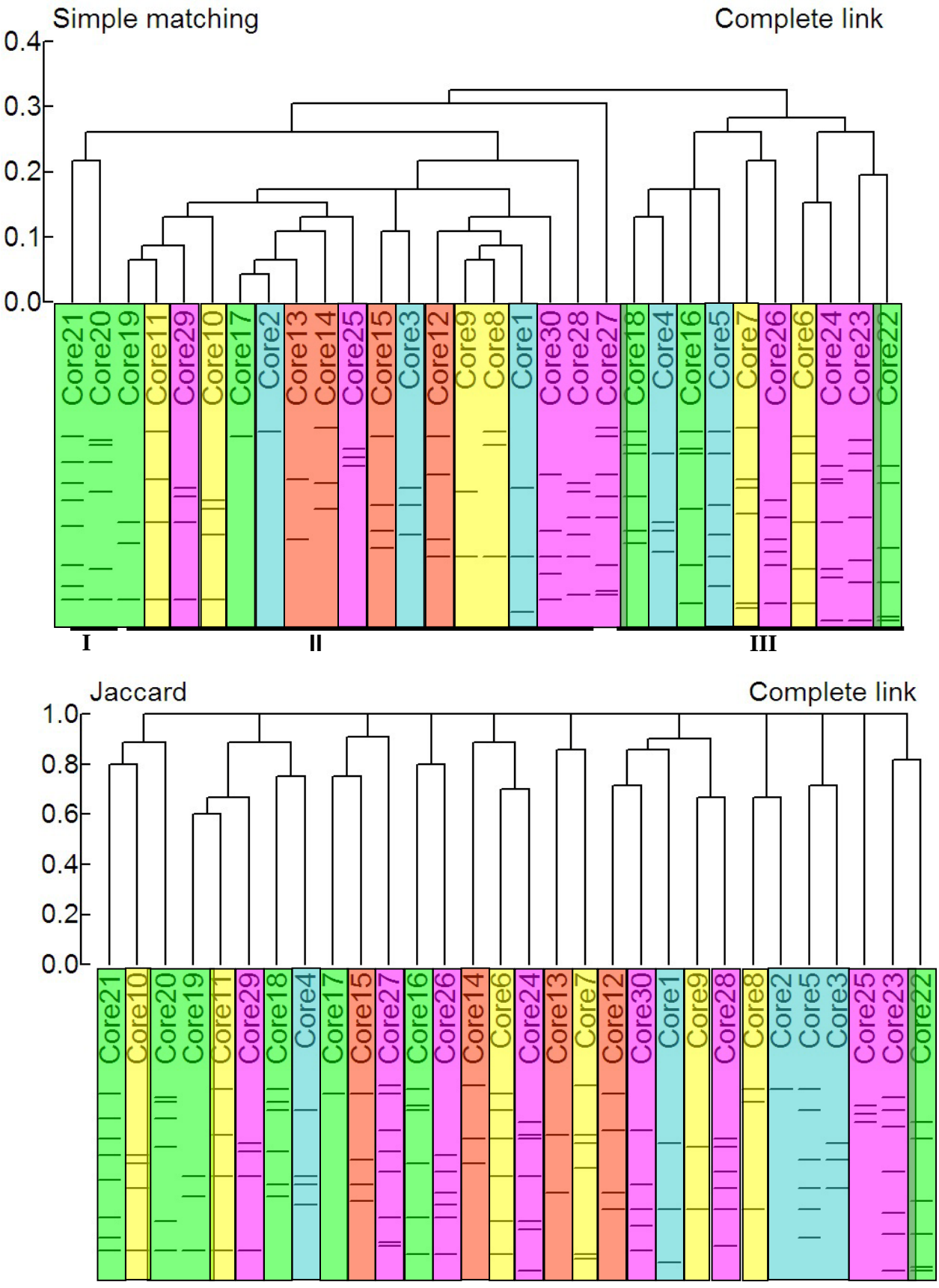


Figure 14: Cluster analysis of the banding pattern, using both a simple matching and a Jaccard setting with a complete link to separate eukaryotic core populations from cores 79, 80, 81, 82 and 83 on the basis of species sequence differences.

## REFERENCES

**Banks MK and Alleman J (2002)**. Microbial indicators of bioremediation potential and success. *Hazardous substance research centres*. Georgia Tech Research Corporation. http://www.hsrc.org/mw-microbial.html.

**Gillan DC (2004)**. The effect of an acute copper exposure on the diversity of a microbial community in North Sea sediments as revealed by DGGE analysis – the importance of the protocol. *Marine Pollution Bulletin* **49**: 504-513.

**Heuer H, Krsek M, Baker P, Smalla K, and Wellington EM (1997)**. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology.* 63, 3233-3241.

**Koizumi Y, Kelly JJ, Nakagawa T, Urakawa H, El-Fantroussi S, Al-Muzaini S, Fukui M, Urushigawa Y and Stahl DA (2002).** Parallel characterisation of anaerobic toluene- and ethylbenzene-degrading microbial consortia by PCR-denaturing gradient gel electrophoresis, RNA-DNA membrane hybridisation, and DNA microarray technology. *Applied and Environmental Microbiology* **68**: 3215-3225.

**McCaig AE, Glover LA and Prosser JI (1999)**: Molecular analysis of bacteria community structure and diversity in unimproved and improved upland grass pastures. *Appl. Environ. Microbiol.* 65, 1721-1730.

**Muyzer G (1999)**: DGGE/TGGE a method for identifying genes from natural ecosystems. *Cur. Opin. Micro.* 2, 317-322.

**Norland S (2004)**: Gel2K gel analysis software. University of Bergen, Norway. http://www.im.uib.no/~nimsn/program/.

**Palleroni NJ, Port AM, Chang H-K and Zylstra GJ (2004)**: *Hydrocarboniphaga effuse* gen. nov., sp. nov., a novel member of the γ-*Proteobacteria* active in alkane and aromatic hydrocarbon degradation. *J. System. Evolu. Micro.* 54, 1203-1207.

**Petrik L, Klink M, Somerset V, Burgers C and White R (2001)**: Simultaneous water recovery and utilisation of two harmful effluents, fly ash leachate and acid mine drainage, for the production of high capacity inorganic ion exchange material useful for water beneficiation. *Coaltech 2020 research report.* Task 6.4.1, K5.1242.

**Shim H, Hwang B, Lee SS and Kong SH (2005)**. Kinetica of BTEX biodegradation by a coculture of *Pseudomonas putida* and *Pseudomonas fluorescens* under hypoxic conditions. *Biodegredation*. 16, 319-27.

**Suyama T, Tokiwa Y, Ouichanpagdee P, Kanagawa T and Kamagata Y (1998).** Pylogenetic affiliation of Soil Bacteria that Degrade Aliphatic Polyesters Available Commercially as biodegradable Plastics. *Appl. Environ.*

*Microbiol.*. 64, 5008-5011.

**Swofford DL (2000)**. PAUP* - Phylogenetic analysis using parsimony (*and Other Methods), Version 4.0b8. Sinauer Associates, Sunderland, Massachusetts, USA.

**Throbäck IN, Enwall K, Jarvis Å and Hallin S (2004)**. Reassessing PCR primers targeting *nirs*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *Applied and Environmental Microbiology* **49**: 401-417.

**Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG (1994)**. The CLUSTAL_X windows interface, flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882.

**White DC., Flemming CA, Leung KT and Macnaughton SJ (1998)**. In situ microbial ecology for quantitative appraisal, monitoring, and risk assessment of population remediation in soils, the sub surface, the rhizosphere and in biofilms. *Journal of Microbiological Methods* 32: 93-105.

**Yeats C, Gillings MR, Davidson AD, Altaville N and Veal DA (1998)**: Methods for microbial DNA extraction from soil for PCR amplification. *Biological Procedures Online.* 1, 40-47.