Chapter 3

Materials and methods

Materials and methods

The objectives of this study were achieved by means of the following procedures:

3.1 Soil samples

Eight soil samples of approximately 50 g each were collected in 2003 in plastic sample bottles from a site in Free State Province (Samples 1-8 in Table 1). Soil samples 1 and 3-7 were collected from unpolluted and polluted top soil in the presence and absence of *Elusine coracana* and *Brantha serratia* plants (Table 1). Samples 2 and 8 were collected at different depths and were known to be polluted with diesel (Table 1). Samples taken from below the soil surface were collected using a soil auger. A further nine soil samples of approximately 50 g each from a pitch/oil/diesel/petrol/tar-polluted site in Mpumalanga Province were collected from approximately 5cm below soil surface in plastic sample bags in February 2004 (Samples 10-18 in Table 1). Soil samples at site 2 were polluted with different PAH-containing compounds and were rhizosphere and non-rhizosphere associated. Soil samples were taken either within the root zone or approximately 10 cm away from plant roots for rhizosphere and non-rhizosphere samples, respectively. The samples were transported to the laboratory and maintained at 4 ºC until total DNA could be extracted (max. 24 h). All soil samples were taken according to the simple random sampling protocol described by Tan (2005), and are considered to be representative of the environments from whence they came. However, broader spectrum sampling according to acknowledged systematic sampling protocols following standard operating procedures should be followed in soil sample collection in the future. A predetermined samlping area, having the same history, soil texture, colour and slope, should be targeted in a random zig-zag pattern and at least 20 samples collected (Zhang 2003).

Table 1: Soil collected for analysis from a site in Free State Province (site 1) and a site in Mpumalanga Province (site 2), South Africa. Soil samples taken at site 1 were collected one month after pollution by a leaking underground diesel pipeline. Soil samples at site 2 were collected from soil persistently polluted for approximately 10 years with different PAH/PCB-containing compounds.

Soil sample number	Area	Description
1	Site 1[c]	Diesel polluted topsoil with no plants growing nearby Total petroleum hydrocarbon concentration 25 000 mg kg^{-1}
2	Site 1[c]	1m deep non-rhizosphere soil polluted with diesel Total petroleum hydrocarbon concentration 8 500 mg kg^{-1}
3	Site 1[c]	Diesel-polluted topsoil with *Elusine coracana* and *Brantha serratia* plants growing nearby Total petroleum hydrocarbon concentration 25 000 mg kg^{-1}
4	Site 1[c]	Diesel-polluted topsoil with no plants growing nearby Total petroleum hydrocarbon concentration 25 000 mg kg^{-1}
5	Site 1[c]	Unpolluted topsoil with no plants growing nearby
6	Site 1[c]	Unpolluted topsoil with *Elusine coracana* and *Brantha serratia* plants growing nearby
7	Site 1[c]	Unpolluted topsoil with no plants growing nearby
8	Site 1[c]	1.5m deep non-rhizosphere soil polluted with diesel Total petroleum hydrocarbon concentration 28 000 mg kg^{-1}
9	Control[d]	Unpolluted reference loamy topsoil from University of Pretoria experiment farm
10	Site 2[d]	Unpolluted soil from *Bidens pilosa* rhizosphere pH 7.8, mineral oil hydrocarbons 3 530 mg (kg dw) $^{-1}$, PAHs 190 mg (kg dw) $^{-1}$, volatile hydrocarbons 2.4 mg kg^{-1}, phenol index (pH 7.0) 96 ul l^{-1}

[c] Soil samples taken to a depth of 10cm unless specified otherwise, the soil had a loamy texture.
[d] Soil samples taken to a depth of 5cm, the soil was a sandy loam (63.4% coarse, 21.1% silt, 13.9% clay).

Table 1 (continued)

Soil sample number	Area	Description
11	Site 2[d]	Unpolluted soil from *Brantha serratia* rhizosphere pH 7.9, mineral oil hydrocarbons 3 530 mg (kg dw) $^{-1}$, PAHs 190 mg (kg dw) $^{-1}$, volatile hydrocarbons 2.4 mg kg^{-1}, phenol index (pH 7.0) 96 ul l^{-1}
12	Site 2[d]	Unpolluted soil from *Cyperus esculentus* rhizosphere pH 7.8, mineral oil hydrocarbons 3 530 mg (kg dw) $^{-1}$, PAHs 190 mg (kg dw) $^{-1}$, volatile hydrocarbons 2.4 mg kg^{-1}, phenol index (pH 7.0) 96 ul l^{-1}
13	Site 2[d]	Polluted soil (pitch/oil/diesel/petrol/tar), 10cm from *C. esculentus* plant pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}
14	Site 2[d]	Polluted soil (pitch/oil/diesel/petrol/tar) from *C. esculentus* rhizosphere pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}
15	Site 2[d]	Polluted soil (pitch/oil/diesel/petrol/tar), 10cm from *B. serratia* plant pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}
16	Site 2[d]	Polluted soil (pitch/oil/diesel/petrol/tar) from *B. serratia* rhizosphere pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}
17	Site 2[d]	Polluted soil (workshop oil) mulched with wood chips 10cm from *B. serratia* plant pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}

Table 1 (continued)

Soil sample number	Area	Description
18	Site 2[d]	Polluted soil (workshop oil) mulched with wood chips from *B. serratia* rhizosphere pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw)$^{-1}$, PAHs 1 200 mg (kg dw)$^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}

3.2 Bacterial isolates

Eight bacterial isolates, representing the dominant culturable taxa from the rhizosphere of weeds and from non-rhizosphere soil at site 2 in Mpumalanga Province, South Africa, with a *ca*. 10-year history of total coal-derived petroleum hydrocarbon pollution, were obtained in pure culture (Molobela 2005). The isolates from polluted soils were randomly designated SA1, SA2 and SA3 from *Bidens pilosa* L. rhizosphere, SA4 and SA8 from *Eleusine coracana* (L.) Geartn. rhizosphere, SA6 and SA7 from *Cyperus esculentus* L. rhizosphere, and SA5 from non-rhizosphere soil.

3.3 DNA extraction

Total soil DNA was extracted directly from soils samples using the BIO101 Fast DNA Spin kit (Soil) (Qbiogene Molecular Biology Products). DNA was maintained at –20 ºC at the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

3.4 Polymerase chain reaction

3.4.1 16S PCR

A portion of 16S bacterial gene of the rDNA was amplified by means of PCR from the total extracted soil DNA, using the 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)

These primers were found to be valuable in molecular ecological and systematics studies focussing on the 16S rRNA gene (Øvreås and Tosrvik 1998). Authentic *Escherichia coli* DNA (courtesy Dr A.K. Drønen[e]) and a reaction with no template DNA were included as positive and negative controls, respectively. Each PCR tube contained a total volume of 50µl: 40.75µl sterile distilled MilliQ water, 5µl PCR buffer with $MgCl_2$ (10x), 2µl dNTPs (2.5µM), 0.5µl primer K (50µM), 0.5µl primer M (50µM), 1µl template DNA (27ng μl^{-1}), 0.25µl hot start Taq (5U μl^{-1}). DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min. at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 51 °C and 1 min. at 72 °C, followed by 10 min. at 72 °C, and then held at 4 °C. The PCR product was analysed on a 1 % TAE (40mM Tris, 20mM acetic acid, 1nM EDTA (pH 8.3)) agarose gel.

[e] A.K. Drønen, University of Bergen, Department of Biology, Bergen, Norway.

3.4.2 16S rDNA colony PCR

The 16S bacterial gene of each isolate was amplified by means of colony PCR, using the following 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)

The M primer was designed specifically for DGGE analysis, hence the GC-clamp for stability. However, these primers were also used in PCR amplification and sequencing of the pure cultures.

A reaction with no template DNA was included as negative control. Each PCR tube contained a total volume of 25µl: 18.7µl sterile water, 2.5µl PCR buffer with $MgCl_2$ (10x), 2µl dNTPs (2.5µM), 0.5µl primer K (50µM), 0.5µl primer M (50µM), 0.5µl 10^{-1} bacterial suspension, 0.3µl Taq (5U μl^{-1}). DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min. at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 54 °C and 2 min. at 72 °C, followed by 10 min. at 72 °C, and then held at 4 °C. The PCR product was analysed on a 1 % 1x TAE agarose gel.

3.4.3 Internal transcribed spacer sequence PCR

A portion of the internal transcribed spacer (ITS) gene sequence of the DNA from each samples was subjected to PCR using the primer set:

ITS1 : 5'CAT CGA GAA GTT CGA GAA GG3'

ITS4 : 5'TAC TTG AAG GAA CCC TTA CC3'

(White *et al*. 1990)

A reaction with no template DNA was included as negative control. Each PCR tube contained a total volume of 25µl: 18.7µl sterile SABAX water, 2.5µl PCR buffer with $MgCl_2$ (10x), 2.0µl dNTPs (2.5µM), 0.5µl primer K (50µM), 0.5µl primer M (50µM), 0.5µl template DNA (27ng μl^{-1}), 0.3µl hot start Taq (5U μl^{-1}). DNA amplification using the K and M primers was performed in a PCR thermal cycler using the following programme: 10 min. at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 54 °C and 2 min. at 72 °C, followed by 7 min. at 72°C, and then held at 4 °C. DNA amplification using the ITS primers was performed in a PCR thermal cycler using the following programme: 1min. at 92 °C, 30 cycles of 1min. at 92°C, 1min. at 50 °C and 1min. at 72 °C, followed by 5min. at 72 °C, and then held at 4 °C. PCR products were analysed on a 1 % TAE agarose gel.

3.4.4 *xylE* and *ndoB* gene fragment PCR

A *ca.* 400bp fragment from the *xylE* gene encoding catechol 2,3-dioxygenase, responsible for aerobic aromatic metabolism, from the *Pseudomonas putida* (ATTC 23973) TOL plasmid was amplified by means of PCR from soil DNA extracted above, using the primers:

Tol1 : 5'GTG-TCT-ATC-TGA-AGG-CTT-GG3'

Tol2 : 5'ATA-GAA-ACC-GAG-CAC-CTT-GG3'

(Milcic-Terzic *et al.* 2001)

A *ca.* 650bp fragment from the *ndoB* gene encoding naphthalene dioxygenase from *P. putida* (ATTC 17484) was amplified by means of PCR from soil DNA, using the primers:

Nah1 : 5'CAC-TCA-TGA-TAG-CCT-GAT-TCC-TGC-CCC-CGG-CG3'

Nah2 : 5'CCG-TCC-CAC-AAC-ACA-CCC-ATG-CCG-CTG-CCG3'

(Milcic-Terzic *et al.* 2001)

A reaction with no template DNA was included as a negative control. Each PCR tube contained a total volume of 25µl: 16.7µl sterile water, 2.5µl PCR buffer with KCl (10x), 2µl $MgCl_2$ (25mM), 2µl dNTPs (2.5µM), 0.5µl primer Tol/Nah 1 (50µM), 0.5µl primer Tol/Nah 2 (50µM), 0.5µl bacterial suspension (10^4cells ml^{-1}), 0.3µl Taq (5U μl^{-1}). DNA amplification was performed in a PCR thermal cycler using the following programme: 3 min at 95 °C, 40 cycles of 45 s at 94 °C, 45 s at 52 °C and 2 min. at 72 °C, followed by 5 min. at 72 °C, and then held at 4 °C. PCR product was cleaned by transferring the entire volume to a 0.5ml Eppendorf tube, adding 2µl of 3M sodium acetate and 50µl 95 % ethanol, and allowing it to stand on ice for 10 min. The suspension was centrifuged at 10 000 rpm for 30 min, the ethanol solution removed and the pellet rinsed in 150µl 70 % ethanol. After further centrifugation at 10 000rpm for 5 min, the ethanol was aspirated and the pellet dried under vacuum for approximately 10min. Following this, the pellet was resuspended in 20µl sterile deionised water. PCR product was analysed on a 1.6 % 1x TBE (89mM Tris, 89mM boric acid, 2mM EDTA (pH 8.0)) agarose gel.

3.4.5 *xylE* and *ndoB* gene fragment colony PCR

A 404bp fragment from the *xylE* gene encoding catechol 2,3-dioxygenase from the *P. putida* (ATTC 23973) TOL plasmid, and a 641bp from the *ndoB* gene encoding naphthalene dioxygenase from *P. putida* (ATTC 17484), were amplified by means of colony PCR from isolated species, according to the method and primers described in 3.4.4. A volume of 0.5µl bacterial suspension (10^4 cells ml^{-1}) was used as a template for PCR, after which the product was analysed as above.

3.4.6 *nifH* PCR

A portion of the *nifH* gene involved in nitrogen fixation was selectively amplified by means of nested-PCR from the total extracted soil DNA and from bacterial colonies, using the degenerate primers:

nifH (Forward A) : 5' GCIWTITAYGGNAARGGNGG 3'

nifH (Forward B) : 5' GGITGTGAYCCNAAVGCNGA 3'

nifH (Reverse) : 5' GCRTAIABNGCCATCATYTC 3'

(Widmer *et al.* 1999)

DNA sequence degeneracies are depicted using the International Union of Pure and Applied Chemistry Conventions (Liébecq 1992):

R : A/G

Y : C/T

W : A/T

V : A/C/G

B : C/G/T

N : A/C/G/T

I : Inosine used to reduce degeneracy in fourfold degenerate positions.

DGGE with product from nested PCR has been proven to be accurate by Bodelier *et al*. (2005), who determined species diversity within methanotrophic microbial communities. Two PCR reactions were performed on each sample, the first using primers *nifH* (Forward A) and *nifH* (Reverse) and the second using *nifH* (Forward B) and *nifH* (Reverse). Soil samples 9-18 (Table 1) and bacterial isolates from Molobela (2005) (See 3.2) were numbered according to their PCR results (Table 2). The PCR reaction component volumes were the same as in 3.4.4 and 3.4.5 using 0.5µl of a 10^{-1} bacterial suspension as template for the first reaction and 0.5 µl of this PCR product (*ca.* 27ng μl^{-1}) as template for the second reaction. DNA amplification was performed in a PCR thermal cycler using the following programme: 11 s at 94 °C, 40 cycles of 15 s at 92 °C, 8 s at 48 °C, 30 s at 50 °C, 10 s at 74 °C, 10 s at 72°C, followed by 10 min. at 72 °C, and then held at 4 °C. The PCR product was viewed on a 1 % TAE agarose gel. All reactions were performed in triplicate to negate possibilities of human or reagent error in PCR protocol.

Table 2: *nifH* PCR productnumbers of soil and bacterial samples collected from an unpolluted control site at the University of Pretoria experiment farm and from polluted and unpolluted areas at site 2 in Mpumalanga Province, South Africa (Table 1), with a history of crude-oil, pitch, diesel, petrol and tar pollution.

***nif* PCR product number**	**Corresponding soil sample or bacterial sequence (SA) number**	**Area state**[f]	**Description/Identification**
1	9	U	Unpolluted control soil from University of Pretoria experiment farm
2	10	U	Unpolluted soil from *Bidens pilosa* rhizosphere
3	11	U	Unpolluted soil from *Brantha serratia* rhizosphere
4	12	U	Unpolluted soil from *Cyperus esculentus* rhizosphere
5	13	P	Polluted soil (pitch/oil/diesel/petrol/tar) 10cm from *C. esculentus* plant
6	14	P	Polluted soil (pitch/oil/diesel/petrol/tar) from *C. esculentus* rhizosphere
7	15	P	Polluted soil (pitch/oil/diesel/petrol/tar), 10cm from *B. serratia* plant
8	16	P	Polluted soil (pitch/oil/diesel/petrol/tar), from *B. serratia* rhizosphere
9	17	P	Polluted soil (workshop oil) mulched with wood chips, 10cm from *B. serratia* plant
10	18	P	Polluted soil (workshop oil) mulched with wood chips from *B. serratia* rhizosphere
11	SA1	P, U	Bacterial isolate from *B. pilosa* rhizosphere in unpolluted soil and from *E. coracana* rhizosphere in polluted soil, groups with *Pseudomonas* genus
12	SA2	P	Bacterial isolate from *B. pilosa* rhizosphere, groups with *Providencia* genus

[f] U = unpolluted and P = polluted

Table 2 (continued)

***nif* PCR product number**	**Corresponding soil sample or bacterial sequence (SA) number**	**Area state[f]**	**Description/Identification**
13	SA3	P	Bacterial isolate from *B. pilosa* rhizosphere, groups with *Providencia* genus
14	SA4	P, U	Bacterial isolate from *E. coracana* and *C. esculentus* rhizospheres and from unpolluted soil void of plants, groups with *Staphylococcus* and *Bacillus* genera
15	SA5	P	Bacterial isolate from polluted soil with no plants growing, groups with *Pseudomonas* genus
16	SA6	P	Bacterial isolate from *C. esculentus* rhizosphere, groups with *Pseudomonas* genus
17	SA7	P	Bacterial isolate from *C. esculentus* rhizosphere, groups with *Pseudomonas* genus
18	SA8	P	Bacterial isolate from *E. coracana* rhizosphere, groups with *Pseudomonas* genus

3.5 DGGE

PCR product was subjected to DGGE according to the method described by Muyzer *et al.* (1993). Ten microlitres containing *ca.* 250ng of the various 16S and ITS PCR products was loaded per lane onto two 25-55 % denaturing gradient gels (Table 3). Similarly, 10µl (*ca.* 250ng) of *xylE* and *ndoB* products were loaded per lane onto a 30-60 % denaturing gradient gel. Finally, *nifH* nested-PCR products of the samples were loaded onto a 30-65 % denaturing gradient gel. Gels were run at 70 V for 17 h at a constant temperature of 60 °C. Image analysis was performed using the Gel2K (Norland 2004) programme and fingerprints were analysed in a cluster investigation using CLUST (Norland 2004).

Table 3: Denaturing gradient table showing volumes in millilitres of DSSA (denaturing stock solution A: 8 % acrylamide in 0.5x TAE (40mM Tris, 20mM acetic acid, 1nM EDTA (pH 8.3) buffer) and DSSB (denaturing stock solution B: 8 % acrylamide, 7M urea, 40 % formamide in 0.5x TAE buffer) mixed to form a gradient within the gel.

Denaturing percentage	DSSA (ml)	DSSB (ml)
25	10.9	3.6
30	10.2	4.4
35	9.4	5.1
40	8.7	5.8
45	8.0	6.5
50	7.3	7.3
55	6.5	8.0
60	5.8	8.7
65	5.1	9.4

Selected bands were picked under blue light from DGGE gels using a sterile micropipette tip. Each band was assigned a number for sequence analysis. The gel fragment was placed into 25µl filter-sterilised deionised water and allowed to stand overnight to dissolve. DNA from bands were then subjected to PCR, with respective primers, for sequencing purposes. Representative final sequences obtained were deposited into GenBank.

3.6 Sequencing

Sequencing the PCR product from the 16S colony PCR using the K and M primers above provided tentative species identification. Each isolate was sequenced in an Eppendorf tube containing 1µl clean PCR product, 2µl "Big Dye" (Roche) sequence mix, 0.32µl primer and 1.68µl filter-sterilised deionised water. The sequence 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 16S eubacterial gene of the rDNA were obtained using the K primer above. Nucleotide sequence order was confirmed by comparison with the sequence obtained from the M primer of the corresponding sample.

Each sequence was subjected to a BLAST analysis on the GenBank database and matching hits, with e-values closest to 0.0 indicating a statistically plausible match, were selected for alignment. For samples 1-8, five matching hits with e-values closest to 0.0, were selected for alignment, whereas three matching hits closest to e 0.0 were selected for alignment from pure cultures SA1-SA8. In both cases, sequences of several species known to catabolise petrol, diesel, oil and other PAH and polyphenol-containing substances were included in the alignments. Sequences were aligned with Clustal X (Thompson *et al.* 1994) and inserted gaps were treated as missing data. Ambiguously aligned regions 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 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 signal in the data sets was assessed by evaluating tree length distributions over 100 randomly generated trees. The consistency (CI) and retention indices (RI) were determined for all data sets.

Phylogenetic trees of sequences from samples 1-8 were rooted with *Bacillus subtilis*, and with *B. subtilis*, *Thermotoga maritima* and *E. coli* as outgroups to the remaining taxa for the non-BLASTed and BLASTed results, respectively. Phylogenetic trees of sequences from pure cultures SA1-SA8 were rooted with *T. maritima* as outgroup to the remaining taxa. Bootstrap analyses were conducted, retaining groups with 70% consistency, to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated. In sequences from soil samples 1-8, this was followed by a distance analysis using *B. subtilis* and *T. maritima* as outgroups to the analysed taxa. Two models of evolutionary base substitutions within PAUP were used to estimate evolutionary distances (Kimura 1981). This model also gives an approximation of evolutionary rates and divergence times, using formulae to determine base-substitution rates at each base of a codon.