BEDWAS COLLIERY AND COKING WORKS REMEDIATIC

BENCH-SCALE BIOTREATABILITY TRIALS ON MATERIAL FROM THE FORMER BEDWAS COLLIERY AND COKING WORKS.

PARSONS BRINCKERHOFF

100 YEARS®

Report Title	:	BENCH-SCALE BIOTREATABILITY TRIALS ON MATERIAL FROM THE FORMER BEDWAS COLLIERY AND COKING WORKS.
Report Status	:	Draft
Job No	:	BEN 45321
Date	:	May 2003
Prepared by	:	Russell Thomas/Alan Beswick (HSL)
Checked by	:	Nigel Snedker
Check Cat	:	C
Approved by	:	

CONTENTS

EXECUTIVE SUMMARY

Parsons Brinckerhoff are acting as joint consulting engineer with Caerphilly County Borough Council (CCBC) in South Wales for the site investigation and remediation of the former Bedwas Colliery and coke works. As part of these work a nominated subcontractor was required to perform biotreatability tests so bioremediation of the site could be adequately assessed.

Based on experiments carried out to assess the biotreatability of samples, it wa concluded that the hydrocarbon contaminated soil from the Bedwas site can be treated by bioremediation, however performance is limited where:

- Heavy metals concentrations are high.
- PAH concentrations are in excess of approximately 25,000 mg/kg
- The presence of contamination is within coal or bitumous fragments, limiting the bioavailability and therefore the biodegradation of PAH.

The microbial composition of the trials was variable, no microorganisms predominated in the trials, though Pseudomonas sp. were the most commonly isolated. The maximum amount of degradation achieved was 89% in terms of percentage degraded (TP 3086) and 33490 mg/kg in terms of amount degraded (TP 3208). Petroleum hydrocarbon contamination was also degraded, with the maximum biodegradation achieved was 79%.

1 INTRODUCTION

1.1 General

1.1.1 Parsons Brinckerhoff are acting as joint consulting engineer with Caerphilly County Borough Council (CCBC) in South Wales for the site investigation and remediation of the former Bedwas Colliery and coke works. The site is a former colliery and coking works in the Bedwas area of Caerphilly. The site is being remediated for reuse as a mixed-use residential/industrial project. As part of the site investigation, biotreatability test were undertaken to examine the potential for bioremediation of the site. These test were undertaken by the HSL.

1.1.2 Bioremediation is capable of remediating a range of hydrocarbons, specifically phenols, BTEX, Polycyclic Aromatic Hydrocarbons (PAH) and petroleum hydrocarbons. The biotreatability tests are carried out on a batch basis in small flasks. The process utilises indigenous bacteria found in the feedstock being treated.

1.1.3 This report details the laboratory scale tests carried out on material taken from the site. Trials were undertaken in shaken flasks at the Health and Safety Laboratories (HSL) according to a protocol previously used on similar projects. Trials lasted a duration of 24 days, with samples taken daily and were also analysed by the HSL.

2 MATERIALS AND METHODS

2.1 Feedstock Material from the Bedwas Site

2.1.1 Sampling

2.1.2 HSL collected approximately 8 - 10kg of each of 5 Bedwas soils from the former colliery and cokeworks site and the Bridgend laboratory. Samples were designated with the following Bedwas codes, and described as follows by the supplier:

Bedwas Code	HSL code	Description
TP9.071	200209961	Brown to grey silty, with sand, fine to coarse gravel, with pockets of oily silt strong contamination at 3m depth.
TP3.086	200209960	Black silty, slightly gravely sand. Gravels fine to coarse with ash, tyres, wood and brick
TP3.124	200209964	Slightly silty, sandy, angular fine to coarse Gravel of ash and slag – strong tar odour
TP3.208	200209962	Soft, brown and grey sandy. Slightly Gravely organic silt, with cobbles and Boulders of concrete. Also some mud Stone and ceramic
TP3.302	200209963	Black, silty gravely sand with some cobbled sandstone and fragments of metal and wood.

2.2 Sample preparation

2.2.1 All soils were heavy with water and / or tar. In view of this, and the coarse gravely nature of some soils, it was not possible to make use of a 'riffler' to homogenize the material during initial processing. As an effective and recognized alternative, we made use of a coning method to obtain representative sub-samples from the bulk soils, for use in flask cultures. Briefly, a central portion – approximately 4kg – of each bulk sample was well mixed in a fume hood. A buffer zone of untouched soil was maintained around the periphery of the bulk container. After 10 minutes of mixing, 800 – 900g of each soil was removed and placed in to a separate container. This was, in turn, well mixed, and 200 – 250g removed and stored in a sterile screw-top container. This latter material was then used for preparation of smaller samples that were used to inoculate culture flasks.

2.3 De-stoning and assessment of percentage gravel.

2.3.1 Pre-weighed portions of the mixed bulk sample were coarse filtered through a 9mm gauge mesh to remove larger gravel components. The sieved material was then passed through a finer filter of 2mm gauge to remove finer gravel. In all cases, soil was worked through the meshes using continuous physical mixing using a spatula. Percentage gravel data were then calculated by the weighing of residual fine material.

2.4 Measurement of percentage moisture of soils

2.4.1 One hundred grams of each complete soil was pre-weighed in to large Petri dishes and well spread to maximize drying effect. Samples, in the partially opened dishes, were left to dry at 25oC under forced evaporation in a class II microbiological cabinet. Re-weighing was performed at 3 days, followed by further breaking up of soil crumb and then a second re-weighing of material at 7 days. Beyond 7 days, no further moisture change was measurable.

2.5 Initial pH of soil

2.5.1 Ten grams of each soil was combined with 5ml of de-ionised water of neutral pH (initial water pH 7.05), and a soil paste formed. pH of soil paste was then established using a calibrated digital pH meter and probe.

2.6 Biotreatability testing and Microbiology analysis

2.7 Culture flask preparations

2.7.1 The soil was prepared as 40% wt/volume flask cultures at neutral pH (established with HCL/NaOH) within Cerniglia minimal salts medium, as recommended in the project background sheet supplied by Parsons-Brinckerhoff. Twenty grams of soil were used in 50ml Cerniglia medium for each flask preparation, and this allowed ample material for all subsequent analyses. PH was checked after addition of soil to media to ensure neutral pH at the commencement of the process. PH was adjustable using filter sterilized NaOH and HCL. High-purity components were used to prepare the medium in order to avoid unnecessary metal contamination from components – something that may occur with some relatively impure media products. As the whole culture-based process was reliant on the activity of the native microorganisms present in the sample, all equipment in contact with active cultures was sterilized or disinfected prior to use. This included the digital pH electrode used for monitoring of culture pH. Flasks were sealed with subaseals to prevent loss of volatile components but were vented every two days – during pH checks - to prevent oxygen starvation of

the aerobic cultures within. Antifoam agents were available but were not necessary.

2.7.2 Media used

2.7.3 The media used to re-suspend the soils was that previously used in tests at Advantica and was an adaptation of 'Cerniglia minimal salts media'. Previous studies carried out at Advantica had shown the superior performance of this media over others tested. This media is modified by replacement of the nitrogen and phosphate sources by ammonium phosphate.

Mineral components

$NH_4H_2PO_4$	1.1 g/l
LiCl	20 µg/l
$(NH_4)_2HPO_4$	1.1 g/l
$MgSO_4\ 7H_20$	0.15 g/l
NaCl	0.3 g/l

Trace metal solutions

$CuSO_4.5H_2O$	80µg/l
$ZnSO_4.7H_2O$	100 µg/l
KBr	30 µg/l
KI	30 µg/l
$MnCl_2.2H_2O$	600 µg/l
$SnCl_2.2H_2O$	40 µg/l
$FeSO_4.7H_2O$	300 µg/l

2.8 Microorganisms used and the method of identification.

2.8.1 The microorganisms used for the bench-scale bioreactor trials were those naturally found within the soil on the Bedwas site. To identify the soil microcosm, microorganisms were isolated from the trial pit soil samples and from samples taken from the bioslurry reactor trials. Microorganisms were also isolated from the samples, according to standard microbial serial dilution procedure, plating out seven drops of each dilution on a solid media.

2.9 Methods of Microbial Enumeration and Identification.

2.9.1 Enumeration of microorganisms was only undertaken on viable colonies, this was undertaken carrying out standard serial dilution plating procedures using 20µl of each dilution on solid media consisting of nutrient agar or minimal agar (C & E medium, as above, with 15 g/l agar noble) with naphthalene (1g) supplied (placed on the inside of the petri dish lid and the plate was inverted) as a carbon source.

2.9.2 16s ribosomal Ribonucleic Acid (RNA) Polymerase Chain Reaction (PCR)-based identification was used to identify predominant colonies observed on the petri dishes. These represented the most commonly seen colony types worthy of further investigation. Sterile pipette tips were used to lift discrete colonies from isolation plates, and these were then suspended in 5 microlitres of sterile water prior to amplification of the 16S rRNA gene of each bacterial isolate. All samples were run in duplicate.

2.9.3 , and of the 9 colony types analysed in this way, all gave PCR products in one or both of the reaction tubes. The results of the initial 16S-based PCR are provided in Figure 1, below. Marker DNA (M) is somewhat unclear from this scan, but the original gel image indicated that the PCR products were of expected size. In addition, weak bands are evident in wells labelled 'H', although these bands are less clear in figure 1, below. Controls ('+' and 'NEG') were as expected.

2.9.4 Nine PCR products identified from the gel (Figure 1) were cleaned using Pharmacia Sephacryl S400 columns, and were sent off for sequencing with a standard 16S primer.

2.9.5 The genetic analysis of the colonies grown from the soil samples taken from the samples involved amplification and analysis of the first third of the 16S rRNA gene. In this region of 16s rRNA - 16S base positions 1 - 530 - contains three valuable 'hyper-variable' regions that differ between different bacterial species. By comparing the DNA sequences of this region to a large database of other 16S sequences (now amounting to over 10,000 sequences), it is possible to infer a genus level identification with accuracy, and to infer a species level identification with some confidence, providing the sequence match is high (95 percent or above). All sequence data were aligned on the Internet using 'Advanced BLAST', through the National Centre for Biotechnology Information, in the USA.

2.10 Sub-sampling culture procedures

2.10.1 Each soil sub-sample was prepared in three sets of duplicate samples, marked 'Day 0', 'Day 14' and 'Day 28'. This resulted in 6 initial sample suspensions generated from each trial soil, and for each of these the liquid component was later separated from the solids, thus duplicating the number of samples to 12 from each original soil sample; 60 from all 5 trial samples. Once flasks from all trial soils are prepared, the 'Day 0' samples were well dispersed and suspended by vigorous shaking (130rpm) within a shaking incubator. They were then removed for immediate analysis. Day 14 samples were removed after 14 days incubation at 25°C, and day 28 samples were removed for analysis after 28 days shaking at 25°C. Ten millilitres of suspension was removed from each flask at the appropriate interval and a dilution series prepared on solid growth media, in duplicate, in order to ensure that accurate microbial quantification could be achieved. For all samples, 0.1ml of suspension was spread on each agar plate. A grain of naphthalene was placed on the inside of each Petri dish lid as a potential carbon source. All plates were incubated at 25°C until mature colonies were countable. Colony forming units (CFU) data are based on a mean count from duplicate plates.

2.11 Preparation of samples for chemical analysis

2.11.1 Following phased completion of microbiological sampling, our organic chemists separated each sample into solid and liquid phases using Soxhlet Thimbles.

2.12 Heavy metals analysis – inorganic chemistry component

2.13 Preparation of soil samples

2.13.1 On receipt of the soil solids and the accompanying leachate solution, the solids were air-dried at 40°C and a 0.5 g aliquot dried down for sample digestion.

2.13.2 Sample digestion EPA 3050B (icp-aes version). Sample digestion involved adding 10 M aqua regia to each soil sample placed in a digestion vessel and refluxed at 95°C until reaction dies down. Samples were then filtered into a 100-ml flask for analysis.

2.14 **Analysis**

2.14.1 Using radial and axial viewing ICP-AES for Group A elements of the ICRCL suite.

2.14.2 Internal Quality Control. NIST Montana soils (normal and elevated) to be run in parallel with samples. NIST samples have 'consensus' leachable metal content as determined by a number of US labs using EPA 3050B.

2.14.3 Hexavalent Chromium was determined on a separate 0.5 g aliquot after leaching in dilute HCL at 37°C and on a separate leachate aliquot supplied to us. Hexavalent Cr was determined as soon as possible after submission of samples from earlier analysis.

2.15 Organic chemistry analysis

2.15.1 Sample preparation and analysis

2.15.2 Each sample was separated into solid and liquid phases. Sub-portions were analysed for organics following solvent extractions. Organic analyses was conducted employing methodologies broadly in line with Environment Agency "Blue Book" methods (Methods for waters and associated materials).

2.15.3 Sample preparation

2.15.4 Whole samples (50 ml containing 20 g of sample – minus the microbiological component used for agar culture) were shaken then transferred to pre-weighed Soxhlet Thimbles. The filtrate (water phase) was collected and 20 ml will be analysed for organics. Five ml were also provided to Inorganics section for metals analysis. The solids remaining in the Soxhlet Thimble were dried at 30°C and 2 g supplied to Inorganics section for metals analysis; the residual solids were analysed for organics.

2.15.5 20 ml of filtrate were spiked with deuterated PAH (d-8 naphthalene, d-10 anthracene and d-12 perylene) then liquid/liquid extracted with dichloromethane (DCM). Following concentration, and sample clean-up (if necessary), the organic extracts were then ready for analysis.

2.15.6 The dried Soxhlet thimbles were reweighed following removal of sample portion for inorganics. This enabled calculation of dry sample weight for extraction. The solids in the thimbles were then be spiked with deuterated PAH (d-8 naphthalene, d-10 anthracene and d-12 perylene) and Soxhlet extracted with DCM for 24 hours. Following concentration, and sample clean up (as necessary), the organic extracts were ready for analysis.

2.16 Organic analysis

2.16.1 Solvent extracted samples were analysed by gas chromatography with mass spectrometric (GC/MS) detection for total PAH, total petroleum hydrocarbon, phenol and BTEX (benzene, toluene, ethyl benzene and xylenes). If required, phenol will be

determined by GC/MS following derivatization. Concentrations to be expressed in terms of weight/volume for water phase and weight/weight for solids.

2.17 Quality issues

2.17.1 Identification of analytes will be confirmed using mass spectrometry. Recovery ranges for PAH will be quoted, but results will not be corrected for recovery.

3 RESULTS

3.1 Results – Microbiology

3.1.1 As can be seen from Table 1, some degree of variation in total microbial numbers was seen both between samples, and within samples at different points in the incubation sequence. In general, concentrations of microorganisms, expressed as colony-forming units per ml of suspension (cfu/ml), were in the range $10^6 - 10^8$ cfu/ml, but some flasks failed to generate colonies at Day 14 and Day 28 analysis points. Two media types were chosen for culture based enumeration; one a comprehensive medium at half strength (Nutrient Agar) and the other a minimal salts medium (Burke's medium).

3.1.2 TP3.071

3.1.3 Over the 28 day period, bacterial numbers were seen to rise from 1.85×10^7 at day 0 to 1.37×10^8 at day 28 on Nutrient Agar (NA). Quantification on Burke's medium recorded counts in the order of 10^7 total cfu/ml throughout the course of incubation.

3.1.4 TP3.086

3.1.5 Variation here was slightly increased on NA, with numbers initially in the order of 108, dropping off at the Day 14 point to $10^6 - 10^7$, with a final rise at the Day 28 point to $10^7 - 10^8$ cfu/ml. Counts on Burke's medium showed less count variation, and an overall maintenance of higher microbial load per ml, with counts in the range 107 – 10^8 cfu/ml across the 28 day period of incubation.

3.1.6 TP3.124

3.1.7 A good degree of consistency was maintained on NA throughout the course of the incubation, with all counts in the order of 10^8 cfu/ml suspension. The data from Burke's medium was more variable. Counts obtained were in the expected range of $10^7 - 10^8$ cfu/ml. However, duplicate flask preparations that we termed 'TP3.124 - B', were seen to fail at the Day 14 and 28 recovery points – reason unclear. All conditions of incubation were identical to other samples, and to the flask 'TP3.124 - A' preparations for the same soil suspension.

3.1.8 TP3.208

3.1.9 Nutrient agar again exhibited variation in the range $10^6 - 10^8$ cfu/ml for this soil suspension. As with sample TP3.086, the lowest cfu count was obtained in the mid term of the incubation, with a progressive recovery by Day 28. In contrast, cfu counts on Burke's were seen to decrease for this soil suspension. Initially, counts in the order of 10^8 were measured, with 10^6 range achieved by the end point at Day 28.

3.1.10 TP3.302

3.1.11 A reduction in microbial counts was observed on NA for this soil suspension, commencing at 10^7–10^8 (Day 0), but down to 106 by Day 28. Recovery of colonies on Burke's showed a similar trend, with consistent counts in the 10^7 cfu/ml range up to and including the Day 14 point, with a fall off in counts by Day 28 to 10^6 cfu/ml.

3.1.12

3.2 Results – inorganic chemistry

3.2.1 All complete inorganic chemistry results can be found in the appendix, the accumulated results for each trial can be found below.

3.2.2 TP3302

Days	As	Cd	Cr	Pb	Hg	Se	Cu	Ni	Zn	Cr6	S	tCN
0	5.84	0.625	15	93	0.81	0.88	90	39	293	0.3	79	
14	5.84	0.625	19	77	0.81	0.88	68	37	188	0.3	79	
28	5.84	0.625	17	101	0.81	0.88	85	35	280	0.8	79	

3.2.3 TP3208

Days	As	Cd	Cr	Pb	Hg	Se	Cu	Ni	Zn	Cr6	S	tCN	
0	47.5	7.8	14.0	186.0	6.0	2.9	41.0	25.0	232.0	0.7	40.0	4	
14	47.5	7.8	10.0	48.0	6.0	2.9	29.0	20.0	127.0	0.0	40.0	4	
28	47.5	7.8	15.0	97.0	6.0	2.9	46.0	29.0	249.0	0.0	40.0	4	

3.2.4 TP3124

As	Cd	Cr	Pb	Hg	Se	Cu	Ni	Zn	Cr6	S	tCN	Days
24	0.54	14	452	1.75	1.27	431	78	1866	1	141	23	
19	0.54	17	348	1.75	1.27	162	45	711	0.3	141	23	
27	0.54	17	461	1.75	1.27	398	70	1735	0.4	141	23	

3.2.5 TP3071

As	Cd	Cr	Pb	Hg	Se	Cu	Ni	Zn	Cr6	S	tCN	Days
7.9	0.12	18	83	0.4	0.58	32	40	138	0.4	1124	0	
7.9	0.12	18	92	0.4	0.58	33	42	133	0.3	1124	0	
7.9	0.12	17	71	0.4	0.58	28	38	125	0	1124	0	

3.2.6 TP3086

As	Cd	Cr	Pb	Hg	Se	Cu	Ni	Zn	Cr6	S	tCN	Days
6.36	0.56	6	29	0.17	0.77	39	21	72	0.2	510	20	
6.36	0.56	6	27	0.17	0.77	39	21	71	0.2	510	20	
6.36	0.56	8	34	0.17	0.77	43	24	90	0	510	20	

3.3 Leachate analysis

3.3.1 Filtered samples were split into two fractions and stored at 4 °C until analysis. The fraction for heavy metal determination was acidified prior to storage.

3.4 Metal determination

3.4.1 Sample aliquots were taken and diluted to 5 ml prior to analysis. Nitric acid was added to matrix-match samples with calibration standards. Samples were analysed using inductively coupled plasma-atomic emission spectrometry (ICP-AES) utilising instrumental conditions specified in EPA method 200.7 Determination of metals and trace elements in water and wastes by inductively coupled plasma-atomic emission spectrometry. Calibration standards were prepared from commercially available standards with values traceable to National Institute of Science and Technology (USA). Standards from alternative suppliers and/or standards with alternative lot numbers were used to cross check the validity of the generated calibration and results were within ± 7.5 at a nominal check concentration of 1.00 mg/L. As a further check a wastewater sample, Aquacheck 231, supplied by the UK Water Research Council (WRc) as a proficiency test sample, was run in parallel with this analysis. The results reported (in the appendix) are the mean of duplicate samples. Contributions from laboratory control blanks have been subtracted.

3.5 Hexavalent chromium determination

3.5.1 Sample aliquots were analysed as received. Samples were analysed using ion chromatography (IC) using an in-house method based upon EPA method 7199 Determination of hexavalent chromium by ion chromatography. Calibration standards were prepared from commercially available standards with values traceable to National Institute of Science and Technology (USA). Standards from alternative suppliers and/or standards with alternative lot numbers were used to cross check the validity of the generated calibration and results obtained were within ±25 % of each other at a set concentration of 0.400 mg/L. The results reported (in the appendix) are the mean of duplicate samples. Contributions from laboratory control blanks have been subtracted.

3.6 Soil analysis

3.6.1 Prepared soil samples were stored in sealed plastic vessels prior to analysis. A nominal 0.5 g aliquot of sample was weighed out in duplicate into clean plastic tubes using a 4-place calibrated microbalance. One set of samples was then processed for metals and the second set processed for hexavalent chromium.

3.7 Metal determination

3.7.1 Sample aliquots were taken and decanted into individually labelled glass digestion vessels and were digested according to EPA method 3050 Determination of metals and trace elements in solid wastes by inductively coupled plasma-atomic emission spectrometry and/or flame atomic absorption spectrometry. The resultant solutions were analysed using inductively coupled plasma-atomic emission spectrometry (ICP-AES) utilising using instrumental conditions specified in EPA method 200.7 Determination of metals and trace elements in water and wastes by inductively

coupled plasma-atomic emission spectrometry. Calibration standards were prepared from commercially available standards with values traceable to National Institute of Science and Technology (USA). Standards from alternative suppliers and/or standards with alternative lot numbers were used to cross check the validity of the generated calibration which were within ± 7.5 of each other at a nominal concentration of 1.00 mg/L. As a further check two reference soil samples were analysed in parallel with the samples; NIST 2710 Montana Soil (heavily elevated trace element concentration) and NIST 2711 2710 Montana Soil (moderately elevated trace element concentration). Consensus values for these two soils were obtained in a round robin testing programme involving 17 laboratories in the US using EPA method 3050. The results reported below are the mean of duplicate samples. Contributions from laboratory control blanks have been subtracted.

3.8 Hexavalent chromium determination

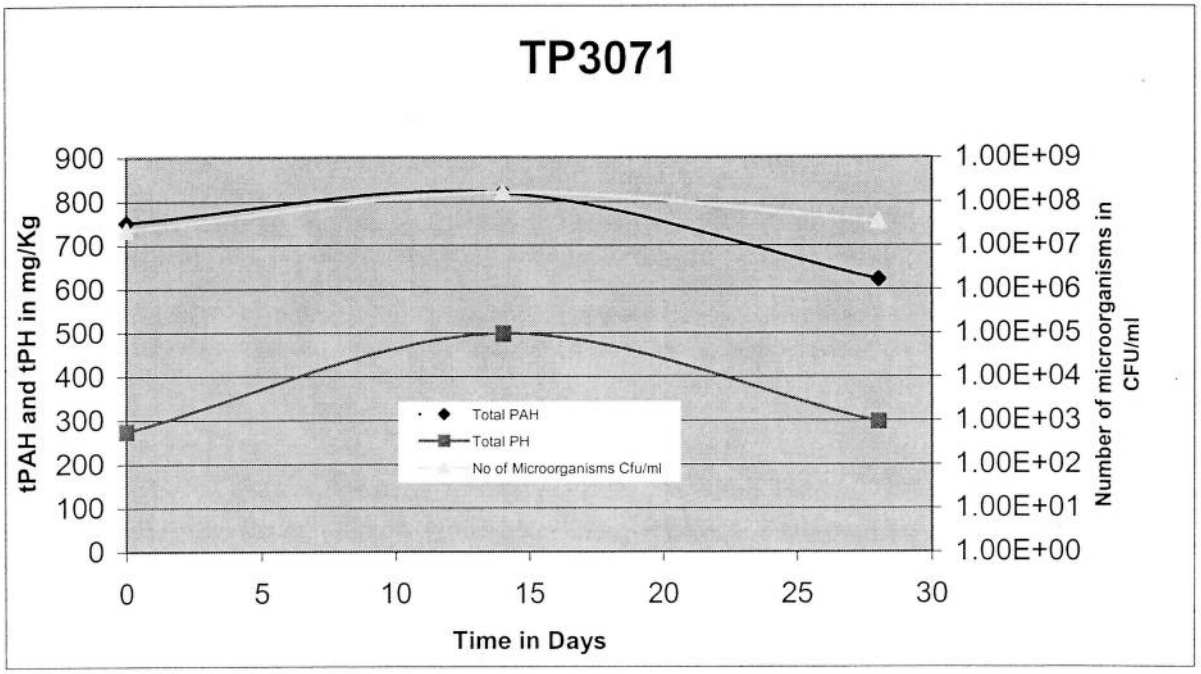
3.8.1 Sample aliquots were taken and leached according to an in-house procedure based upon EPA method 3060A Alkaline digestion for hexavalent chromium but using a heated ultrasonic extraction procedure rather than a hotplate extraction procedure. Samples solutions were subsequently filtered and analysed using ion chromatography (IC) using an in-house method based upon EPA method 7199 Determination of hexavalent chromium by ion chromatography. Calibration standards were prepared from commercially available standards with values traceable to National Institute of Science and Technology (USA). Standards from alternative suppliers and/or standards with alternative lot numbers were used to cross check the validity of the generated calibration and results obtained were within ± 25 % of each other at a nominal concentration of 0.400 mg/L. The results reported below are the mean of duplicate samples. Contributions from laboratory control blanks have been subtracted.

1 INTERPRETATION AND DISCUSSION

1.1 Biotreatability performance

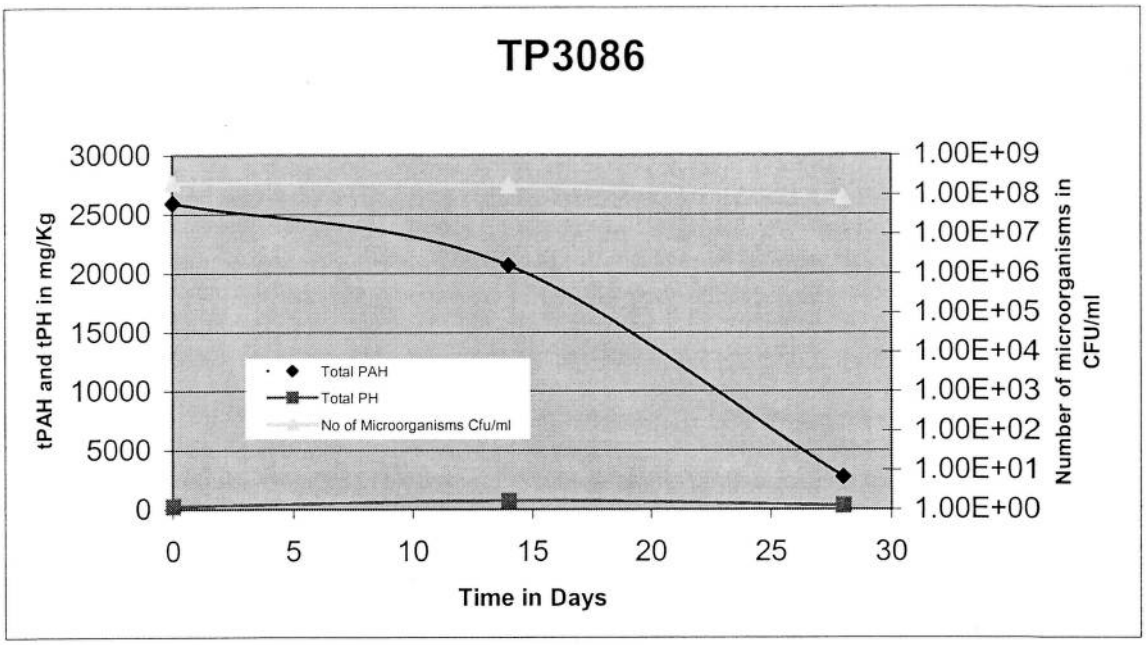
1.1.1 Sample TP3071.

1.1.2 The sample was described as a brown to grey silt, with sand and fine to coarse gravel, with pockets of oil. This material was not heavily contaminated with the main contaminant PAH only reaching a maximum concentration of 900 mg/kg total PAH. This level of contamination would normally be degraded quite rapidly in bench-scale biotreatability test, a degradation of about 60-80% would normally be expected in the first 7-10 days. Though some decrease was noted in the total PAH concentration during the experiment the decrease was only about 130 mg/kg or 17%, a poor result. The number of microorganisms did not significantly decrease suggesting that the soil was not particularly toxic; this was also supported by a lack of heavy metal contamination. However, elevated concentrations of sulphur (1124 mg/kg) were identified and this may have had an inhibitory if not toxic effect on the microorganisms, through acidification and alteration of the redox potential of the media. In addition to this it is probable that a significant amount of the contamination identified was trapped within bitumous particles or coal fragments and was not readily available for biodegradation (bioavailable) and therefore not degraded.



1.1.3 Sample TP3086

1.1.4 The sample was described black silty, slightly gravely sand. Gravels fine to coarse with ash, tyres, wood and brick. This was waste tip material which



1.1.5 This material was heavily contaminated with PAH to about 25,000 mg/kg, the concentration of petroleum hydrocarbons was relatively low reaching a maximum concentration of 750 mg/kg. This concentration of PAH contamination would be expected to pose a significant challenge to biodegradation. A significant decrease in the total PAH concentration was noted during the trials the decrease was about 23184 mg/kg tPAH or 89%, a very good result. The number of microorganisms did not increase significantly as would be expected from such significant degradation, in fact a gradual decrease in the number of microorganisms was noted, suggesting a toxic effect of the high PAH concentration. Again a significant concentration of sulphur (510 mg/kg) was detected but this would appear to have no inhibitory effect on biodegradation. A slight increase in the concentration of petroleum hydrocarbons was noted, though this was not particularly significant by comparison to the tPAH concentration.

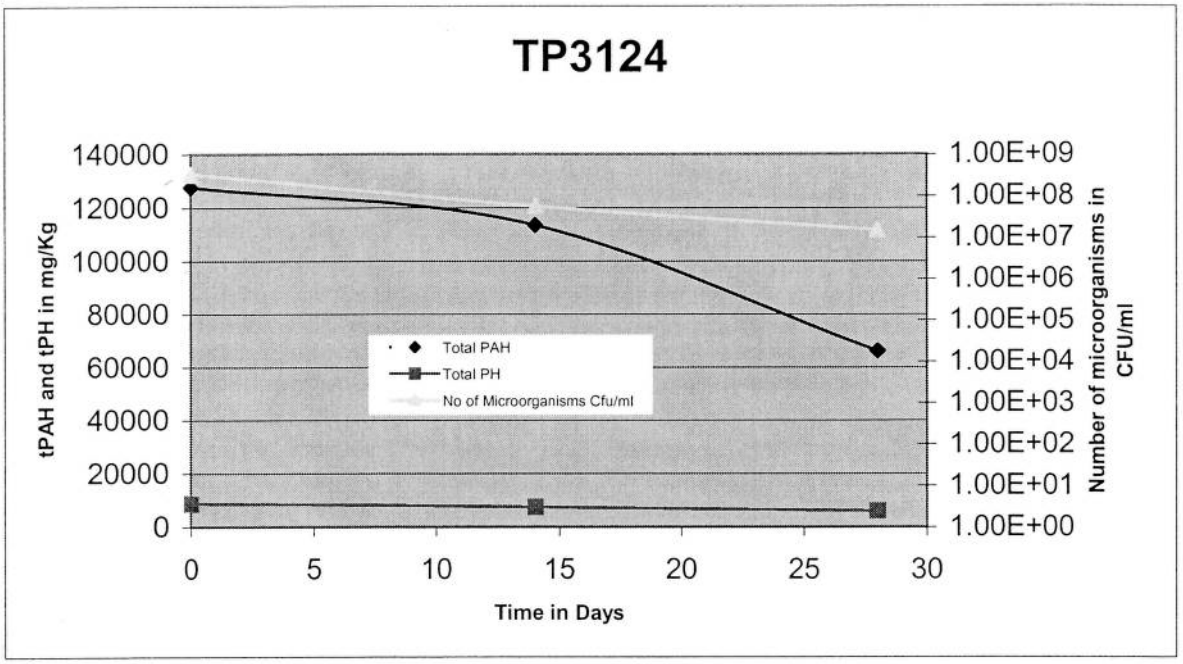
1.1.6 Sample TP 3124

1.1.7 The sample was described as a slightly silty, sandy, angular fine to coarse gravel of ash and slag – strong tar odour.

1.1.8 This material was heavily contaminated with the main contaminant PAH reaching a maximum concentration of 127650 mg/kg total PAH. This level of contamination could normally be degraded effectively in bench-scale biotreatability test, a degradation of about 60-80% would normally be expected in the first 7-10 days. A decrease of about 61460 mg/kg PAH or 48% was noted during the trial. The concentration of petroleum hydrocarbons decreased significantly also by 2780 mg/kg tPH or 31%. The number of microorganisms did not increase significantly as would be expected from such significant degradation, in fact a gradual decrease in the number of microorganisms was noted again, suggesting a toxic effect of the high PAH concentration.

1.1.9 The number of microorganisms significantly decreased during the trial suggesting that the contaminated material was toxic to the microorganism, either from the high

concentration of hydrocarbons or from the elevated concentrations of lead, copper, nickel, zinc and cyanide.



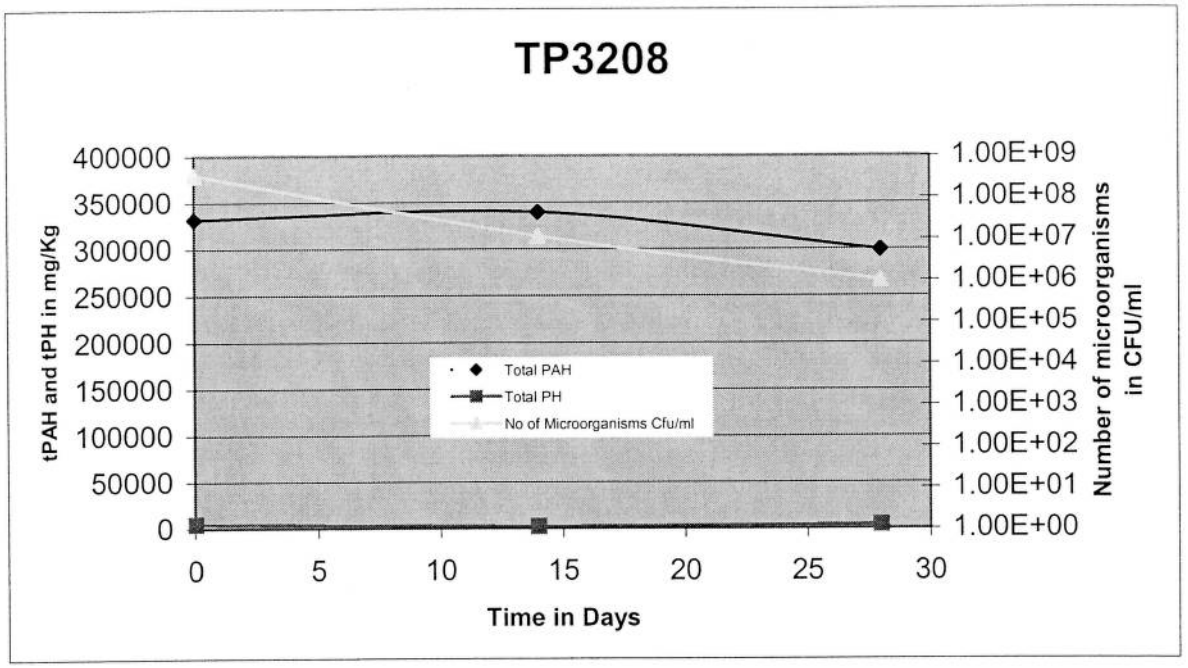
1.1.10 It is possible that the reason for the partial degradation of both the tPH and PAH were due to the gradually reducing number of microorganisms and therefore the ability to degrade the contamination reduced throughout the trial.

1.1.11 Sample TP3208

1.1.12 The sample was described as soft, brown and grey sandy, slightly gravely organic silt, with cobbles and boulders of concrete.

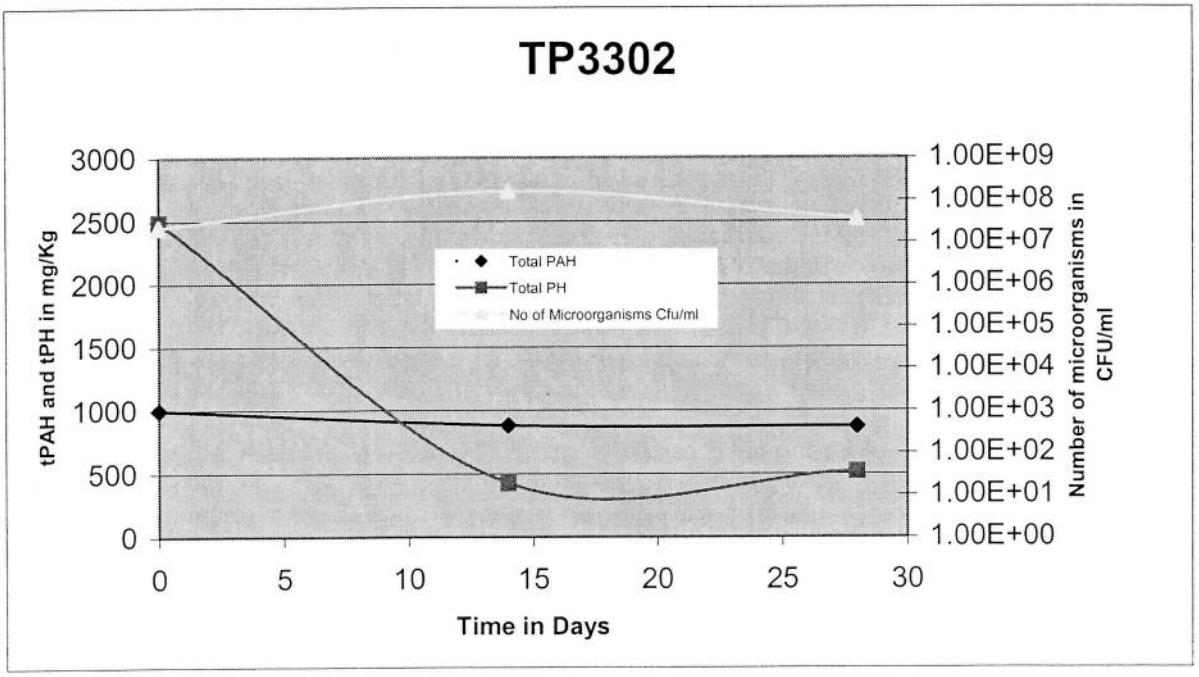
1.1.13 This material was very heavily contaminated with the main contaminant PAH reaching a maximum concentration of 331940 mg/kg total PAH. This level of contamination would normally be inhibitory and toxic to microorganisms. Though some decrease was noted in the total PAH concentration during the experiment the decrease was quite significant considering the amount degraded about 33040 mg/kg, but in relation to the starting concentration it was just a 10% decrease.

1.1.14 The number of microorganisms significantly decreased suggesting that the very high concentration of hydrocarbons was particularly toxic. A slight decrease in the concentration of petroleum hydrocarbons by 16% was also noted.



1.1.15 Sample TP3302

1.1.16 The sample was described as black, silty gravely sand with some cobbled sandstone and fragments of metal and wood.



1.1.17 This material was low to moderately contaminated with PAH or petroleum hydrocarbons only reaching a maximum concentration of 900 mg/kg total PAH and 2500 mg/kg petroleum hydrocarbons. This level of contamination would normally be

degraded quite rapidly in bench-scale biotreatability test, a degradation of about 60-80% would normally be expected in the first 7-10 days. Though some decrease was noted in the total PAH concentration during the experiment the decrease was only about 123 mg/kg or 12%, a poor result, however the degradation of the petroleum hydrocarbons was more successful at 1974 mg/kg or 79%. The number of microorganisms did not significantly decrease suggesting that the soil was not particularly toxic; this was also supported by a lack of heavy metal contamination. It is probable that a significant amount of the PAH contamination identified was trapped within or coal fragments and was not readily available for biodegradation (bioavailable) and therefore not degraded. The petroleum hydrocarbons were probably not associated with the coal fragments and as a result, available for biodegradation resulting in the significant degradation observed.

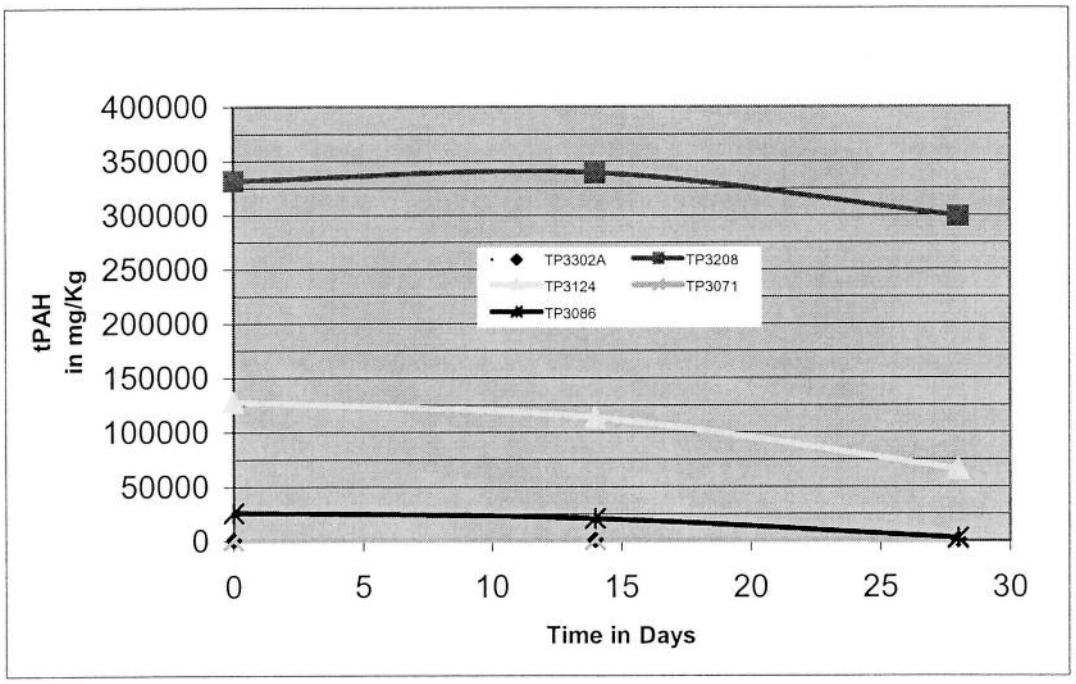


Figure 6. Relative Performance of Each Trial at Degrading tPAH in Terms of tPAH Degraded in mg/kg Over Time.

1.1.18 From Figure 6 its can be seen that relative to each other the material from TP3124 (61460 mg/kg) showed the greatest amount of tPAH in mg/kg degraded, followed by TP3208 (33040 mg/kg), TP3086 (23184 mg/kg), TP3302A (128 mg/kg), and TP3071 (123 mg/kg).

1.1.19 From Figure 7 it can be seen that relative to each other in terms of percentage tPAH degraded material from TP3068 (89%) showed the greatest degradation followed by TP3124 (18%) TP3071 (17%), TP3302 (12%), TP3208 (10%).

1.1.20 Examination of these results show that hydrocarbon in all the soils was biodegradable providing it was bioavailable. Both the results from the material with the lowest concentrations of tPAH in TP3371 and TP3302 indicated that the material wa biodegradable, however a significant amount of the hydrocarbon was not bioavailable

and even after biodegradation, it would leave a residual hydrocarbon contamination risk.

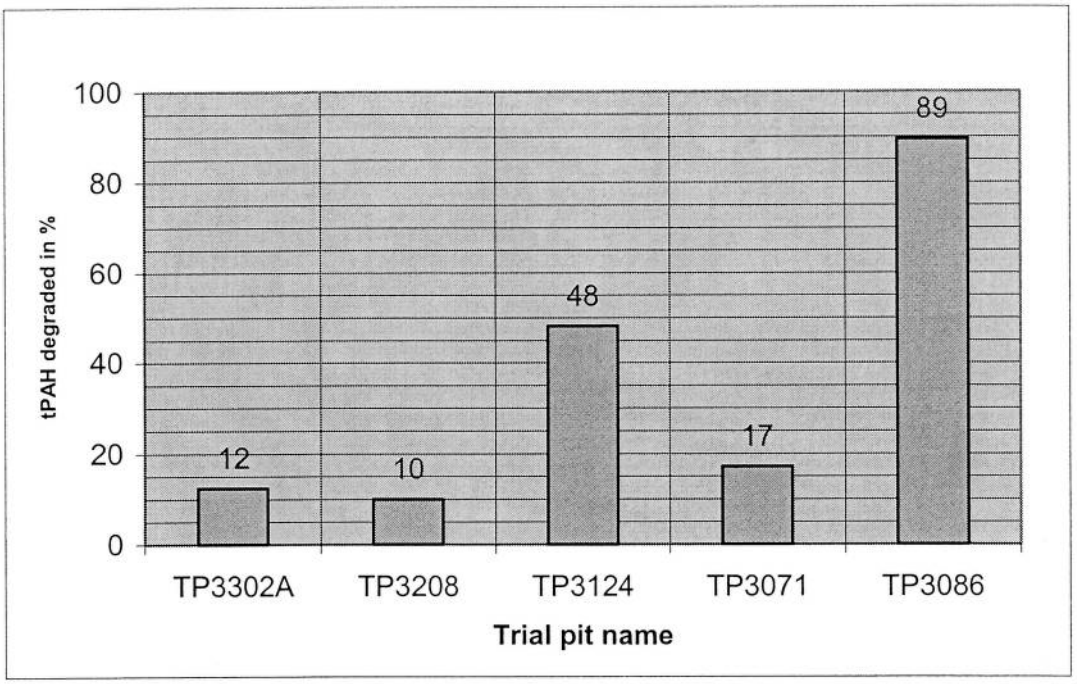


Figure 7. Relative Performance of Each Trial at Degrading tPAH in Terms of % Degraded

1.2 Microbiology

1.2.1 The Identification from colonies cultured from the Bedwas soils, with respective soil codes are given in the table below. Some of the named bacteria are of some interest, and give some indication of what the microbial composition of the soil is. There is a wide range of species, and the profiles for each soil vary quite markedly with time progression, without any obvious pattern. Many of the organisms are well known, including such species as *Pseudomonas sp., Acidovorax sp., Flavobacterium sp* and *Bacillus sp.* Each of these has been implicated in the biodegradation of hydrocarbon contaminants previously and provides further evidence that biodegradation occurred. Though many of the species are common, some of the other bacteria are unusual and probably unique to the site.

Table 1. Microbial composition of the soils from the Bedwas site.

Bedwas Soil Code	Stage of flask incubation	Description of colony	Closest match from sequencing
TP3.071	Day 0	Orange colony	*Flavobacterium sp.*
	Day 14	White colony	*Bacillus sp.*, closest named match *Paenibacillus amylolyticus*
		Orange colony	*Uncultured Bacteroidetes bacterium*
	Day 28	Orange colony	*Uncultured bacterium MK12*, most likely to be *Flavobacterium sp.*
		Pink colony	Uncultured bacterium, closest named species is *Cytophaga sp.*
TP3.086	Day 0	Cream-coloured colony	Uncultured *Pseudomonas sp.* closest named match is *Pseudomonas syrin*
	Day 14	Cream-coloured colony	*Acidovorax sp.*, closest named match is *Acidovorax defluvii*
	Day 28	Cream-coloured colony	*Pseudomonas agarici* - plant pathogenic species
TP3.124	Day 0	Pale orange colony	*Pseudomonas sp.*, could be *Pseudomonas borealis* or *Pseudomonas frederiksbergensis*
	Day 14	White colony	Best match is an uncultured bacterium from benzene contaminated groundwater, closest named match is *acidovorax defluvii*
		Yellow colony	*Micrococcus sp.*, closest named match is *Micrococcus luteus*
	Day 28	White colony	*Nostocoida limicola* - filamentous bacterium from activated sludge environments
TP3.208	Day 0	White colony	Glacial ice bacterium, closest named match is *Bacillus macroides*
	Day 14	White colony	*Pseudomonas agarici*
	Day 28	Cream-coloured colony	*Pseudomonas fluorescens*
TP3.302	Day 0	Purple colony	*Janthinobacterium lividum* - a denitrifying bacterium
		Orange colony	*Flavobacterium sp.*, closest named match is *Flexibacter columnaris*
	Day 14	Cream-coloured colony	Glacial ice bacterium, most likely *bacillus sp.*, closest named match is *Bac macroides*
		Pale purple colony	Unable to identify - good sequence, but failed to align to any bacterial mat
	Day 28	Cream-coloured colony	*Corynebacterium xerosis*

1.2.2 A phylogenetic tree of the organisms isolated from the Bedwas site soil is shown in Figure 8. This shows the diverse nature of the microbial ecology and indicates no dependence on any specific type or bacteria.

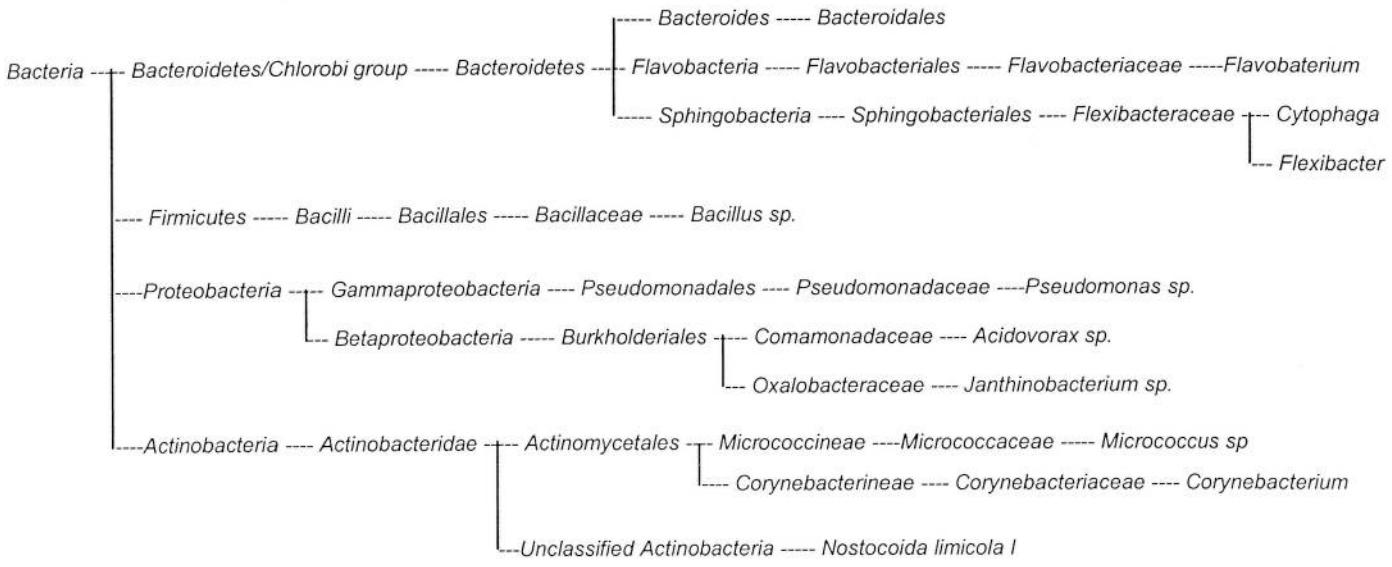


Figure 8. Phylogenetic tree of the microorganisms found at the former Bedwas colliery and coking works site.

1.2.3 The predominant microorganisms, (*Bacillus sp.*, Pseudomonas sp. and *Acidovorax delafluvii*), found within the initial samples are similar to those previously identified as capable of degrading complex organic compounds, including PCB, benzene(1,2) Phenol (2) and petroleum hydrocarbons (3, 4, 5). The identification of *Pseudomonas species* on contaminated sites is very common, they are probably the most regularly identified microorganisms implicated in the biodegradation organic compounds (14-21).

1.2.4 Unlike previous studies on soil from gasworks sites where most of the organisms were from the Phylum proteobacteria (including the *alpha beta* and *gamma* subgroups), the bedwas samples showed no specific group of bacteria dominated the soils

1.2.5 Most of the microorganisms were only identified with a high degree of certainty to the genus level, suggesting that the soils probably contained novel species which had not been previously isolated and identified

2 CONCLUSIONS

2.1 Conclusions from experimental data

2.1.1 Based on experiments carried out to assess the biotreatability of samples from Bedwas, the following conclusions have been obtained.

2.1.2 The hydrocarbon contaminated soil from the Bedwas site can be treated by bioremediation, however performance is limited where:

- Heavy metals concentrations are high.
- PAH concentrations are in excess of approximately 25,000 mg/kg
- The presence of coal or bitumous fragments limits the bioavailability and therefore the biodegradation of PAH.

2.1.3 The microbial composition of the trials was variable, no microorganisms predominated in the trials, though *Pseudomonas sp.* were the most commonly isolated.

2.1.4 The maximum PAH concentration found in the trials was 331940 mg/kg tPAH.

2.1.5 Biodegradation was observed at concentration of PAH which would normally be thought toxic to microorganisms (over 25,000 mg/kg).

2.1.6 The maximum amount of degradation achieved was 89% in terms of percentage degraded (TP 3086) and 33490 mg/kg in terms of amount degraded (TP 3208).

2.1.7 Petroleum hydrocarbon contamination was also degraded, with the maximum biodegradation achieved was 79%.

3 APPENDIX

1, Rooney-Varga, Juliet N., Anderson, Robert T., Fraga Jocelyn L.,Ringelberg David, & Lovley, Derek., Microbial Communities Associated with Anaerobic Benzene Degradation in a Petroleum-Contaminated Aquifer, Appl. Environ. Microbiol, July 1999, p. 3056–3063

2. Fries, Marcos R., Gary D. Hopkins, Perry L. McCarty, Larry J. Forney and James M. Tiedje. 1997. Microbial Succession During a Field Evaluation of Phenol and Toluene as the Primary Substrates for Trichloroethene Cometabolism. Appl. Environ. Microbiol. 63(4):1515-1522.

3, Morse, William R., Brochu, William J., Carter, Sean R., Gibbs, Julie A., Begley, James F., Perriello, Felix A. and George A. DiCesare Treatment of MTBE Impacted Sites Using Biostimulation, NPRA 2002 Environmental Conference, New Orleans, Louisiana September 9-10, 2002

4, Mallard, G.E., Baedecker, M.J., and Cozzarelli, I.M., Anaerobic biodegradation of methyl esters by Acetobacterium woodii and Eubacterium limosum: Journal of Industrial Microbiology, v. 13, p. 321-327. 1994.

5. Rafii, Fatemeh and Cerniglia, Carl E., Reduction of Azo Dyes and Nitroaromatic Compounds by Bacterial Enzymes from the Human Intestinal Tract, Environmental Health Perspectives Volume 103, Supplement 5, June 1995.

3.1 TABULATED RESULTS

3.1.1 Table 1 – Microbiological data and physical properties of soils

Bedwas Soil Code	PH of soil	% Moisture loss (@ 7days)	% Gravel 2-8mm grade	% Gravel 9mm & greater grade	Stage of flask incubation	CFU/ml on ½ str. Nutr. Agar	CFU/ml on Burke's Medium
TP3.071	pH 6.75	23.70	10.9	17.4	Day 0	A. 1.85×10^{7}	A. 1.95×10^{7}
						B. 2.40×10^{7}	B. 2.45×10^{7}
					Day 14	A. 3.15×10^{8}	A. 6.50×10^{7}
						B. 3.70×10^{8}	B. 9.60×10^{7}
					Day 28	A. 1.22×10^{8}	A. 3.35×10^{7}
						B. 1.37×10^{8}	B. 3.75×10^{7}
TP3.086	pH 7.16	18.80	29.0	40.2	Day 0	A. 1.21×10^{8}	A. 1.90×10^{8}
						B. 2.95×10^{8}	B. 2.10×10^{8}
					Day 14	A. 2.00×10^{7}	A. 2.87×10^{8}
						B. 7.00×10^{6}	B. 5.50×10^{7}
					Day 28	A. 2.81×10^{8}	A. 1.13×10^{8}
						B. 5.50×10^{7}	B. 5.30×10^{7}
TP3.124	pH 7.08	22.80	42.8	17.9	Day 0	A. 4.65×10^{7}	A. 8.55×10^{7}
						B. 3.65×10^{7}	B. 5.75×10^{8}
					Day 14	A. 5.25×10^{7}	A. 5.60×10^{7}
						B. 4.00×10^{7}	B. NG
					Day 28	A. 2.50×10^{7}	A.1.50×10^{7}
						B.1.70×10^{7}	B. NG
TP3.208	pH 7.03	32.40	8.7	11.4	Day 0	A. 4.30×10^{7}	A. 4.55×10^{8}
						B. 5.20×10^{7}	B. 2.35×10^{8}
					Day 14	A. 8.50×10^{6}	A. 2.00×10^{7}
						B. 1.77×10^{8}	B. 2.50×10^{6}
					Day 28	A. 6.85×10^{7}	A. 1.00×10^{6}
						B. 8.40×10^{7}	B. 1.00×10^{6}
TP3.302	pH 7.60	15.65	32.5	21.7	Day 0	A. 2.40×10^{7}	A. 1.45×10^{7}
						B. 4.50×10^{8}	B. 1.80×10^{7}
					Day 14	A. 3.63×10^{8}	A. 1.30×10^{7}
						B. 4.00×10^{6}	B. 1.40×10^{7}
					Day 28	A. 5.50×10^{6}	A. 1.00×10^{6}
						B. 7.70×10^{6}	B. 5.00×10^{6}

3.1.2 Table 2 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#071		mg/L		
Group A	As	< LloQ	< LloQ	< LloQ
	Cd	< LloQ	< LloQ	< LloQ
	Cr	0.06	< LloQ	< LloQ
	Pb	< LloQ	< LloQ	< LloQ
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	< LloQ	< LloQ	< LloQ
	Ni	< LloQ	< LloQ	< LloQ
	Zn	< LloQ	< LloQ	< LloQ

3.1.3 Table 3 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#086		mg/L		
Group A	As	< LloQ	< LloQ	< LloQ
	Cd	< LloQ	< LloQ	< LloQ
	Cr	< LloQ	< LloQ	< LloQ
	Pb	< LloQ	< LloQ	< LloQ
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	< LloQ	< LloQ	< LloQ
	Ni	< LloQ	0.06	0.11
	Zn	< LloQ	< LloQ	< LloQ

3.1.4 Table 4 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#124		mg/L		
Group A	As	0.08	< LloQ	< LloQ
	Cd	< LloQ	< LloQ	< LloQ
	Cr	< LloQ	< LloQ	< LloQ
	Pb	< LloQ	< LloQ	< LloQ
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	< LloQ	< LloQ	< LloQ
	Ni	0.04	0.27	0.06
	Zn	< LloQ	< LloQ	< LloQ

3.1.5 Table 5 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#208/211		mg/L		
Group A	As	< LloQ	< LloQ	< LloQ
	Cd	< LloQ	< LloQ	< LloQ
	Cr	< LloQ	< LloQ	< LloQ
	Pb	< LloQ	< LloQ	< LloQ
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	< LloQ	< LloQ	< LloQ
	Ni	< LloQ	0.04	< LloQ
	Zn	< LloQ	< LloQ	< LloQ

3.1.6 Table 6 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#302		mg/L		
Group A	As	< LloQ	0.11	0.10
	Cd	< LloQ	< LloQ	< LloQ
	Cr	< LloQ	< LloQ	< LloQ
	Pb	< LloQ	< LloQ	< LloQ
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	< LloQ	< LloQ	< LloQ
	Ni	< LloQ	0.04	0.04
	Zn	< LloQ	< LloQ	< LloQ

3.1.7 Table 7 – Instrumental Lower limit of Quantification

Lower limit of Quantification (LLoQ)	mg/L	Comment
As	0.06	Measured at HSL using the 10 sigma on a blank sample rule, except for mercury (Hg) which was taken from EPA 200.7 detection limit table
Cd	0.01	
Cr	0.004	
Pb	0.04	
Hg	0.02	
Se	0.07	
Cu	0.004	
Ni	0.04	
Zn	0.01	

3.1.8 Table 8 – Results for Aquacheck 231 waste water sample

	Measured value	Reference value	Comment
	mg/L		
As	0.024	0.040	Below LLoQ
Cd	0.035	0.033	
Cr	0.145	0.137	
Pb	0.169	0.166	
Se	0.049	0.040	Below LLoQ
Cu	0.111	0.109	
Ni	1.04	1.00	
Zn	2.12	1.99	

3.1.9 Table 9 – Sample results

Sample ID		Day 0	Day 14	Day 28
		mg/L		
LloQ	Hexavalent Cr	0.005		
TP 3#071		< LLoQ	< LLoQ	< LLoQ
TP 3#086		< LLoQ	< LLoQ	< LLoQ
TP 3#124		< LLoQ	< LLoQ	< LLoQ
TP 3#208/211		< LLoQ	< LLoQ	< LLoQ
TP 3#302		< LLoQ	0.009	< LLoQ

3.1.10 Table 10 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#071		mg/kg		
Group A	As	< LloQ	< LloQ	< LloQ
	Cd	< LloQ	< LloQ	< LloQ
	Cr	18	18	17
	Pb	83	92	71
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	32	33	28
	Ni	40	42	38
	Zn	138	133	125

3.1.10.1.1 Table 11 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#086		mg/L		
Group A	As	< LloQ	< LloQ	< LloQ
	Cd	< LloQ	< LloQ	< LloQ
	Cr	6	6	8
	Pb	29	27	34
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	39	39	43
	Ni	21	21	24
	Zn	72	71	90

3.1.11 Table 12 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#124		mg/L		
Group A	As	24	19	27
	Cd	< LloQ	< LloQ	< LloQ
	Cr	14	17	17
	Pb	452	348	461
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	431	162	398
	Ni	78	45	70
	Zn	1866	711	1735

3.1.12 Table 13 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#208/211		mg/L		
Group A	As	< LloQ	< LloQ	< LloQ
	Cd	< LloQ	< LloQ	< LloQ
	Cr	14	10	15
	Pb	186	48	97
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	41	29	46
	Ni	25	20	29
	Zn	232	127	249

3.1.13 Table 14 – Sample results

Sample ID		Day 0	Day 14	Day 28
TP 3#302		mg/L		
Group A	As	< LloQ	< LloQ	< LloQ

	Cd	< LloQ	< LloQ	< LloQ
	Cr	15	19	17
	Pb	93	77	101
	Hg	< LloQ	< LloQ	< LloQ
	Se	< LloQ	< LloQ	< LloQ
Group B	Cu	90	68	85
	Ni	39	37	35
	Zn	293	188	280

3.1.14 Table 15 – Instrumental Lower limit of Quantification

Lower limit of Quantification (LLoQ)	mg/L	mg/kg	CRL trigger Value mg/kg	CRL action Value mg/kg	Comment
As	0.06	13	10	69	LloQ based upon a nominal 0.5 g soil sample and a final dilution volume of 100 ml
Cd	0.01	2	3	15	
Cr	0.004	1	600	664	
Pb	0.04	9	500	813	
Hg	0.02	4	1	10	
Se	0.07	13	3	17	
Cu	0.004	1	130	423	
Ni	0.04	7	70	376	
Zn	0.01	2	300	1665	

3.1.15 Table 16 – Results for Reference soil NIST 2710

	Measured value Mean ± sd (n = 5)	Reference median value	Reference range
	mg/kg		
As	643 ± 17	590	490 – 600
Cd	20 ± 1	20	13 – 26
Cr	25 ± 1	19	15 – 23
Pb	5324 ± 175	5100	4300 – 7000
Hg	2 ± 1	32	27 – 37
Cu	2902 ± 70	2400	2400 – 3400
Ni	14 ± 0	10.1	8.8 – 15
Zn	6188 ± 266	5900	5200 - 6900

3.1.16 Table 17– Results for Reference soil NIST 2711

	Measured value Mean ± sd (n = 5)	Reference median value	Reference range
	mg/kg		
As	96 ± 2	90	88 – 110
Cd	40 ± 1	40	32 – 46

Cr	26 ± 1	20	15 – 25
Pb	1145 ± 50	1100	930 – 1500
Cu	110 ± 3	100	91 – 110
Ni	18 ± 0	16	14 – 20
Zn	344 ± 7	310	290 - 340

3.1.17 Table 18 – Sample results

Sample ID		Day 0	Day 14	Day 28
		mg/kg		
LloQ	Hexavalent Cr			
TP 3#071		0.4	0.3	< LLoQ
TP 3#086		0.2	0.2	< LLoQ
TP 3#124		1.0	0.3	0.4
TP 3#208/211		0.7	< LLoQ	< LLoQ
TP 3#302		0.3	0.3	0.8
LloQ	0.5 g sample/20 ml	0.2		
CRL Trigger		25		

Because of the size of the organic chemistry data tables, these data are presented on 2 separate Excel spreadsheets