

MICROBIAL DEGRADATION OF LINEAR
ALKYLBENZENE CABLE OIL IN SOIL AND AQUEOUS
CULTURE UNDER AEROBIC AND ANAEROBIC
CONDITIONS

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2003

Declaration

I hereby declare that:

- (a) this thesis has been composed by me,
- (b) the work is my own,
- (c) the work has not been submitted for any other degree or professional qualification.

(Signed)

Stephen Johnson

Abstract

Linear alkylbenzene (LAB) is a light non-aqueous phase liquid used as insulating oil in buried electricity transmission cables, from where it may enter soil and groundwater in the event of damage to the cable casing or joints. LAB is also found in the aquatic environment due to its use as a precursor in the manufacture of linear alkylbenzene sulphonate (LAS) detergents, in which it remains as a trace contaminant.

LAB is readily biodegraded under aerobic conditions, but because of its use in buried cables, it may be released into anoxic environments where it can persist for some time. Most previous work has focussed on the effects of LAB in freshwater and estuarine sediments, with little published data on terrestrial ecotoxicity and fate. LAB has been found to be acutely toxic to the free-living soil nematode, *Caenorhabditis elegans*.

Aqueous enrichment cultures of organisms from cable oil-contaminated soil show that LAB can be degraded under nitrate-reducing conditions. Isomeric analysis of the remaining LAB is a good indicator of the degree of degradation. However, soil column experiments and anaerobic respirometry of soil slurries using ¹⁴C-labelled LAB do not show appreciable degradation even after 5-6 months. Degradation under iron-reducing, sulphate-reducing and methanogenic conditions has not been demonstrated.

Anaerobic respirometry using a radiolabelled LAB homologue indicates limited primary degradation of LAB in soil slurry. Paradoxically, the inclusion of a commercial oxygen-releasing preparation of magnesium peroxide and magnesium phosphate resulted in greatly reduced degradation, possibly due to toxic effects or enhanced sorption.

An investigation using denaturing gradient gel electrophoresis (DGGE) of eubacterial rDNA polymerase chain reaction (PCR) products has provided an indication that changes in microbial diversity occur in soil following contamination. Furthermore, anaerobic contaminated soils showed rapid LAB degradation upon exposure to aerobic conditions, suggesting that a community of facultative anaerobes capable of degrading the oil had developed.

Acknowledgements

I gratefully acknowledge the guidance and support of my academic supervisors Professor D. Andrew Barry of the Institute for Infrastructure and Environment, the University of Edinburgh and Professor Nick Christofi of the School of Health and Life Sciences, Napier University.

I would like to thank National Grid Transco plc. for providing me with both a stimulating subject for my doctoral studies, and the funding to enable me to carry it out. Special thanks are due to a number of individuals at National Grid, including my first industrial supervisor, Dr Daxaben Patel, her successor Mrs Sally Aikenhead, Professor Steve Swingler and Dr Ian Glover.

Thanks to the technical staff at the School of Health and Life Sciences, Napier University, especially the inestimable Dr. John Kinross.

I would also like to express my thanks to the following:

Dr Sophie Foley, Dr Martin Tangney and Dr Lorna Proudfoot, academic staff at Napier University in whose courses I demonstrated. As well as a welcome additional income, the experiences led down avenues of investigation that I would not otherwise have considered.

Dr Lisa McIver of the Department of Chemistry, University of Edinburgh, for the use the radionuclide facilities and Dr Bill Gregory at the Institute of Cell, Animal and Population Biology (ICAPB), University of Edinburgh, for *Caenorhabditis elegans* and *Escherichia coli* OP50.

Dr Bob Rees and Mr John Parker at the Scottish Agricultural College for soil and analytical services

Dr Kirk Semple and Mr Philip Lee at the Environmental Science Department, Lancaster University for providing radiolabelled material, use of a sample oxidiser and help and advice on experimental design and interpretation.

Dr Fred Davies and Ms Rosie Hyuhn at Reading University for training and advice on GC-MS methods and interpretation.

Undergraduate and visiting project students who contributed so much time and effort as poorly- or un-paid technicians: Señorita Beatriz Martin, Ms Alison Docherty, Mlle Magali Castan, Mlle Dominique Bastiani and Herr Kai Temming.

Dr Hideshige Takada of Tokyo University of Agriculture & Technology for providing raw data used in the statistical analysis in Chapter 6.

Dr Anita Lewis and Dr Paul Bardos of r³ Environmental Technology Ltd for providing samples of ORC[®]

Finally, thanks to Ms Teresa MacDonald for encouragement, proof reading and generally putting up with me.

Table of Contents

Declaration	1
Abstract	2
Acknowledgements	3
Table of Contents	4
List of Tables	8
List of Figures	9
List of Plates	13
Nomenclature and Abbreviations	14
1 Introduction	17
1.1 Thesis Structure	18
1.2 Background	20
1.3 Aims	22
1.4 Properties of cable oil	22
1.4.1 Chemical structure	22
1.5 Naming conventions	26
1.6 Manufacture and use of LAB	27
1.7 Legislation in the UK	29
1.8 Guidelines for acceptable levels of cable oil in soil	32
1.9 Fate of cable oil in contaminated soil	34
2 Literature review	36
2.1 Aerobic biodegradation	36
2.2 Anaerobic degradation of hydrocarbons	42
3 Extraction and analytical techniques	63
3.1 Extraction	64
3.2 Gas chromatography (GC)	65
3.2.1 Choice of GC column	66
3.2.2 Quantification	68
3.3 Detectors	68
3.3.1 Mass spectrometry (MS)	68
3.3.2 Flame ionisation detection (FID)	69
3.3.3 Thermal conductivity detection (TCD)	70
3.3.4 Electron capture detection (ECD)	70
3.3.5 Compound-specific isotope analysis	70
4 GC-MS analysis of cable oil	72
4.1 Analytical instruments	72
4.2 Gas chromatography of unused cable oil	73
4.3 Identification of GC peaks	74
5 Toxicology	77
5.1 Bioavailability of linear alkylbenzenes	77
5.2 Toxicity of linear alkylbenzenes	78
5.3 Environmental toxicology using <i>Caenorhabditis elegans</i>	82
5.4 Materials and methods	85
5.4.1 Summary of experimental method	85
5.4.2 Test organisms	86

5.4.3	Preparation of media and solutions	86
5.4.4	Manipulation of <i>C. elegans</i>	88
5.4.5	Test soils	89
5.4.6	Test chambers	92
5.4.7	Retrieval of test organisms	94
5.4.8	Scoring	95
5.4.9	Calculations	95
5.4.10	Verification of the LAB content of the soils	96
5.5	Results	96
5.5.1	Quality control	96
5.5.2	Recovery and mortality	97
5.5.3	LC50 by trimmed Spearman-Kärber method	98
5.5.4	LC50 by Probit method	99
5.5.5	LC50 by linear interpolation	100
5.5.6	Comparison of LC50 by different methods	100
5.6	Discussion	101
5.7	Conclusions	103
6	Aqueous culture	104
6.1	Introduction	104
6.2	Materials and methods	108
6.2.1	Batch cultures	108
6.2.2	Inoculum	109
6.2.3	Experimental systems	110
6.2.4	LAB extraction	112
6.2.5	GC-MS Analysis	113
6.2.6	Nitrate/nitrite analysis	115
6.3	Results and discussion	116
6.3.1	Extraction efficiency	116
6.3.2	Enrichment cultures	116
6.3.3	Isolates from enrichment cultures	124
6.3.4	Pure <i>G. metallireducens</i> cultures	127
6.4	Conclusions	129
7	Anaerobic ¹⁴ C LAB Respirometry	131
7.1	Introduction	131
7.2	Materials and methods	132
7.2.1	Respirometer design	132
7.2.2	Preparation of test soils	132
7.2.3	Inoculum	133
7.2.4	Assembly	133
7.2.5	Addition of radiolabelled LAB	136
7.2.6	Incubation	137
7.2.7	Analysis	137
7.2.8	Calculations	139
7.3	Results and discussion	140
7.3.1	Activity added to respirometers	140
7.3.2	First respirometry experiment	140
7.3.3	Second respirometry experiment	143
7.4	Conclusions	155

8	Soil column experiments	156
8.1	Materials and methods	157
8.1.1	Column design.....	157
8.1.2	Soils.....	158
8.1.3	Sampling.....	159
8.1.4	Extraction	162
8.1.5	Analysis	162
8.2	Results and discussion	162
8.2.1	Appearance.....	162
8.2.2	LAB content	165
8.3	Conclusions.....	170
9	Analysis of GC data from a cable-oil contaminated site	172
9.1	Introduction.....	173
9.2	Methods.....	173
9.3	Results.....	174
9.4	Discussion and conclusions	180
10	Molecular Techniques	183
10.1	Introduction.....	183
10.1.1	Isolation of DNA from soil	183
10.1.2	Polymerase chain reaction.....	184
10.1.3	Electrophoresis.....	186
10.2	Materials and methods.....	187
10.2.1	Extraction of DNA from soil.....	187
10.2.2	Extraction of DNA from bacterial cultures.....	188
10.2.3	16S rDNA Polymerase Chain Reaction	188
10.2.4	Electrophoresis.....	190
10.3	Results and discussion	198
10.3.1	Method optimisation	198
10.4	PCR and confirmation of products	200
10.4.1	DGGE of PCR products.....	202
10.5	Conclusions.....	211
10.5.1	Soil columns.....	212
10.5.2	Enrichments and isolates.....	212
11	Discussion	214
11.1	Extraction and analysis of cable oil	214
11.2	Toxicology.....	216
11.3	Culture methods.....	216
11.4	Anaerobic respirometry	217
11.5	Soil columns	218
11.6	Analysis of data from a cable oil-contaminated site.....	218
11.7	Molecular techniques.....	219
11.7.1	Soil columns.....	220
11.7.2	Enrichments and isolates.....	220
12	Conclusions	222
13	Suggestions for further work.....	224
13.1	Field monitoring	224
13.2	Other cable oils.....	224
13.3	DGGE	225

13.4	Oxygen Release Compound (ORC [®]).....	225
14	References	226
15	Appendices	246
15.1	Names, synonyms and molecular structures of C ₁₀ – C ₁₃ linear alkylbenzenes	247
15.2	GC-MS report	259
15.3	Analysis of <i>C. elegans</i> toxicity data	280
15.3.1	Trimmed Spearman-Kärber analysis.....	280
15.3.2	Probit analysis	284
15.3.3	Linear interpolation.....	290
15.4	Publications.....	292
15.4.1	Land Contamination and Reclamation, 2001	292
15.4.2	European Geophysical Society XXVII General Assembly, 2002	306
15.4.3	2nd European Biodegradation Conference, 2003	308
15.4.4	Engineering Geology, 2003	314

List of Tables

Table 1.1 Physical properties of linear alkylbenzene (LAB) cable oil.....	25
Table 1.2 Capacity of major manufacturing facilities of LAB in 1999 (adapted from Burrige (2002))	28
Table 2.1 Factors limiting biodegradation (after Providenti <i>et al.</i> (1993))	43
Table 2.2 Summary of available research on anaerobic degradation of hydrocarbons (with selected aerobic references).....	46
Table 5.1 Summary of available toxicity data for C ₁₀₋₁₃ LAB. Sources cited in Heinze (2001b).....	79
Table 5.2 Environmental Risk phrases (European Commission 1993).....	81
Table 5.3 Timetable for soil toxicology test with <i>C. elegans</i>	85
Table 5.4 M9 buffer (Brenner 1974)	86
Table 5.5 Alkaline hypochlorite solution (to axenise 1 Petri dish)	87
Table 5.6 Ficoll solution.....	87
Table 5.7 Properties of soil used in toxicology, anaerobic respirometry and soil column experiments	90
Table 6.1 <i>Geobacter</i> medium 579 (German Collection of Microorganisms and Cell Cultures)	111
Table 6.2 Linear regression of % biodegradation of LAB vs. log ₁₀ internal:external (I:E) nC ₁₂ LAB isomer ratio with different terminal electron acceptors	123
Table 7.1 Summary of respirometer contents for first experiment	135
Table 7.2 Summary of respirometer contents for second experiment.....	135
Table 9.1 Isomeric composition of LAB by % of recovered oil	175
Table 10.1 Requirements for PCR of bacterial 16S rDNA	189
Table 10.2 Thermal cycler program used for PCR amplification of bacterial 16S rDNA	190
Table 10.3 Solutions for 6% polyacrylamide denaturing gradient gel.....	193
Table 10.4 Optimisation of extraction and PCR parameters (see Plate 10.8)	199
Table 10.5 PCR amplification of 16S rDNA from a variety of sources (see Plate 10.9)	200
Table 10.6 PCR amplification of 16S rDNA from different sources (see Plate 10.10)	201
Table 10.7 Samples applied to first DGGE gel (see Plate 10.11)	203
Table 10.8 DGGE samples (see Plate 10.12)	204
Table 10.9 DGGE samples (see Plate 10.13)	205

List of Figures

Figure 1.1 Transverse section of an oil-filled paper-wrapped cable (photograph courtesy National Grid Transco plc.).....	21
Figure 1.2 General structure of linear alkylbenzenes found in cable oil (N.B. terminal isomers not found).....	23
Figure 1.3 Partitioning of cable oil in soil (not to scale). Arrows indicate preferred partition.....	24
Figure 1.4 Structure of a representative homologue of LAB.....	26
Figure 1.5 Global consumption and manufacturing capacity of LAB by region in 1999 and projected consumption for 2010 (data from Burrige (2002)).....	27
Figure 1.6 Pollution linkage.....	29
Figure 2.1 Generalised pathway for the aerobic catabolism of alkylbenzenes (Smith & Ratledge 1989).....	38
Figure 2.2 Beta oxidation pathway (Stryer 1995).....	39
Figure 2.3 Possible end points for β -oxidation of LAB.....	40
Figure 2.4 Routes to bioremediation, adapted from Spain (2001).....	42
Figure 2.5 Comparison of aerobic and anaerobic hydrocarbon biodegradation.....	56
Figure 2.6 Initial microbially catalysed transformations of toluene under anaerobic conditions (Grbic-Galic 1991) – bold arrows indicate reactions known to occur.....	58
Figure 2.7 Proposed pathway for anaerobic toluene degradation (Heider <i>et al.</i> 1999).....	58
Figure 2.8 Proposed pathway for anaerobic toluene mineralization (Chee-Sanford <i>et al.</i> 1996).....	59
Figure 2.9 Proposed pathway for anaerobic ethylbenzene degradation (Heider <i>et al.</i> 1999).....	59
Figure 2.10 Proposed pathways for anaerobic degradation of alkylbenzenes (Elshahed <i>et al.</i> 2001).....	60
Figure 2.11 Proposed pathways for anaerobic benzoate/benzoyl CoA degradation (Koch <i>et al.</i> 1993; Harwood & Gibson 1997).....	61
Figure 4.1 Typical gas chromatogram of cable oil showing resolution of individual homologues of form nC_m where n = length of alkyl chain and m = position of phenyl group. The $1C_{12}$ LAB internal standard (not shown) elutes between $3C_{13}$ LAB and $2C_{13}$ LAB.....	73
Figure 4.2 Summary of mass spectral fragments from LAB, indicating probable sources. Molecular weights in BOLD are diagnostic. Structures for MW = 105, 119, 133, 147, 161 and 175 are conjectural.....	76
Figure 5.1 Life cycle of <i>Caenorhabditis elegans</i> . (L1 = First larval stage etc. Stages are marked by a moult of the epidermis and each stage differs from the previous one by size. Only L5 individuals are sexually mature).....	83
Figure 5.2 Verification of oil content and isomeric composition. Error bars = 1 SD, $n = 3$	97
Figure 5.3 Experiment 3 - Plot of recovery and mortality vs. LAB % dry weight soil. Error bars = 1 SD, $n=30$	97
Figure 5.4 Experiment 6 - Plot of recovery and mortality against LAB % dry weight soil. Error bars = 1 SD, $n = 30$	98

Figure 5.5 Concentration of LAB required to kill X % of a population of <i>C. elegans</i> . Calculated by Probit analysis. Error bars = 95 % confidence intervals, n = 180 (individual experiments) or 360 (pooled data). Upper 95 % confidence limits for mortalities above 80 % for experiment 3 are effectively infinite.	99
Figure 5.6 Comparison of LC50 (24h, <i>C. elegans</i> in soil) calculated by different methods. Data from experiments 3, 6 and pooled data. Error bars = 95% confidence intervals, n = 180 (for individual experiments) or 360 (pooled data).	101
Figure 6.1 Relative abundance of individual homologues of LAB found by GC-MS of typical undegraded cable oil (author’s own data, error bars = 1 standard deviation, n = 33).	105
Figure 6.2 Amount of LAB recovered from individual CON cultures (filled markers) and I:E ratios (open markers) plotted against time.	117
Figure 6.3 Amount of LAB recovered from individual ANA cultures (filled markers) and I:E ratios (open markers) plotted against time.	117
Figure 6.4 Amount of LAB recovered from individual AER cultures (filled markers) and I:E ratios (open markers) plotted against time.	118
Figure 6.5 Amount of LAB recovered from individual NIT cultures (filled markers) and I:E ratios (open markers) plotted against time.	118
Figure 6.6 Amount of LAB recovered from individual SUL cultures (filled markers) and I:E ratios (open markers) plotted against time.	119
Figure 6.7 Amount of LAB recovered from individual MET cultures (filled markers) and I:E ratios (open markers) plotted against time.	119
Figure 6.8 Total oxidised inorganic nitrogen relative to CON (error bars = 1 standard deviation, n = 3).	120
Figure 6.9 Total oxidised inorganic nitrogen relative to MET (error bars = 1 standard deviation, n = 3).	121
Figure 6.10 Nitrite evolution (error bars = 1 standard deviation, n = 3) N.B. Apparent elevation of nitrite in the NIT treatments is an artefact due to interference from molybdate	121
Figure 6.11 Plot of % biodegradation of LAB vs. log ₁₀ internal:external (I:E) nC12 LAB isomer ratio with linear regression (heavy) line, 95% confidence (light) and 95 % prediction intervals (broken lines). Data points are from individual bottles: aerobic (◇), anaerobic containing multiple TEAs (□) or nitrate alone (▲) and aerobic data from the literature (Takada & Ishiwatari 1990) (×)	124
Figure 6.12 LAB recovered from enrichment cultures (NIT, ANA, MET) after 589 d and isolates (CON, ANA W1, ANA C1, MET W1, MET C1) after 386 d. Error bars = 1 SD, n= 3	125
Figure 6.13 nC12 LAB I:E ratio of cable oil recovered from enrichment cultures (NIT, ANA, MET) after 589 d and isolates (CON, ANA W1, ANA C1, MET W1, MET C1) after 386 d. Error bars = 1 SD, n= 3	126
Figure 6.14 Variation of LAB recovered from uninoculated cultures. Treatments differed in terminal electron acceptors: Fe citrate (open triangles), FeCl (filled diamonds) or KNO ₃ (×). Error bars = 1 SD, n = 3.	127
Figure 6.15 Variation of LAB recovered from cultures inoculated with <i>Geobacter metallireducens</i> . Treatments differed in terminal electron acceptors: Fe citrate (open triangles), FeCl (filled diamonds) or KNO ₃ (×). Error bars = 1 SD, n = 3	128

Figure 6.16 Variation of I:E ratio in uninoculated cultures. Treatments differed in terminal electron acceptors: Fe citrate (open triangles), FeCl (filled diamonds) or KNO ₃ (×). Error bars = 1 SD, n = 3.....	128
Figure 6.17 Variation of I:E ratio in cultures inoculated with <i>Geobacter metallireducens</i> . Treatments differed in terminal electron acceptors: Fe citrate (open triangles), FeCl (filled diamonds) or KNO ₃ (×). Error bars = 1 SD, n = 3.....	129
Figure 7.1 Radiolabelled 3 ¹⁴ C ₁₂ LAB to show location of labelled atom.....	131
Figure 7.2 Fate of radiolabelled 3C ₁₂ LAB in first respirometry experiment. Treatments are described in Table 7.1.....	140
Figure 7.3 Volume of total 3C ₁₂ LAB represented by activity in NaOH in first respirometry experiment. Data presented with and without two anomalous points.....	141
Figure 7.4 Activity detected in soil slurry under different conditions as a percentage of the total activity added. This represents undegraded or only partially degraded LAB remaining in the soil slurry.....	144
Figure 7.5 Activity detected in NaOH under different conditions as a percentage of the total activity added. This represents CO ₂ from mineralised LAB and the soluble fraction of volatilised LAB and breakdown products.....	144
Figure 7.6 Activity detected in a polyurethane bung under different conditions as a percentage of the total activity added. This was included to detect volatile LAB and breakdown products.....	146
Figure 7.7 Activity not detected under different conditions as a percentage of the total activity added. Calculated by summing the activity detected from soil, NaOH and bungs, and subtracting from the total activity added to each respirometer.....	146
Figure 7.8 Summary of ¹⁴ C activity found in different compartments of the second respirometry experiment. Data corrected for systematic losses seen in control respirometers due to sorption to glassware and loss during degassing of respirometers. Error bars = 1 SD, n = 8.....	147
Figure 7.9 Changes in volume of LAB recovered from sealed and unsealed flasks over time.....	148
Figure 7.10 nC ₁₂ I:E ratio of LAB extracted from soil slurry under different conditions.....	151
Figure 7.11 Mean nC ₁₂ LAB I:E ratios seen in soil slurry under different conditions. Error bars = 1 SD, n = 6 for Control or 7 for other treatments).....	152
Figure 7.12 Relationship between degree of degradation of 3C ₁₂ LAB and total LAB degradation seen in aqueous cultures under a range of conditions.....	153
Figure 8.1 LAB vs. depth after 253d (error bars = 1 SD, n = 3).....	165
Figure 8.2 LAB recovery vs. after 253 d anaerobic + 7 d aerobic (error bars = 1 SD, n = 3).....	166
Figure 8.3 Mean LAB recovery (error bars = 1 SD, n = 3).....	166
Figure 8.4 nC ₁₂ I:E ratio vs. depth after 253 d anaerobic (error bars = 1 SD, n = 3).....	168
Figure 8.5 nC ₁₂ I:E ratio vs. depth after 253 d anaerobic + 7 d aerobic (error bars = 1 SD, n = 3).....	168
Figure 8.6 Mean nC ₁₂ I:E ratio (error bars = 1 SD, n = 3).....	169
Figure 8.7 I:E vs. LAB recovery after 253 d anaerobic (error bars = 1 SD, n = 3)..	169

Figure 8.8 I:E vs. LAB recovery after 253 d anaerobic + 7 d aerobic (error bars = 1 SD, n = 3).....	170
Figure 9.1 Raw data GC data from “new” cable oil and hydrocarbons extracted from a contaminated site, from data provided by National Grid.....	174
Figure 9.2 Total ion current chromatogram of a sample of unused “old” cable oil .	176
Figure 9.3 Chromatogram of "old" cable oil (courtesy National Grid Transco plc)	176
Figure 9.4 Isomeric composition of “old” oil, calculated from data provided by National Grid	177
Figure 9.5 Total ion current chromatogram of unused "new" LAB cable oil	177
Figure 9.6 Isomeric composition of “new” unused cable oil, calculated from data provided by National Grid	178
Figure 9.7 Isomeric composition of cable oil recovered from excavation water, from data provided by National Grid	178
Figure 9.8 Isomeric composition of cable oil recovered from stream water, from data provided by National Grid	179
Figure 9.9 Isomeric composition of cable oil recovered from stream sediment, from data provided by National Grid	179
Figure 10.1 Bioline Hyperladder I.....	191
Figure 10.2 Bioline Hyperladder IV.....	191
Figure 10.3 Bands seen in DGGE of 16S rDNA PCR products from autoclaved soil columns.....	206
Figure 10.4 Bands seen in DGGE of 16S rDNA PCR products from unsterile soil columns.....	207
Figure 10.5 Bands seen in DGGE of 16S rDNA PCR products from columns containing unsterile soil plus Bushnell-Haas medium.....	207
Figure 10.6 Bands seen in DGGE of 16S rDNA PCR products from soil columns containing unsterile soil plus B-H plus additional nitrate	208
Figure 10.7 Bands seen following DGGE of 16S rDNA PCR products from ANA enrichment culture LAB-contaminated soil.....	208
Figure 10.8 Bands seen following DGGE of 16S rDNA PCR products from MET enrichment culture from LAB-contaminated soil.....	209
Figure 10.9 Bands seen following DGGE of 16S rDNA PCR products from isolate BH/NIT/ANA C1 from LAB-contaminated soil	209
Figure 10.10 Bands seen following DGGE of 16S rDNA PCR products from isolate BH/NIT/MET C1 from LAB-contaminated soil	210
Figure 10.11 Bands seen following DGGE of 16S rDNA PCR products from isolate BH/NIT/MET W1 from LAB-contaminated soil	210
Figure 10.12 Bands seen following DGGE of 16S rDNA PCR products from isolate BH/NIT/MET W1 from LAB-contaminated soil	211

List of Plates

Plate 5.1 Apparatus used to mix soil and LAB	91
Plate 5.2 Handling of individual <i>Caenorhabditis elegans</i> (arrowed) using a platinum pick. Image shows an adult worm as well as larvae and eggs	93
Plate 5.3 Adult <i>Caenorhabditis elegans</i> (arrowed) on soil	94
Plate 6.1 CPSN enrichments from ANA (left) and MET (right) cultures. Blue colour and gas in Durham tube and at liquid surface indicates nitrate reduction	109
Plate 6.2 Nitrite test on various dilutions of NaNO ₂ and NaMO ₄ to show interference from molybdate.....	122
Plate 7.1 Soil respirometer under preparation. The bubbles on the surface of the soil slurry are a result of the vacuum being applied via the needle piercing the Teflon liner through a hole in the cap. Also visible are the stainless steel rod and crocodile clip	134
Plate 7.2 Stages in sample oxidation (a) paper thimble in platinum basket prior to oxidation, (b) during oxidation, (c) non-combustible material remaining after combustion.....	139
Plate 7.3 Appearance of PU bungs from respirometers (a) killed control, (b) anaerobic and (c) ORC. Numerals indicate time in d since start of experiment	145
Plate 8.1 Soil columns prior to incubation	157
Plate 8.2 Freezing soil columns.....	160
Plate 8.3 Sectioning frozen columns	161
Plate 8.4 Appearance of soil columns before and after 253 d incubation: autoclaved soil before (a) and after (b), saturated with distilled water (c & d), Bushnell-Haas (e & f) and B-H plus nitrate (g & h)	164
Plate 10.1 Assembling DGGE gel sandwich.....	192
Plate 10.2 Clamping glass plates.....	192
Plate 10.3 Casting denaturing gradient gel. Note gradient former in foreground ...	194
Plate 10.4 Gradient gel incorporating bromophenol blue to visualise gradient	194
Plate 10.5 Electrophoresis tank and power supply.....	195
Plate 10.6 Staining with ethidium bromide	197
Plate 10.7 Gels are fragile but may be handled on a filter paper support.....	197
Plate 10.8 Extraction efficiency of MoBio soil DNA extraction kit, with and without IRS (see Table 10.4 for legend).....	199
Plate 10.9 Agarose gel electrophoresis of 16S rDNA from a variety of sources (see Table 10.5 for legend).....	200
Plate 10.10 Agarose gel electrophoresis of 16S rDNA from a variety of sources (see Table 10.6 for legend).....	201
Plate 10.11 DGGE of selected PCR products (see Table 10.7 for legend). Gradient is 0% denaturant at top to 100% at bottom of gel.	203
Plate 10.12 DGGE gel (see Table 10.8 for legend). Gradient is 30% denaturant at top of gel to 100% at bottom.	204
Plate 10.13 DGGE gel (see Table 10.9 for legend). Gradient is 30% denaturant at top of gel to 100% at bottom.	205

Nomenclature and Abbreviations

16S rDNA	DNA coding for the small RNA subunit of the ribosome
°C	Degrees Celsius
$\delta^{13}\text{C}$	Carbon isotopic composition
A	Adenine
AP	Ammonium persulphate
ASTM	American Society For Testing And Materials
BAB	Branched alkylbenzene
BAT	Best available technology
BATNEEC	Best available technology not entailing excessive cost
BCF	Bioconcentration factor
B-H	Bushnell-Haas
BLAST	Basis local alignment search tool
bp	Base pair(s)
BTEX	Benzene, toluene, ethylbenzene, xylenes
C	Cytosine
CDM	Cable oil-degrading microorganism
CFU	Colony forming unit
CHO	Chinese hamster ovary
CLARRC	Contaminated land assessment and remediation research centre
CLEA	Contaminated land exposure assessment
CoA	Coenzyme A
CPSN	Casein, peptone, starch and nitrate medium
CSTEE	Scientific Committee on Toxicity, Ecotoxicity and the Environment
DDB	Dodecylbenzene
DEFRA	Department for environment, food and rural affairs (previously DETR)
DETR	Department of transport and the regions
DGGE	Denaturing gradient gel electrophoresis
dH ₂ O	Deionised water
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
dpm	Disintegrations per minute
<i>e</i>	Euler's number, $e \approx 2.718281828459045$
EA	Environment agency, Environment act 1995
EC	European council, European commission, European community
ECD	Electron capture detection
ECETOC	European Centre For Ecotoxicology And Toxicology Of Chemicals
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
EPA	Environment protection act 1990
FID	Flame ionisation detector/detection
FW	Fresh water
G	Guanine
GC	Gas chromatography
GC-FID	Gas chromatography – flame ionisation detection

GC-MS	Gas chromatography – mass spectroscopy
HC	Hydrocarbon
HMSO	Her Majesty's stationery office
ICAPB	Institute of cell, animal and population biology
ICRCL	Inter-departmental committee on the redevelopment of contaminated land
i.d.	Internal diameter
I:E	Internal:external isomeric ratio
IPC	Integrated pollution control
IPPC	Integrated pollution prevention and control
IRS	Inhibitor removal solution
IUPAC	International union of pure and applied Chemistry
K _{oc}	Organic carbon matter partition coefficient
K _{ow}	Octanol-water partition coefficient
LAAPC	Local authority air pollution control
LAB	Linear alkylbenzene
LAS	Linear alkylbenzene sulphonate
LC50	Concentration of a substance required to kill 50% of a population
log	Logarithm to base 10
ln	Natural logarithm, logarithm to base <i>e</i>
LNAPL	Light non-aqueous phase liquid
M	Adenine/thymine degeneracy in a nucleotide sequence
MENA	Monitored enhanced natural attenuation
MNA	Monitored natural attenuation
Mol	Mole, molar
MS	Mass spectrometry
MSD	Mass selective detector
MS-DOS	Microsoft disk operating system
m/z	Mass/charge ratio
n	Number of samples
NA	Nutrient agar
NAPL	Non-aqueous phase liquid
NCBI	National center for biotechnology information
nC _m LAB	LAB homologue, composed of an alkyl chain m carbons in length with a phenyl substitution at position n
NGC	National grid company plc (now national grid transco plc)
NEDD	<i>N</i> -(1-naphthyl)-ethylenediamine dihydrochloride
NIST	National institute of standards and technology
NMR	Nuclear magnetic resonance
NOAEL	No observed adverse effect concentration
NOEL	No observed effect concentration
OECD	Organisation for economic co-operation and development
ORC [®]	Oxygen release compound. ORC [®] is a registered trademark of Regenesi Bioremediation Products
ORP	Oxidation-reduction potential
PAH	Polyaromatic hydrocarbons
PC	IBM compatible personal computer
PCR	Polymerase chain reaction
PEC	Probable effect concentration, predicted environmental concentration

PHREEQC	A computer program for speciation, batch-reaction, one-dimensional transport, and inverse geochemical calculations
plc	Public limited company
PNEC	Predicted no-effect concentration
PPCA	Pollution prevention and control act 1990
ppm	Parts per million
PTFE	Polytetrafluoroethylene, “Teflon”
PU	Polyurethane
PVC	Polyvinylchloride
RNA	Ribonucleic acid
rpm	Revolutions per minute
SCCWRP	Southern California coastal water research project
SD	Standard deviation
SURRC	Scottish universities research and reactor centre
T	Thymine
T _½	Half life
TAE	Tris(hydroxymethyl)aminomethane /acetic acid/EDTA
TEA	Terminal electron acceptor
TCD	Thermal conductivity detection
TOF	Time-of-flight
TEMED	Tetramethylethylenediamine
TEX	Toluene, ethylbenzene and xylenes
TIC	Total ion current
TOC	Total organic carbon
TON	Total oxidised inorganic nitrogen
UK	United Kingdom of Great Britain and Northern Ireland
USEPA	United States Environmental Protection Agency
w/v	Weight/volume
w/w	Weight/weight
XLPE	Cross-linked polyethylene

1 Introduction

Underground electricity transmission cables in England and Wales, operated by National Grid Transco plc, are insulated using, predominantly, linear alkylbenzene (LAB) cable oils. These oils are occasionally released into the soil following mechanical damage to the cable or failure of a cable joint due to thermal movement. Once released the oil migrates to the local water table, and becomes partitioned between sorbed and bulk liquid phases, with only a very small proportion in the aqueous phase. Microcosm investigations (Johnson 2000; Herbath 2003) indicate that conditions in the upper saturated zone rapidly become anaerobic following such contamination though it is likely that in the field, conditions may alternate between aerobic and anaerobic as the water table fluctuates.

While repairing the cable, the immediate backfill is excavated and removed to controlled landfill, though investigations are underway into a soil-washing process that would allow this backfill to be reused (S. Aikenhead, pers. comm.). It is not usually practicable to excavate and replace soil from the wider area that may be contaminated, because of the existence of surface features and buried services (most buried cables are in urban areas). An effective solution to the remediation of this soil is sought.

National Grid Transco plc are moving to the use of highly cross-linked polyethylene (XLPE) as a solid insulator in new cables, which will pose a minimal risk of pollution. The new system is in use at 132kV and 275kV, and is due to be trialled at 400kV shortly. However, there does not yet exist a technology for interfacing the two

types of cable so damaged sections of oil-filled cable will continue to be replaced by the same type.

1.1 Thesis Structure

The project has involved a variety of approaches to assessing the toxicity, biodegradability and fate of cable oil. Accordingly, this thesis is split into chapters, each addressing a different facet of the project:

- Introduction
 - The present chapter describes the structure of the thesis, the background to the project and places it in the context of current EC and UK legislation.
- Literature review
 - A comprehensive review of the literature pertaining to the occurrence and fate of linear alkylbenzenes and related contaminants in the environment.
- Extraction and analysis of cable oil
 - This chapter describes the major analytical tool used in the project for quantifying LAB in a wide range of samples. Mass fragments detected following electron-impact ionisation of LAB homologues in a quadropole mass spectrometer are described.
- Toxicology
 - A review of the available toxicological data followed by the calculation of LC50 of cable oil to *Caenorhabditis elegans*, a common soil organism.

- Culture methods
 - Enrichment cultures and isolates are used to investigate the degradation of cable oil in both aqueous and soil cultures with a variety of electron acceptors. A relationship between isomeric composition and degree of biodegradation is described and shown to hold under both aerobic and anaerobic conditions.
- Anaerobic respirometry
 - Anaerobic soil slurries were contaminated with a radiolabelled LAB homologue. The radiolabel was recovered from soil, volatile traps and CO₂ traps to investigate the fate of the LAB.
- Soil columns
 - Soil columns are constructed to mimic conditions found in a contaminated site to investigate whether the degradation seen in conditions that are more artificial is likely to occur in the field.
- Analysis of data from a cable oil-contaminated site
 - The isomeric composition of linear alkylbenzenes extracted from a leaking cable joint bay is compared to that found in a nearby stream to assess the usefulness of isomeric composition as a measure of biodegradation in the field.
- Molecular techniques
 - A preliminary investigation of the biodiversity of enrichment cultures, isolates and soil columns using denaturing gradient gel electrophoresis (DGGE) of eubacterial 16S rDNA polymerase chain reaction (PCR) products.

- Discussion and conclusions
 - The findings from each of the chapters are summarised and synthesised to yield a picture of the effects and fate of LAB cable oil following a release, along with recommendations on the most appropriate remedial approach.

1.2 Background

Linear alkylbenzenes (LAB) are found naturally in crude oil (Wilhelms *et al.* 2001; Dutta & Harayama 2001) and are produced synthetically for use in the manufacture of linear alkylbenzene sulphonate (LAS) detergents, from where they enter aquatic environments (Ishiwatari *et al.* 1983). They are used in varnishes and, significantly for this study, as electrical transmission cable oil. They are also used as lubricants in refrigeration systems (Takigawa *et al.* 2002) and were major components of synthetic drilling oils in the first half of the 1990s (M-I. *LLC* 1998). They are thus potentially a significant contaminant both in aquatic sediments and in soils.

Alkylbenzenes were reported in sewage extracts as early as 1974 (Manka *et al.* 1974) and LAB was identified as an environmental contaminant by Crisp *et al.* (1979), who suggested that they may be derived from LAS detergents, either as unreacted feedstock or by microbial desulphonation. Eganhouse *et al.* (1982) identified LAB in marine sediments and discussed their likely source, concluding that unreacted LAB residue in LAS detergent was not sufficient to account for the amount seen and suggesting that since the sulphonation process is reversible, desulphonation of the LAS might occur during use of the product (Eganhouse *et al.* 1983). It was

considered that microbial desulphonation was unlikely since, while it had been observed during biodegradation of LAS, this was always accompanied by ω -oxidation and cleavage of the side chain. This was supported by Ishiwatari *et al.* (1983), who found them associated with LAS detergent contamination in sediments in Tokyo Bay.

A number of studies have been carried out into the potential for bioremediation of cable oil (Cheston 1997; Tebbutt 1998; Koussia 1999). They indicate good potential for aerobic attenuation/bioremediation of linear alkylbenzenes but less is known about possible anaerobic mechanisms. This is significant since cable oil releases tend to be into soil where anaerobic conditions may prevail (Johnson 2000).

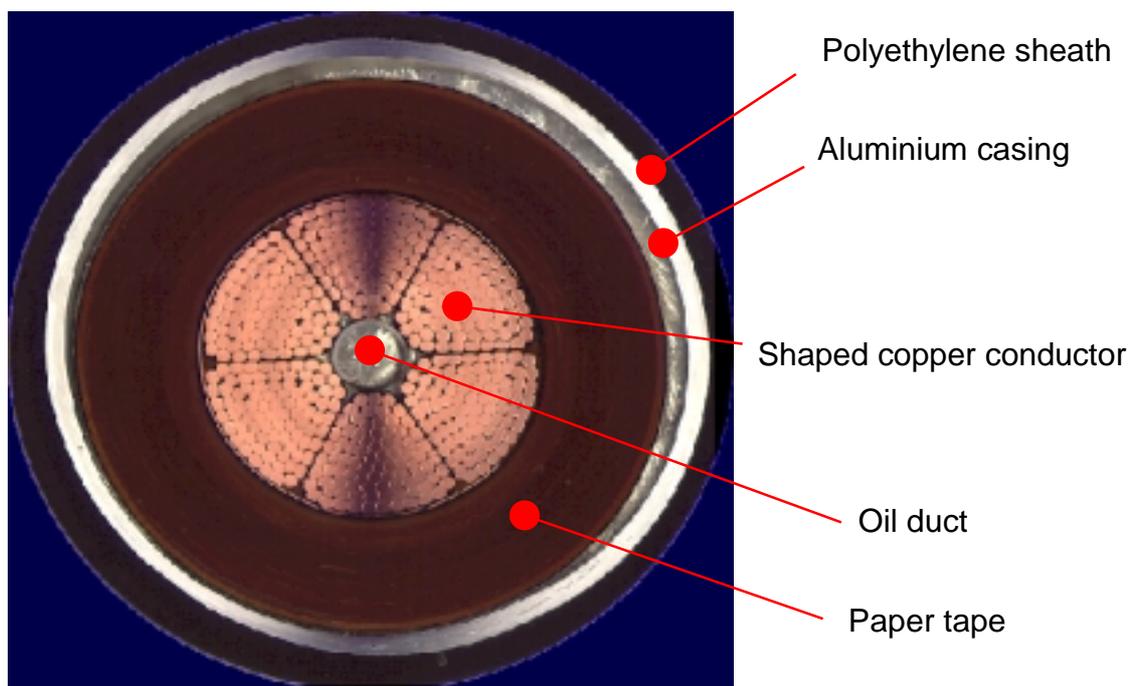


Figure 1.1 Transverse section of an oil-filled paper-wrapped cable (photograph courtesy National Grid Transco plc.).

1.3 Aims

Data generated during the course of this project should provide a clearer understanding of the effects, chemistry and microbiology associated with cable oil in the subsurface. These data could potentially be used to model the degradation of cable oil in the PHREEQC environmental modelling application and the resulting information should lead to the identification of a viable strategy for the effective remediation of soil and groundwater contaminated with LAB.

1.4 Properties of cable oil

Cable oils used in England and Wales are synthetic mixtures of linear alkylbenzenes. They are obtained from a number of sources (trade names include C14B, T3788, P1) and while variable in composition, the mixture used at present has been characterised as being 99% C₁₀-C₁₃ alkylbenzenes (Rowland 1996).

1.4.1 Chemical structure

The generalised chemical structure of linear alkylbenzene cable oil is shown in Figure 2. It can be visualised as a C₁₀-C₁₃ alkyl chain, with a single phenyl group attached at some point along its length. Note that, in contrast to the BTEX (benzene, toluene, ethylbenzene and xylenes) compounds, terminal isomers (*n*-alkylbenzenes) are absent in cable oil. This is thought to be due to the existence of an energetically unstable carbonium intermediate in the synthetic pathway (Swisher *et al.* 1961). Modern manufacturing methods favour 2C_m congeners and produce a mixture that is

almost entirely LAB, with few side-reaction products (Zhang *et al.* 2003; Knifton *et al.* 2003).

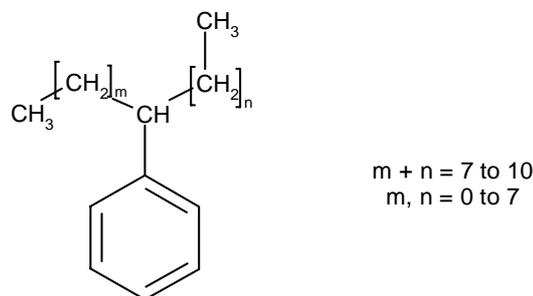


Figure 1.2 General structure of linear alkylbenzenes found in cable oil (N.B. terminal isomers not found)

In the opinion of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE), LAB has moderate affinity for water, high affinity for air and high affinity for soil, sediments and organic matter (Scientific Committee for Toxicity, Ecotoxicity and the Environment 1998).

Values given in the literature for physical properties (Table 1.1) should be treated with caution. For instance, published data for aqueous solubility range from 5×10^{-11} to 7×10^{-9} mol l⁻¹, and Log K_{ow} from 4.97 to 9.12. Not only do these cover several orders of magnitude, but if published data for each are used to estimate the other by using the general solubility equation (Equation 1.1), the calculated figures are in the ranges 3×10^{-5} to 5×10^{-10} mol l⁻¹ and 8.9 to 10.8, respectively. While these figures are broadly consistent, it is clear that the properties have not been measured to a high degree of accuracy or precision.

$$\log S_w = 0.5 - 0.01(MP - 25) - \log K_{ow}$$

Equation 1.1 General solubility equation relating molar aqueous solubility (S_w), octanol-water coefficient (K_{ow}) and melting point (MP °C) of an organic non-electrolyte. For liquids, MP is set at 25°C (Ran *et al.* 2002).

Vapour pressure measurements are more precise, covering a range of an order of magnitude (0.065 – 0.17 Pa). The partitioning of LAB between the various environmental compartments in soil is summarised diagrammatically in Figure 1.3.

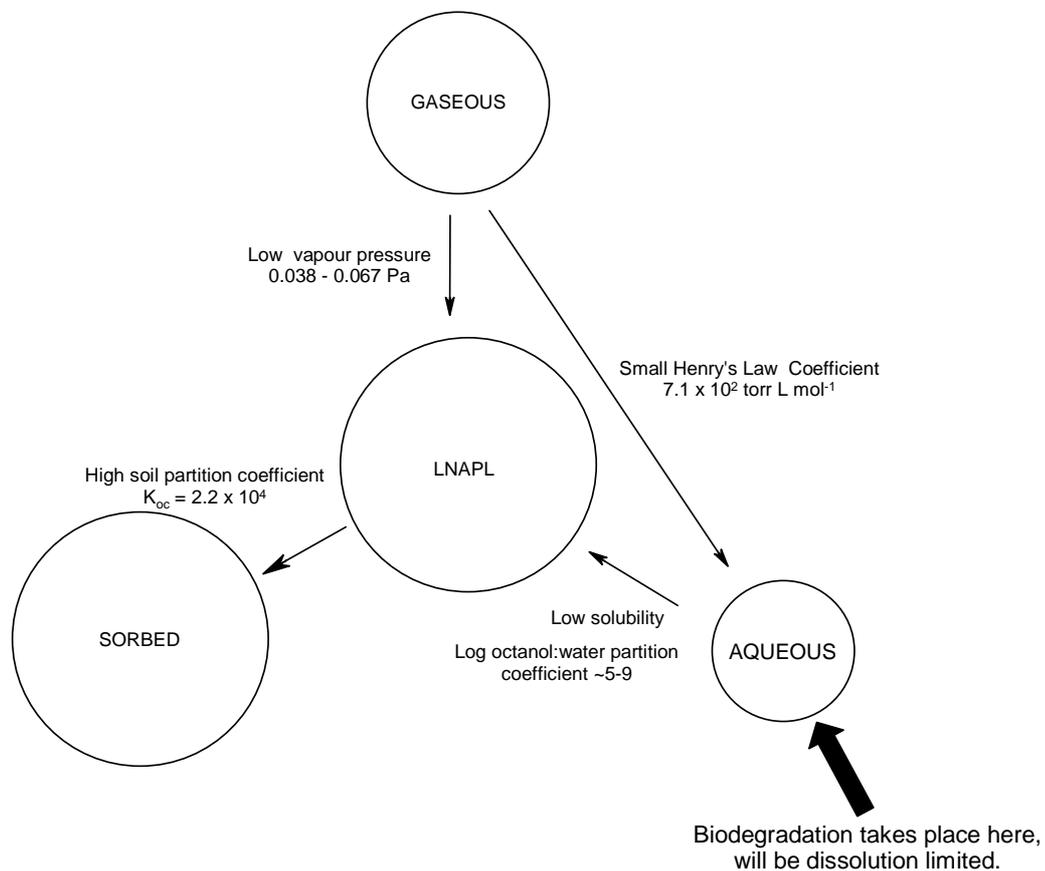


Figure 1.3 Partitioning of cable oil in soil (not to scale). Arrows indicate preferred partition.

Table 1.1 Physical properties of linear alkylbenzene (LAB) cable oil

Property	Value
Appearance	Clear colourless liquid ⁽¹⁾
Density at 20°C	0.86 kg l ⁻¹ ⁽²⁾
Melting point	< -70°C ⁽³⁾
Boiling point	≥ 260°C ⁽¹⁾ 726°C ⁽²⁾ 278-314°C ⁽⁴⁾
Kinematic viscosity at 20°C	7.5-8.5 mm ² s ⁻¹ ⁽¹⁾ 8.1 mm ² s ⁻¹ ⁽²⁾
Flashpoint	≥ 130°C ⁽¹⁾ 150°C ⁽²⁾
Aqueous solubility	Immiscible ⁽¹⁾ < 0.01 mg l ⁻¹ ⁽²⁾ 0.41 mg l ⁻¹ ⁽⁵⁾ 4-7 nmol l ⁻¹ ⁽⁶⁾
Vapour pressure at 25°C	4.9 × 10 ⁻⁴ mmHg ⁽⁵⁾ 0.038 – 0.067 Pa ⁽⁶⁾ 0.0017 hPa ⁽⁷⁾
Henry's Law constant	7.1 × 10 ² torr L mol ⁻¹ ⁽⁵⁾
Soil partition coefficient, K _{oc}	2.2 × 10 ⁴ ⁽⁵⁾
Log octanol:water partition coefficient, K _{ow}	5.72-5.75 ⁽⁵⁾ 4.97-5.08 ⁽⁶⁾ 7.5-9.12 ⁽⁸⁾
Photodegradation	< 1 % after 14 days ⁽⁵⁾

Source:

- 1 – BICC Cables safety data sheet, July 1994
- 2 – Shell health, safety and environment data sheets
- 3 – Wibarco (1993), cited in Heinze (2001b)
- 4 – EniChem (1993), cited in Heinze (2001b)
- 5 – Gledhill et al. (1991)
- 6 – Sherblom et al. (1992)
- 7 – Calculated value from EPIWIN, cited in Heinze (2001b)
- 8 – Calculated value from Hansch and Leo (1979) , cited in Heinze (2001b)

1.5 Naming conventions

A variety of nomenclatures has been applied to LABs. Some authors name them according to the length of the longest aliphatic chain, while others treat them as fundamentally aromatic molecules with a single functional substitution consisting of unbranched (in the case of terminal isomers) or single-branched (at the carbon nearest the attachment to the benzene ring) alkyl chains.

Thus, the isomer of $C_{18}H_{30}$ depicted in Figure 1.4 may be referred to in the literature as dodecyl-2-benzene, 2-phenyldodecane or (1-methylundecyl)-benzene. The latter, indicating a branched molecule, is the current IUPAC name. Nevertheless, the term LAB is still in common usage to distinguish these molecules from more highly branched species. Some authors reserve the title LAB for terminal isomers, including toluene and ethylbenzene.

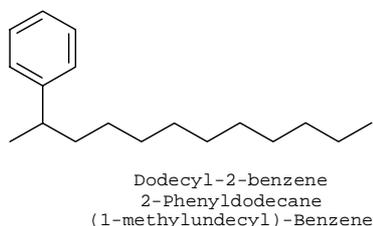


Figure 1.4 Structure of a representative homologue of LAB

It is common to use the shorthand nC_m LAB where n = position of the benzene ring and m = number of carbon atoms in the longest aliphatic chain. The isomer in Figure 1.4 would be referred to as $2C_{12}$ LAB in this nomenclature. This leads to a situation in which each of the ~20 isomers may have as many as four designators. There is

also variable use of, e.g., dodecylbenzene (DDB) to mean all $C_{18}H_{30}$ monoalkylated phenyls, only the terminal substitution of *n*-dodecane with a phenyl group or, colloquially, the entire mixture of synthetic LAB. Industrially, the mixture is known as benzene, 10-13 alkylate. Since there is ambiguity in the use of some of the terminologies, it is difficult to identify all the literature on the subject.

1.6 Manufacture and use of LAB

The main global consumer of LAB is the detergent industry (Hansen *et al.* 2000). Demand totalled 2.3×10^6 tonnes in 1999 and is projected to outstrip the current manufacturing capacity, reaching 3.4×10^6 tonnes y^{-1} by 2010 (Burrige 2002).

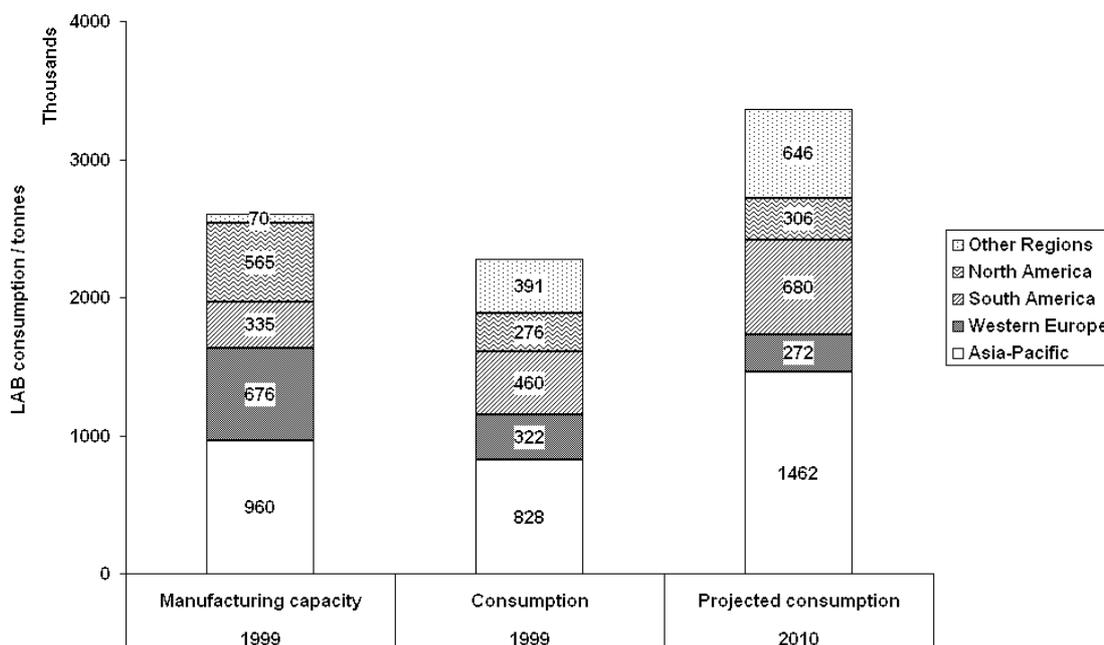


Figure 1.5 Global consumption and manufacturing capacity of LAB by region in 1999 and projected consumption for 2010 (data from Burrige (2002))

Table 1.2 Capacity of major manufacturing facilities of LAB in 1999 (adapted from Burrige (2002))

Company	Facility	Country	Capacity × 10³ tonnes
Europe			
Chevron Onite	Goneville	France	100
Lukoil Neftochim	Burgas	Bulgaria	5
Petresa	San Roque	Spain	220
Petrobrazil	Brazi	Romania	15
Prva Iskra	Baric	Serbia	50
Sasol	Augusta	Italy	220
Sasol	Porto Torres	Italy	100
Wibarco	Ibbenburen	Germany	36
			746
Americas			
Deten Quimica	Camacari	Brazil	220
Huntsman	Chocolate Bayou, TX	USA	180
Petresa	Becancour	Canada	135
Quimica Venoco	Guacara	Venezuela	70
Repsol YPF	Ensenada	Argentina	45
Sasol	Baltimore, MD	USA	130
Sasol	Lake Charles, LA	USA	120
			900
Asia			
Formosa Union Chemical	Lin Yuan	Taiwan	90
Fushun Petrochemical	Fushun	China	200
Indian Petrochemicals	Baroda	India	45
Iran Chemical Industries Investment	Esfahan	Iran	50
Isu Chemical	Ulsan	South Korea	190
Jin Tung Petrochemical	Nanjing	China	100
Nippon Petrochemicals	Kawasaki	Japan	0.090
Nirma	Baroda	India	0.075
Reliance Industries	Patalganga	India	100
Tamilnadu Petroproducts	Manali	India	75
Unggul Indah Cahaya	Merak	Indonesia	110
			960
		TOTAL	2606

Because of this projected shortfall, a number of plants are being expanded, converted from branched alkylbenzene (BAB) production, or built from scratch, especially in

countries where demand is predicted to be highest. These include Saudi Arabia, United Arab Emirates, Qatar, Egypt, Syria, India, Vietnam and China. In fact, expansion has been so rapid that there is a danger that capacity will exceed demand, with attendant effects on the profitability of LAB manufacture.

It has been suggested that while demand continues to grow by 4 % y^{-1} from 2001 to 2006, manufacturing capacity will increase by 8 % y^{-1} over the same period. As a result of this, both Saudi Arabia and Kuwait are reconsidering their plans for expansion (Anonymous 2003).

1.7 Legislation in the UK

Contaminants in soil are subject to a wide range of influences (Jones *et al.* 1996). The definition of pollution is based on the concept of a pollution linkage (Figure 1.6). A pollution linkage consists of a source (such as a leaking underground cable), a target or receptor (usually human, though it may be another organism, structure, or an ecosystem - under UK legislation, practically any body of water is considered a target), and a pathway linking the two. If such a linkage does not exist, then a substance is not considered a risk. Pollution prevention and control measures seek to break the linkage by addressing one or more of its components.

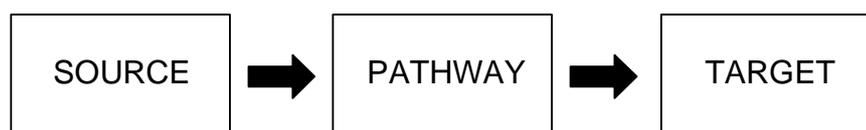


Figure 1.6 Pollution linkage

Current environmental legislation in the United Kingdom is aimed at meeting the requirements of European Council Directive 96/61/EC on Integrated Pollution Prevention and Control (European Council 1996), the IPPC Directive. This Directive was based on a number of key principles, set out in the Rio Declaration (United Nations 1992):

“Principle 3

The right to development must be fulfilled so as to equitably meet developmental and environmental needs of present and future generations.”

“Principle 15

... the precautionary principle shall be widely applied ... Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation.”

This can be extended to include the reduction or elimination of a potential pollutant at its source by replacement with a more benign substance or by the use of adequate containment techniques.

“Principle 16

... the polluter should ... bear the cost of pollution.”

That is, a person (actual or legal) responsible for a potentially polluting activity should bear the economic costs of its prevention and remediation.

The requirements of the IPPC Directive are particularly enacted in the Pollution Prevention and Control Act (HMSO 1999), replacing the Integrated Pollution Control (IPC) regime and Local Authority Air Pollution Control (LAAPC) regime

from Part I of the Environment Protection Act (HMSO 1990) (EPA). The PPCA is mainly aimed at specific, highly polluting industries and waste disposal operatives, and so is not applicable to accidental cable oil releases, though its central tenets inform other legislation.

The 1999 Act also replaced the concept of “Best Available Techniques Not Entailing Excessive Cost” (BATNEEC) with the snappier, and more exacting, “Best Available Techniques” (BAT).

The Environment Act 1995 (EA) inserted a new Part IIA into the EPA, defining contaminated land as:

“... any land which appears to the local authority in whose area it is situated to be in such a condition, by reason of substances in, or under the land that,

- (a) significant harm is being caused or there is a significant possibility of such harm being caused; or
- (b) pollution of controlled waters is being, or is likely to be caused” (HMSO 1995)

This definition can be interpreted to include soil and groundwater contaminated by cable oil. Practical implications of the Environment Act were covered by the Contaminated Land Regulations (England) (HMSO 2000). While cable oil is not considered a significant pollutant, and it is not released to the environment in the normal course of its use, National Grid is funding research into its remediation. This is in accordance with the policy of the Environment Agency as stated in the Part IIA EPA (1990) Process Documentation:

“to encourage voluntary action by polluters or other appropriate persons”

and

“to encourage the use of remedial technologies which are effective in changing, fixing or transforming contaminants into less harmful substances” (Environment Agency 2000).

Among the treatment strategies considered acceptable by the Environment Agency are monitored natural attenuation (MNA) and monitored enhanced natural attenuation (MENA). In order for these strategies to be accepted over more invasive techniques such as excavation and removal to a controlled landfill (itself a less-than-sustainable option) a good understanding of the physical, chemical and biological systems involved is required.

1.8 Guidelines for acceptable levels of cable oil in soil

A variety of guidelines exist to aid in the decision making process for remediation of contaminated land. None of the commonly consulted UK guidance refers specifically to linear alkylbenzenes.

The guidelines produced for the Greater London Council, initially used by them and quickly adopted by many waste regulation authorities in the UK, as well as the Health & Safety Executive makes no mention of LAB or oil (Kelly 1980).

In the UK, the Interdepartmental Committee for the Redevelopment of Contaminated Land (ICRCL) has been providing guidance on contaminated land and

redevelopment issues since 1976. There is no mention of LAB or other oils in their guidance on contaminants associated with former coal carbonization sites (ICRCL 1983), though polycyclic aromatic hydrocarbons (PAH) are considered as markers for coal tar.

The ICRCL guidance has been superseded by the Contaminated Land Exposure Assessment (CLEA) model (Environment Agency 2002).from the Department of the Environment, Transport and the Regions (DETR) – now the Department for Environment, Food and Rural Affairs (DEFRA).

DEFRA formally withdrew the guidance on the health effects of contaminated land given in ICRCL Guidance Note 59/83 (2nd edition) (ICRCL 1983) on 14 January 2003. The source information is still available from DEFRA on request to aid understanding of historical decisions on the remediation of contaminated sites but it should not be used in the assessment of new sites. All new decisions should be based on CLEA 2002.

After a protracted labour, the CLEA 2002 model was finally released in March 2002. This is an evolution of the CLEA 1997 model developed by the late Professor Colin Ferguson, first at Nottingham Trent University, and later at Nottingham University (after the entire Land Management Team moved to the latter institution).

In common with other UK strategies, this approaches the problem of contaminated land with the idea of fitness for a particular purpose. It is based on human

toxicological data, and uses Monte Carlo simulations to model the potential pathways for human exposure, taking into account the intrinsic uncertainties.

DEFRA has begun to release a new set of guideline values, calculated using the CLEA 2002 model. These indicate a level below which the site is considered safe. Above the guideline value, further investigation is required. Thus, the CLEA guidelines provide an objective basis for decision-making, based on an assessment of risk to human health.

Not all of the CLEA guidance documents have been released. These will not address LAB but will cover PAH and BTEX compounds.

Another set of widely used guidelines is the Dutch Intervention Values. The original guidelines (Alders 1994) did not mention LABs specifically, but gave a target value for oil in soil of 50 mg kg^{-1} , and an intervention value of 5000 mg kg^{-1} . When they were updated (Pronk 2000), they included a level indicating serious contamination by dodecylbenzene in soil of 1000 mg kg^{-1} , and $0.02 \text{ } \mu\text{g l}^{-1}$ in groundwater. As previously discussed, there is some ambiguity over the term “dodecylbenzene”, however this appears to be the only statutory guidance on LAB contamination.

1.9 Fate of cable oil in contaminated soil

Studies show that attenuation in aquatic sediments alters the isomeric composition of linear alkylbenzene mixtures (Bayona *et al.* 1986; Takada & Ishiwatari 1991; Holt & Bernstein 1992; Bhatia & Singh 1996; Mangas *et al.* 1998). Chromatographic study

(Peng *et al.* 1992) indicates that external isomers have higher retention indexes. While in theory it is possible that the observed shift in isomeric composition in sediments could be due to preferential adsorption of external isomers, Preston & Raymundo (1993) found that, unlike the more polar linear alkylbenzene sulphonate (LAS) molecules, LAB sorption to particulates in estuarine sediments was not a function of alkane chain length. The isomeric shift is thus an indicator of a biologically mediated transformation.

It was decided to design experiments using aqueous cultures and sterile/non-sterile soils under Fe(III), SO_4^{2-} and NO_3^- -reducing conditions. In order to generate meaningful data from such columns, and other proposed experiments, it was vital that an appropriate means of analysing the isomeric composition of the oil was developed.

LAB is broken down relatively easily in aerobic conditions. Recently, there have been indications that anaerobic breakdown of cable oil may occur (Herbath 2003). This is of great interest since subsurface conditions near cable oil releases have been shown to be predominately anaerobic (Johnson 2000).

It was proposed to prepare enrichment cultures of organisms from cable oil-contaminated soil under a variety of redox regimes. Controls were to include sterilised cultures and cultures lacking a terminal electron acceptor (TEA), mineral salts and carbon source. Cultures were to be inoculated with soil from an existing contaminated system.

2 Literature review

Linear alkylbenzene is completely mineralised relatively easily in aerobic conditions. Since so little is known about the ability of microorganisms to degrade LAB anaerobically, a review of the literature relating to aerobic degradation of LAB and to anaerobic degradation of hydrocarbons that share structural features with LAB will give an insight into the likelihood that similar degradative pathways exist for LAB.

2.1 Aerobic biodegradation

Aerobic biodegradation refers to the degradation of compounds by organisms using oxygen as the terminal electron acceptor (TEA) in the respiratory electron transport chain. The degrading organisms are usually prokaryotes (bacteria) or fungi.

Existing studies of the biochemistry of LAB have largely been on terminal isomers (commercially available monoisomeric standards are all terminal isomers). It is unclear what the relevance of these studies is to LABs in the environment since commercially synthesised LABs do not include these isomers (Eganhouse 1986).

Aerobic biodegradation of 1-phenylalkanes has been demonstrated in the bacteria *Nocardia* (Sariaslani *et al.* 1974), *Acinetobacter* (Amund & Higgins 1985) and *Pseudomonas* (Bayona *et al.* 1986; Smith & Ratledge 1989). Dodecylbenzene cable oil is considered readily biodegradable according to OECD guidelines 301D (OECD

1992). Indeed, cable oil is thought to act as a carbon source in contaminated soil (Reid *et al.* 2000).

Studies on biodegradation rates of hydrocarbons indicate a hierarchy of ease of biodegradation in which *n*-alkanes > branched alkanes > low molecular weight aromatics > cycloalkanes (Leahy & Colwell 1990). The trend is not so obvious for higher molecular weight aromatic compounds because of the existence of many different isomers (Huesmann 1995). Nevertheless, this hierarchy is visible in features of LAB degradation that have been noted in the literature: there have been a number of studies of the isomeric composition of LAB in sediments from Tokyo Bay (Takada & Ishiwatari 1990; Takada & Ishiwatari 1991; Chalaux *et al.* 1995). These indicated preferential degradation of certain isomers. Other studies, of LAB in soils amended with anaerobically digested sewage sludge (Holt & Bernstein 1992; Mangas *et al.* 1998) and of LAB exposed to *Nocardia amarae* isolated from crude oil- and petroleum-contaminated soils (Bhatia & Singh 1996), found similar patterns of isomeric changes. They all found that external isomers of LABs are degraded in preference to internal isomers. This was also observed by Bayona *et al.* (1986) and Angley *et al.* (1992), both of whom also found better biodegradation if the chain was long. This suggests that access to a methyl terminus is significant in the initial degradation of LABs.

Smith & Ratledge (1989) and Smith (1990) elucidated aerobic biodegradation routes for C₁-C₇ terminal alkylbenzenes, 1-phenyldodecane and 1-phenyltridecane by *Pseudomonas* sp. (Figure 2.1). In the compounds with shorter chain lengths (up to 7

carbons), the initial attack on the molecule was an oxidative cleavage of the phenyl ring. With longer chain lengths initial attack was via ω - or β -oxidation of the methyl terminus of the alkyl chain. It is possible that both methyl termini are attacked in non-terminal isomers.

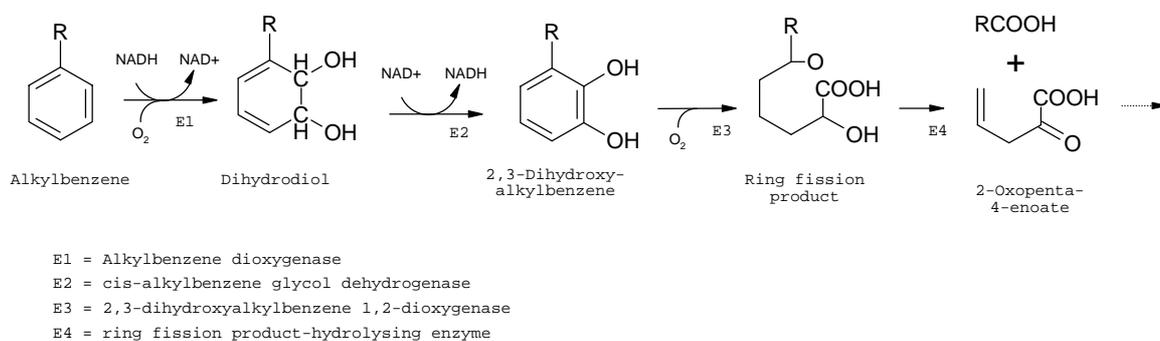


Figure 2.1 Generalised pathway for the aerobic catabolism of alkylbenzenes (Smith & Ratledge 1989)

Bhatia & Singh (1996) examined the aerobic biodegradation of commercial LAB by *Nocardia amarae*. They found that the position of the benzene ring affected ease of degradation, with external isomers being more readily degraded than internal ones. They examined the breakdown products and found that the *cis*, *cis*-muconic acid pathway was most significant, with the phenyl acetic acid pathway also being significant where the alkyl chain was an odd number of carbons long and the phenyl substitution was at an even carbon. In these cases, *trans*-cinnamic acid formation provided a minor pathway.

Recently, work has been undertaken into the fate of cable oil in soil. Terrestrial subsoils are generally composed of chemically weathered minerals and thus have a lower organic carbon content than most aquatic sediments. Redox conditions vary

over longer distances and the microbial flora is significantly different, especially when compared to marine environments. However, studies have produced promising results for cable oil bioremediation. Cheston (1997) and Tebbutt (1998) found that uncontaminated soils contained aerobic organisms that could degrade cable oil at low concentrations. Koussia (1999) found removal rates of $0.15 \mu\text{l ml}^{-1} \text{ week}^{-1}$ in unamended soil, with a 50% increase in rate when nutrients were added.

Aerobic degradation of alkane chains can be likened to fatty acid degradation by β -oxidation (Figure 2.2). Indeed actinomycetes, already implicated in the aerobic biodegradation of LAB (Johnson 2000), have been shown to convert a variety of compounds containing an alkyl side chain to their respective fatty acids as the first step in the assimilative/degradative process (Alvarez 2003).

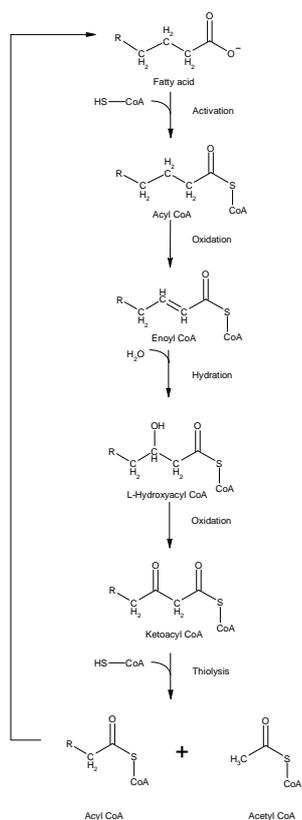


Figure 2.2 Beta oxidation pathway (Stryer 1995)

If a terminal isomer of LAB were degraded via this pathway, the result would be either toluene or ethylbenzene, depending on whether the number of carbon atoms in the original alkane chain was odd or even. However, since the pathway requires the addition of molecular oxygen (at two points in each cycle) it will not occur in anaerobic conditions. In any event, the terminal isomers are absent in synthetic LAB. β -oxidation of the isomers found in cable oil may lead to one of the structures in Figure 2.3. This will be dependant on whether the initial chain(s) are odd or even in length, whether both ends of the chain are attacked, and on how close to the phenyl group the relevant enzyme can operate before the charge or physical size of the group interferes too much.

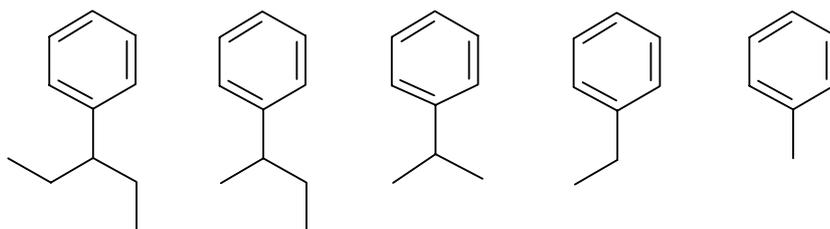


Figure 2.3 Possible end points for β -oxidation of LAB

However, β -oxidation does require molecular oxygen and so is unlikely to occur in strictly anaerobic conditions.

Aekersberg *et al.* (1991) were the first to show that hexadecane and other long chain alkanes could be degraded to CO_2 by a bacterial strain under sulphate-reducing conditions. There was some evidence that this strain produced membrane lipids with an odd number of carbon atoms when fed alkanes with an even number of carbon atoms. This suggests that the alkane chain undergoes the removal or addition of an

odd number of carbon atoms in this organism, which contrasts with the strictly even removal of β -oxidation, though it is consistent with ω -oxidation, possibly followed by β -oxidation. This even-to-odd transformation has not been seen in subsequently identified alkane-degrading anaerobes and each species is specific to a limited range of chain lengths, indicating that a range of novel pathways are used (Heider *et al.* 1999).

To date, much of the work done assumes that attenuation of LAB in the environment is due to a combination of physical process and aerobic biodegradation. However, oxygen is not always available, while other terminal electron acceptors may be available in soil.

For instance, electron acceptors are used preferentially by microbial communities according to availability in a BTEX plume ($O_2 > NO_3^- > Fe(III) > SO_4^{2-}$ (Appelo & Postma 1993)). Hence, it is important to consider potential degradation routes under a variety of redox regimes to understand whether degradation will occur in the absence of oxygen, and if so, whether it will continue as each electron acceptor is depleted, or whether it is necessary to manipulate conditions to maintain a particular set of conditions.

2.2 Anaerobic degradation of hydrocarbons

Biodegradation of xenobiotic hydrocarbons is dependent on the existence of microorganisms capable of utilising the contaminant as a carbon and/or electron donor, availability of the contaminant and appropriate terminal electron acceptor, and availability of other nutrients.

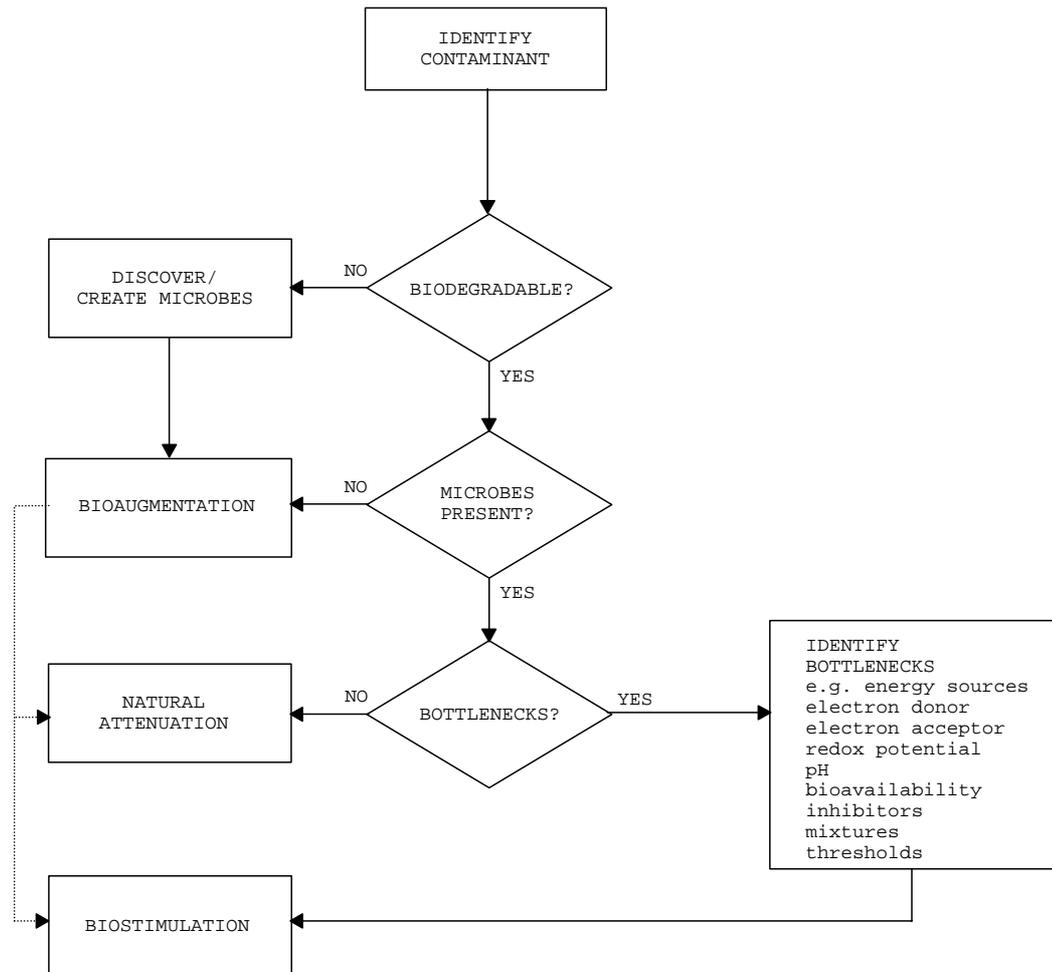


Figure 2.4 Routes to bioremediation, adapted from Spain (2001)

Many compounds have been shown in the laboratory to be readily biodegraded, yet still persist in the environment. Spain (Spain 2001) suggests that this may be addressed by manipulating conditions or bacterial communities (Figure 2.4). A number of factors (Table 2.1) can limit biodegradation (Providenti *et al.* 1993).

Table 2.1 Factors limiting biodegradation (after Providenti *et al.* (1993))

FACTOR	EXAMPLE
Soil moisture	Water required for microbial metabolism and movement In excess, can reduce oxygen availability
Soil type	Availability of nutrients Sorption of contaminants
Aeration	Oxygen required for aerobic metabolism May be needed as a substrate for oxygenases
Redox potential	Terminal electron acceptors for microbial respiration
pH	Affects microbial metabolism Solubility and sorption of contaminants
Temperature	Metabolic rates Contaminant solubility, sorption, viscosity, volatilisation
Availability of contaminant	Low solubility Uptake problems Sorption to soil/sediment Restricted movement of microbes Low concentration of contaminants
Metabolic constraints	Lack of appropriate enzymes Requirements for cometabolites Preferential metabolism of alternate C sources Toxicity of contaminant
Predation of bacteria by protozoa	May reduce bacterial numbers to unsustainable levels

Where xenobiotic hydrocarbons are found under anaerobic conditions, there are two major routes to bioremediation. The most commonly used method is to try and modify conditions by adding oxygen – either by treating the soil *ex situ* (land farming, windrows or composting) or *in situ* by sparging the soil with air/oxygen, or injecting aerated water or hydrogen peroxide (H₂O₂). Organisms that can couple

oxidation of hydrocarbons to reduction of chlorate (ClO_3) or perchlorate (ClO_4) can also degrade chlorite (ClO_2) to O_2 and Cl^- . Addition of chlorate to a population of such organisms thus leads to the *in situ* production of molecular O_2 , which can then be used by aerobic microorganisms (Coates & Anderson 2000). Alternatively, a commercially available slow release oxygen releasing preparation may be used. These methods have problems with access, mechanical stability, biofouling etc. and an alternative is the investigation of potential anaerobic degradation mechanisms.

It should be noted that field evidence for anaerobic degradation of hydrocarbons is called into question because of intermittent recharge with O_2 , heterogeneity in soil etc. (Salanitro *et al.* 1997). However, there is growing evidence of anaerobic degradation of hydrocarbons previously thought not to be susceptible. It has been shown that monoaromatic hydrocarbons are transformed to organic acids in anoxic groundwater (Cozzarelli *et al.* 1990).

There are a number of advantages of anaerobic remediation: the most obvious being that it does not need oxygen as an electron acceptor (though it may be needed as a substrate for oxygenases). The delivery of oxygen to an oxygen-limited soil environment without excavating the soil can be difficult and costly.

Anaerobic bacterial growth typically results in low biomass production compared to aerobic growth (Holliger *et al.* 1997). This reduces the likelihood of soil pores becoming clogged with bacterial cells and compromising hydraulic flow.

Most published work in the field is on BTEX compounds and so will not be directly applicable to cable oil. Where data are available for LABs they relate to terminal isomers, not found in cable oils. However, LABs share a number of structural features with BTEX compounds and with *n*-alkanes so it is useful to review research on a variety of compounds and electron acceptors.

Table 2.2 summarises the published work on anaerobic hydrocarbon biodegradation. It includes selected work under aerobic conditions where appropriate.

Table 2.2 Summary of available research on anaerobic degradation of hydrocarbons (with selected aerobic references)

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
Aromatics (general)	Extensive literature – readily degraded Hydrogen peroxide (H ₂ O ₂) used as supplementary O ₂ source in soil bioremediation (Fiorenza & Ward 1997)	(Gersberg <i>et al.</i> 1995) No significant degradation over 14 months <i>in situ</i> (Barbaro & Barker 2000) Degradation in mixed aerobic/nitrate reducing conditions (Wilson & Bouwer 1997) (Harms <i>et al.</i> 1999) TEX degraded but benzene still recalcitrant (Thomas <i>et al.</i> 1997)	None found	Fe(III) reduced in sediments where BTEX compounds were being oxidised with SO ₄ ²⁻ reduction. Either H ₂ S from sulphate reduction reacting with Fe(III) or Fe(III) reduction coupled to BTEX degradation (Schmitt <i>et al.</i> 1996) <i>Ferroglobus placidus</i> oxidised aromatics at 85°C. May be significant for deep subsurface and hydrothermal zones (Tor & Lovley 2001) (Anderson <i>et al.</i> 1998)	Fe(III) was reduced in sediments where BTEX compounds were being oxidised with sulphate reduction. Two hypotheses – either H ₂ S from sulphate reduction reacting with Fe(III) or Fe(III) reduction coupled to BTEX degradation (Schmitt <i>et al.</i> 1996) (Elshahed <i>et al.</i> 2001)	None found

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
Benzene	T _{1/2} = 11 d at 0.25-0.35 m (where neither O ₂ or carbon limiting) rises to 173 years at 2.6-2.8 m (Franzmann <i>et al.</i> 1999)	(Morgan <i>et al.</i> 1993) Needs O ₂ (Anid <i>et al.</i> 1993) Benzoate degraded under denitrifying conditions (Harwood & Gibson 1997) Benzene degraded in microaerobic condition – O ₂ needed for oxygenases (Durant <i>et al.</i> 1999) First report of a single organism capable of degrading benzene under nitrate-reducing conditions (Coates <i>et al.</i> 2001)	None found	Fe(III) ligands stimulate anoxic degradation (Lovley <i>et al.</i> 1994) (Kazumi <i>et al.</i> 1997) <i>Geobacter</i> spp. Community - many primitive bacteria (hyperthermophiles), previously thought to require SO ₄ ²⁻ , can grow using Fe(III) (Anderson <i>et al.</i> 1998; Rooney-Varga <i>et al.</i> 1999) Fe(II) reoxidised to Fe(III) in toluene degrading incubations (needs NO ₃ ⁻) (Caldwell <i>et al.</i> 1999)	Complete mineralization in microcosms containing both contaminated sediment and mineral media (Edwards & Grbic-Galic 1992) PATHWAY (Chaudhuri & Wiesmann 1995) Benzene C ₆ H ₆ + 3.75SO ₄ ²⁻ → 6CO ₂ + 3.75S ²⁻ + 3H ₂ O within single cells (Lovley <i>et al.</i> 1995; Anderson & Lovley 2000a) (Chapelle <i>et al.</i> 1996) (Kazumi <i>et al.</i> 1997) Bacterial consortium (Phelps <i>et al.</i> 1998)	(Vogel & Grbic-Galic 1986) (Grbic-Galic & Vogel 1987) (Kazumi <i>et al.</i> 1997)

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
Toluene	Extensive literature – readily degraded Obligate aerobic <i>Sphingomonas</i> isolated from deep subsurface (Fredrickson <i>et al.</i> 1995)	PATHWAY (Kuhn <i>et al.</i> 1988) (Evans <i>et al.</i> 1991) Partial pathway see Figure 2.6 (Grbic-Galic 1991) (Morgan <i>et al.</i> 1993) (Anid <i>et al.</i> 1993) <i>Thauera</i> sp. Inhibited by benzyl alcohol (Biegert & Fuchs 1995) Uses Acetyl and Cinnamoyl CoA – Proposed pathway see Figure 2.8 (Chee-Sanford <i>et al.</i> 1996) $C_7H_8 + 6NO_3 \rightarrow 7CO_2 + 4H_2O + 3N_2$ (Mester & Kosson 1996)	Toluene readily transformed under a range of redox conditions (Langenhoff <i>et al.</i> 1996)	Fe(III) ligands stimulate anoxic degradation (Lovley <i>et al.</i> 1994) Many monoaromatics degraded using Fe(III) as sole electron acceptor (Lovley 1995) Toluene readily transformed under a range of redox conditions (Langenhoff <i>et al.</i> 1996) (Heider <i>et al.</i> 1999) <i>Geothrix fermentans</i> isolated from toluene oxidising sediments (Coates <i>et al.</i> 1999)	(Haag <i>et al.</i> 1991) (Rueter <i>et al.</i> 1994) (Chapelle <i>et al.</i> 1996) (Nay <i>et al.</i> 1999b) (Heider <i>et al.</i> 1999) Toluene readily transformed under a range of redox conditions (Langenhoff <i>et al.</i> 1996) Degradation under sulphate-reducing conditions accelerated by presence of iron. Not a biological process – probably a reaction with biogenic H ₂ S (Beller <i>et al.</i> 1992) (Edwards <i>et al.</i> 1992)	(Vogel & Grbic-Galic 1986) (Grbic-Galic & Vogel 1987) PARTIAL PATHWAY (Grbic-Galic 1991) ² H enrichment (Ward <i>et al.</i> 2000) (Elshahed <i>et al.</i> 2001)

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
		<p>Toluene readily transformed under a range of redox conditions (Langenhoff <i>et al.</i> 1996)</p> <p>Proposed pathway see Figure 2.7 (Heider <i>et al.</i> 1999)</p> <p>(Caldwell <i>et al.</i> 1999)</p> <p>(Nay <i>et al.</i> 1999b)</p> <p>PATHWAY (Ellis 2001)</p> <p>Toluene dioxygenase still expressed at low DO. Suggests that aerobic biotransformation can occur even if aerotolerant</p>			(Rabus <i>et al.</i> 1993)	

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
		anaerobic respiration predominates (Costura & Alvarez 2000)				
Ethylbenzene	Extensive literature – readily degraded	(Morgan <i>et al.</i> 1993) Needs O ₂ (Anid <i>et al.</i> 1993) Proposed pathway see Figure 2.9 (Heider <i>et al.</i> 1999)	None found	None found	None found	None found
m-Xylene	Extensive literature – readily degraded	(Kuhn <i>et al.</i> 1988) (Evans <i>et al.</i> 1991) (Morgan <i>et al.</i> 1993) Needs O ₂ (Anid <i>et al.</i> 1993) (Heider <i>et al.</i> 1999)	None found	None found	(Heider <i>et al.</i> 1999) (Edwards <i>et al.</i> 1992)	None found

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
p-Xylene	Extensive literature – readily degraded	(Morgan <i>et al.</i> 1993) Needs O ₂ (Anid <i>et al.</i> 1993)	None found	None found	(Edwards <i>et al.</i> 1992)	None found
o-Xylene	Extensive literature – readily degraded	Transformed (Evans <i>et al.</i> 1991) (Anid <i>et al.</i> 1993)	None found	None found	(Rueter <i>et al.</i> 1994) (Heider <i>et al.</i> 1999) (Edwards <i>et al.</i> 1992)	None found
Alkanes	Long chain alkanes (e.g. octadecane) recalcitrant due to poor availability. Needed O ₂ (Bajpai <i>et al.</i> 1998) (Koma <i>et al.</i> 2001)	Facultative anaerobes degraded saturated hydrocarbons under anaerobic conditions, both with and without nitrate as an electron acceptor - probably by fermentation (Grischenkov <i>et al.</i> 2000) (Rabus <i>et al.</i> 2001)	None found	None found	C ₁₂ -C ₂₀ alkanes (Aekersberg <i>et al.</i> 1991) Hexadecane → CO ₂ (Coates <i>et al.</i> 1997) marine C ₁₅ -C ₃₅ alkanes degraded in marine sediments (molybdate reduction inhibited so sulphate is TEA) (Caldwell <i>et al.</i> 1998)	Hexadecane and pentadecane (but not decane or hexane) → CH ₄ in absence of sulphate (Zengler <i>et al.</i> 1999) Hexadecane → CH ₄ without sulphate reduction (So & Young 1999; Anderson & Lovley 2000b)

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
Alkene	Extensive literature – readily degraded	None found	None found	None found	1-Hexadecene (Aekersberg <i>et al.</i> 1991) 1-Hexadecene (Heider <i>et al.</i> 1999)	None found
LAB	1-phenylalkanes (Amund & Higgins 1985) 67% degraded after 28 d by modified Sturm test after OECD guideline 301B (OECD 1984): (Huls 1987) 64% degraded after 28 d by manometric respirometry test after OECD guideline 301F (OECD 1992): (Istituto Guido	Current work suggests that LAB degraded under nitrate-reducing conditions No indication of redox conditions but manufacturers claim anaerobic biodegradation of 85-95% after 42 d in activated sludge from a sewage treatment works receiving domestic effluent according to ECETOC method (1988) Anaerobic	None found	None found	Anaerobic degradation of cable oil with evidence of sulphate reduction. Anaerobic cable-oil degrading microorganisms isolated (Herbath 2003)	None found

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
	<p>Donegani 1995) 51-61% degraded after 35 d by Shake Flask Carbon Evolution Procedure: (Gledhill <i>et al.</i> 1991)</p> <p>PATHWAY see Figure 2.1 (Smith & Ratledge 1989)</p> <p>LAB degradation faster if chain long (Anglely <i>et al.</i> 1992)</p> <p>Aerobic degrading organisms isolated (Nichols 1996; Cheston 1997; Koussia 1999; Johnson 2000)</p> <p>Cable oil acts as carbon source (Reid <i>et al.</i> 2000)</p> <p>LAB from crude oil degraded in seawater (Dutta &</p>	<p>biodegradation > 70% by ECETOC method (1988) claimed by manufacturer (Petresa 1994)</p>				

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
	Harayama 2001) (Hartmann <i>et al.</i> 2000) LAB from crude oil degraded in seawater (Dutta & Harayama 2001)					
PAH	Extensive literature – readily degraded	Naphthalene, phenanthrene, biphenyl (Rockne & Strand 2001)	None found	None found	PAH → CO ₂ in marine sediments (Coates <i>et al.</i> 1996)	None found
Others	Extensive literature – readily degraded	Benzoate → Benzoyl CoA → Acetyl CoA – Proposed pathways see Figure 2.11 (Harwood & Gibson 1997) <i>Rhodopseudomonas</i> , <i>Azoarcus</i> , <i>Thauera</i> spp.	Acetate, ethanol, butyrate, propionate degraded using Fe(III) or Mn(IV) (Lovley & Philips 1988)	Fe(III) → Fe(II) by bacteria after oxygen, nitrate and Mn(IV) reduced (Munch & Ottow 1980) Fe(III) used after NO ₃ ⁻ by facultative anaerobes (Sorensen 1982) Acetate (Lovley &	1-hexadecanol, 2-hexadecanol, palmitate, stearate (Aekersberg <i>et al.</i> 1991) Acetate, butyrate, propionate used by organisms introduced to oil wells in drilling muds (Spark <i>et al.</i> 2000)	None found

	Aerobic	NO₃⁻ reduction	Mn(IV) → Mn(II)	Fe(III) → Fe(II)	SO₄²⁻ → H₂S	Methanogenesis
				Phillips 1986) (Lovley & Phillips 1988) (Lovley <i>et al.</i> 1995) Fe(III) depleted in HC-contaminated soil (Tuccillo <i>et al.</i> 1999)		

Where information on the degradation of a specific compound is unavailable, it may be possible to predict the likelihood that it will be degraded under particular conditions by referring to known degradation of other compounds with similar structures (Wackett & Ellis 1999). Unlike aerobic metabolism, where there are a relatively few pathways available for degradation of a compound, the exact pathways of anaerobic breakdown of hydrocarbons are usually novel. There is a general pattern, however (see Figure 2.5). Acyl Coenzyme A structures (benzoyl-CoA in the case of BTEX compounds) are important intermediates anaerobic pathways.

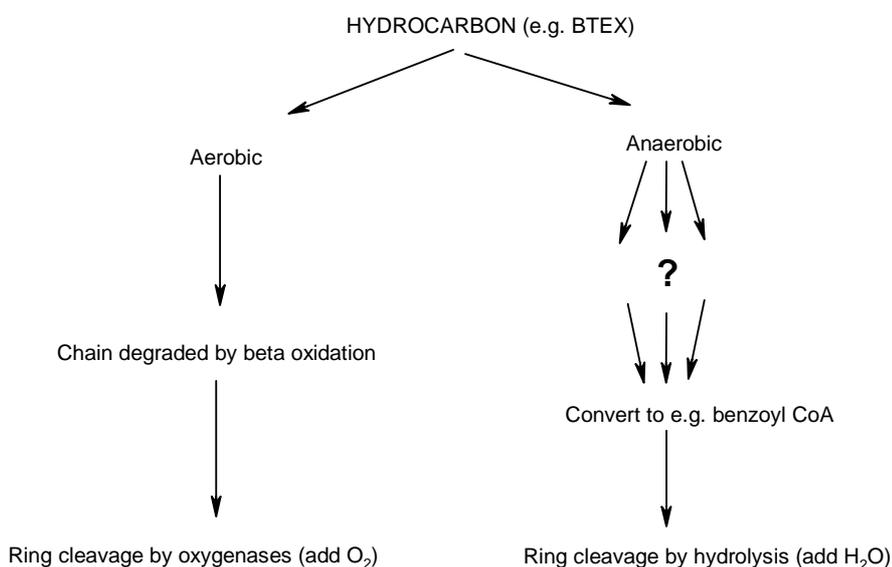


Figure 2.5 Comparison of aerobic and anaerobic hydrocarbon biodegradation

Anaerobic degradation is generally slower than aerobic degradation of the same compound, for instance ethylbenzene degradation has been shown to be slow in anaerobic conditions (Borden *et al.* 1995)

Degradation of some compounds may be inhibited if other, more readily degraded carbon/energy sources are available: benzene degradation is inhibited in the presence of other hydrocarbons (Krumholz *et al.* 1996).

Anaerobic degradation of BTEX compounds often proceeds via the formation of an acyl-CoA. Denitrifying bacteria have been shown to have appropriate CoA ligases (Villemur 1995). Enzymes have also been isolated from denitrifying bacteria capable of further conversion of benzoyl-CoA under anaerobic conditions (Boll *et al.* 2000).

Nitrate-, sulphate- and iron-reduction have all been shown to play a part in degradation of numerous hydrocarbons. Long-chain alkanes seem to be most often degraded in sulphate reducing conditions while, overall, BTEX is more susceptible to nitrate reducing conditions. Fe(III) reduction has been shown to be significant in the degradation of a number of BTEX compounds (Lovley & Anderson 2000).

There are no published pathways for the anaerobic catabolism of linear alkylbenzenes. However, it may be useful to review the known and proposed pathways for other hydrocarbons. Toluene is attacked (Figure 2.6) by hydrolysis (Grbic-Galic 1991).

Many BTEX compounds, when degraded anaerobically, produce benzoyl-CoA as an intermediate. Several pathways have been proposed for toluene (Figure 2.7, Figure 2.8).

The initial attack on ethylbenzene is expected to be at the side chain (Figure 2.9).

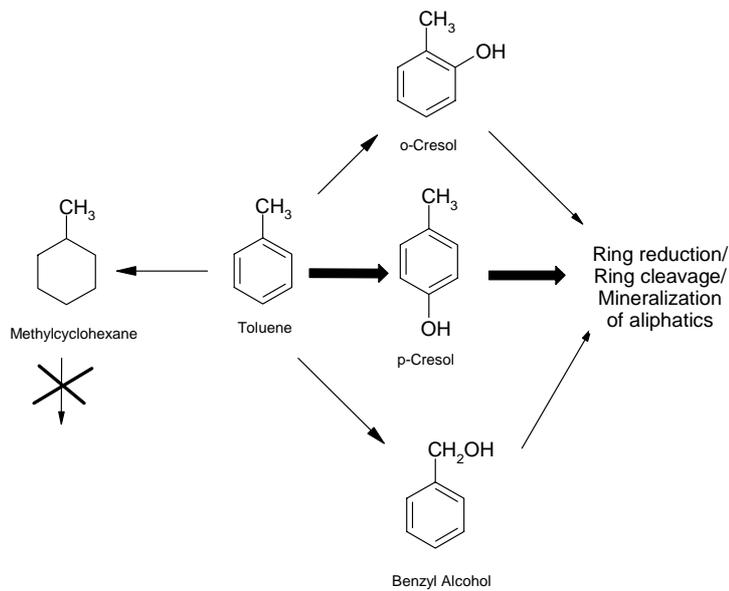


Figure 2.6 Initial microbially catalysed transformations of toluene under anaerobic conditions (Grbic-Galic 1991) – bold arrows indicate reactions known to occur

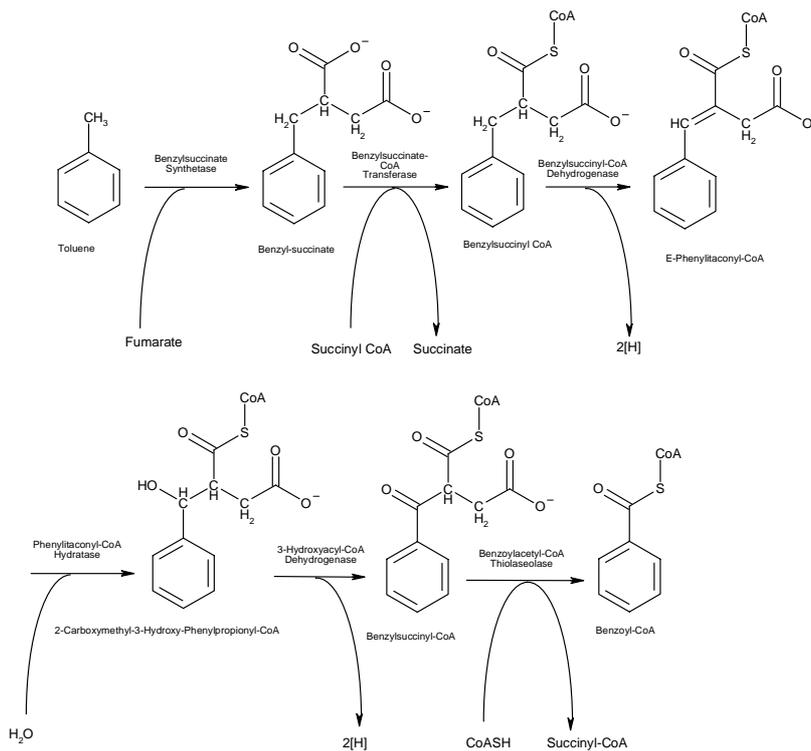


Figure 2.7 Proposed pathway for anaerobic toluene degradation (Heider *et al.* 1999)

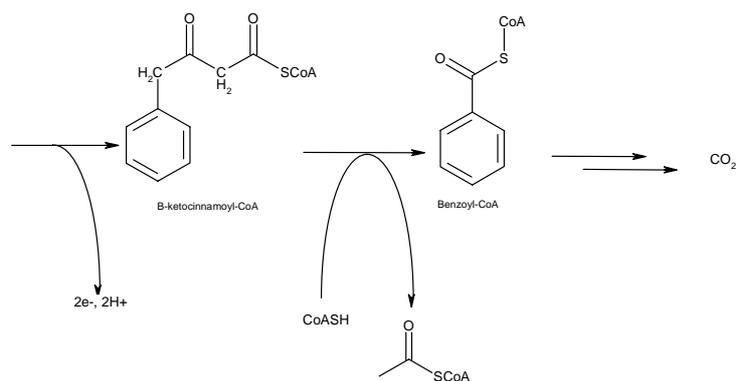
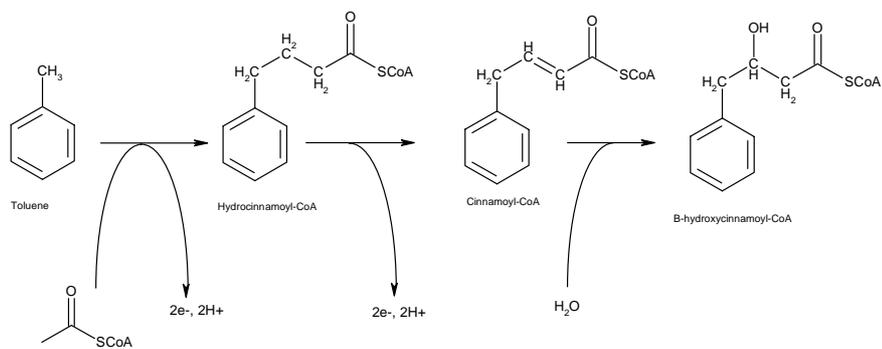


Figure 2.8 Proposed pathway for anaerobic toluene mineralization (Chee-Sanford *et al.* 1996)

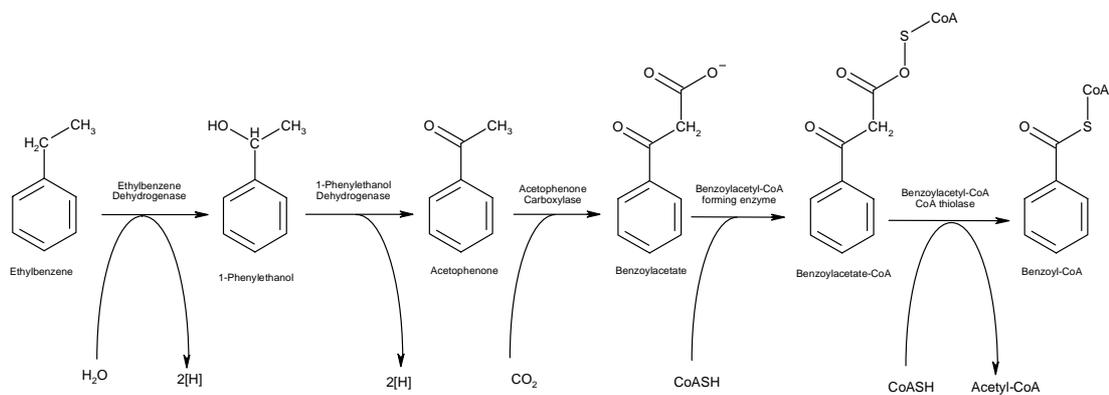


Figure 2.9 Proposed pathway for anaerobic ethylbenzene degradation (Heider *et al.* 1999)

Recent work on toluene, ethylbenzene and xylenes in aquifers (Elshahed *et al.* 2001) indicate that all are degraded to benzoate, with the production of methane under sulphate-reducing conditions (Figure 2.10). It was suggested that the presence of intermediates from these pathways in the environment might be used to confirm that the contaminants are being degraded *in situ*.

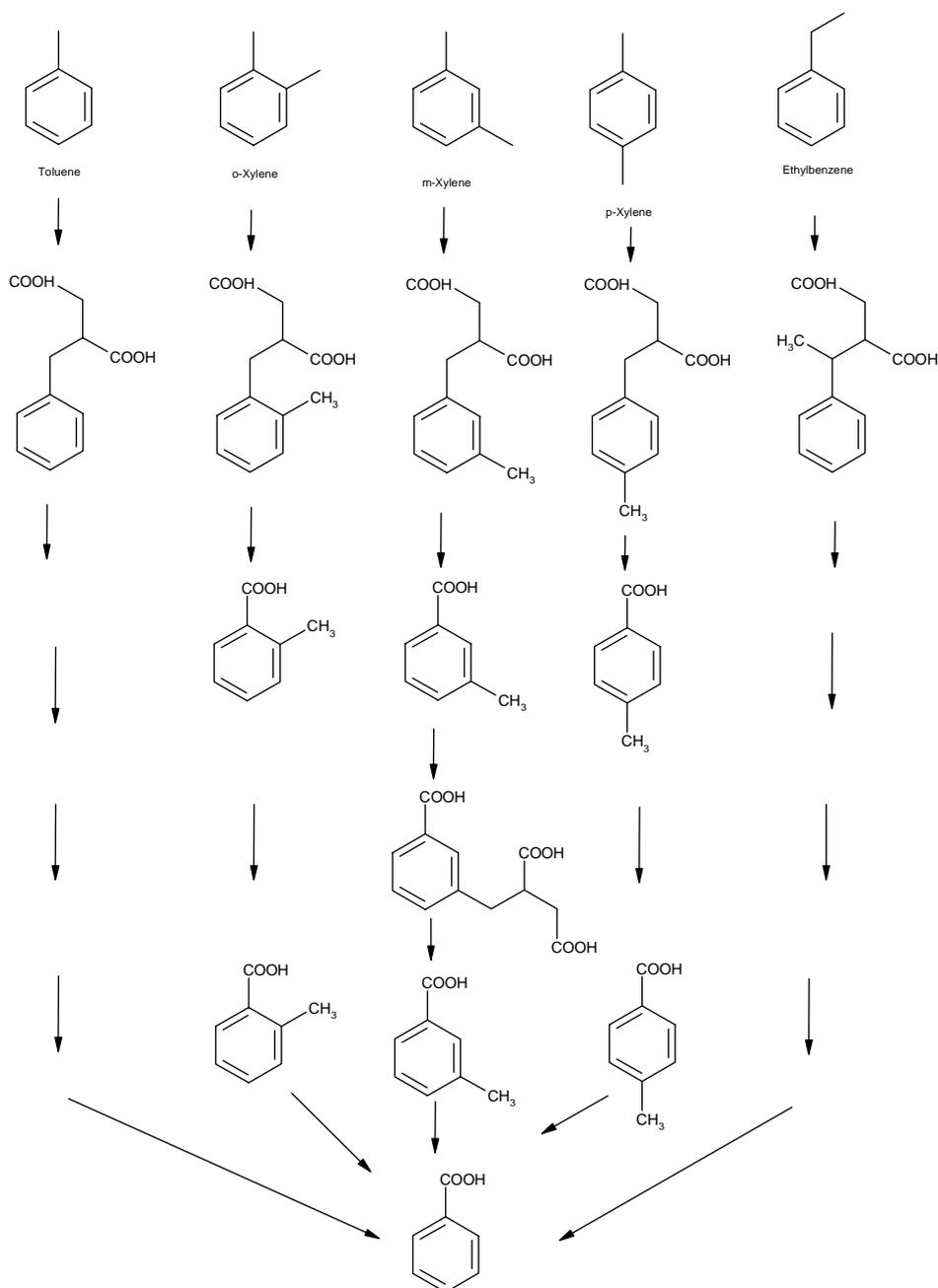


Figure 2.10 Proposed pathways for anaerobic degradation of alkylbenzenes (Elshahed *et al.* 2001)

Studies on the degradation of benzoate by the phototropic bacterium *Rhodospseudomonas palustris* and the denitrifiers *Thauera aromatica* (formerly *Pseudomonas* sp. strain K172) and *Azoarcus evansii* (formerly *Pseudomonas* sp. strain K740) have led to the partial elucidation of a number of pathways (Koch *et al.* 1993; Harwood & Gibson 1997). The pathways are summarised in Figure 2.11. The studies, using nuclear magnetic resonance (NMR) have demonstrated the sequence of breakdown products, though the enzymes involved have yet to be confirmed.

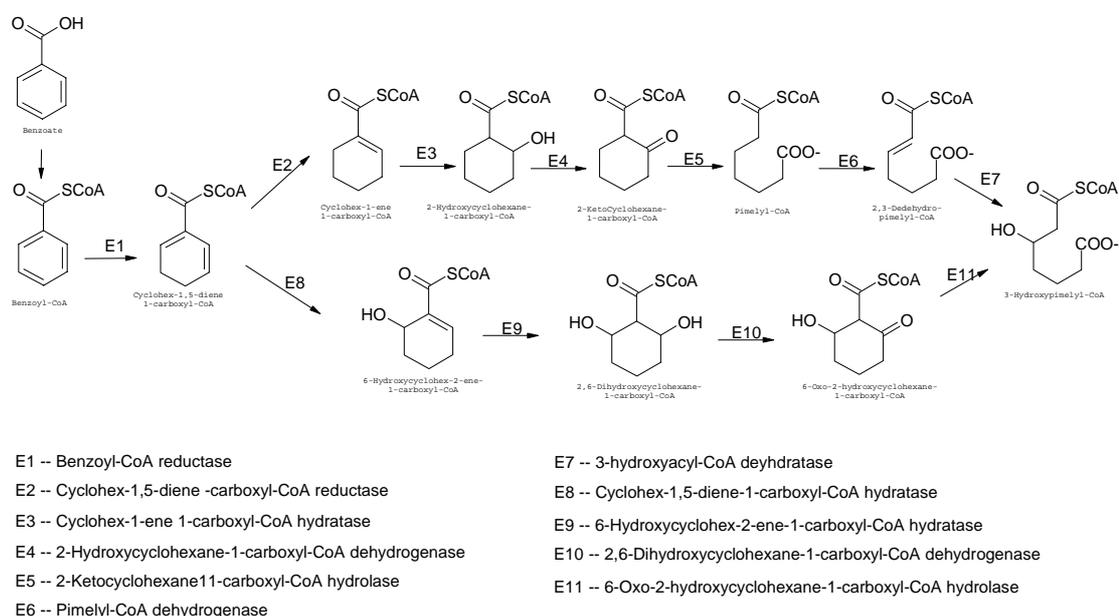


Figure 2.11 Proposed pathways for anaerobic benzoate/benzoyl CoA degradation (Koch *et al.* 1993; Harwood & Gibson 1997)

The most likely route for anaerobic degradation of LABs will probably be an initial attack on the alkyl chain(s) to form an acyl-CoA, followed by a hydrolytic ring cleavage. This is supported by findings that the enzyme, benzylsuccinate synthase, which is responsible for the production of benzylsuccinate from toluene and fumarate in *Azoarcus*, can function using a variety of toluene surrogates (Beller & Spormann 1999).

In the case of the long-chain LABs found in cable oil, it is likely that poor solubility will ultimately limit the rate of degradation. Conversely, limited instantaneous bioavailability may actually improve degradation rates by limiting toxic effects.

3 Extraction and analytical techniques

Most of the published literature on the fate of LAB in the environment has been on aquatic sediments. There does not seem to be any knowledge of the isomeric composition and fate of LAB in contaminated soil and groundwater.

There has been a limited amount of work done on the analysis of environmental LAB samples (SCCWRP 1994a; SCCWRP 1994b). This has been hampered by the fact that the only available standards are terminal isomers, not found in cable oil.

In order to investigate the fate of LAB it is necessary to identify an analytical technique that will provide quantitative and qualitative data on both fresh cable oil and on oil recovered from experimental systems and field sites. The available techniques were reviewed and it was decided that the primary analytical tool would be gas chromatography-mass spectrometry (GC-MS)

Linear alkylbenzenes demonstrate a systematic increase in boiling point with increasing alkyl chain length (Baumann *et al.* 1965) and with increasing externalization of the phenyl group (Heinzen & Yunes 1993; Heinzen & Yunes 1996) and this may be used to separate the homologues in a gas chromatograph (GC).

GC-MS (Barcelo 1992) was used to obtain an isomeric breakdown of cable oil before and after exposure to (i) soil and (ii) enrichment cultures. GC- flame ionisation detection (FID) was also a candidate technique due to its suitability for quantitative

work but GC-MS, despite its lower sensitivity, is better suited to the qualitative analysis of complex mixtures. GC-FID is useful for routine analysis but since Napier University has an HP 5890 Series II GC with an HP 7673 GC/SFC injector and an HP 5972 mass selective detector (MSD), and more recently an Agilent 6890 GC with a 7683 injector and a 5973 MSD, these were the main analytical tools for the project.

3.1 Extraction

Gas chromatography requires that the sample be either a liquid or dissolved in an appropriate solvent. LAB is poorly soluble in water but dissolves readily in non-polar solvents. The solvent can also be used to recover the LAB from complex samples such as aqueous cultures or soil samples.

A simple hexane extraction, previously used to extract cable oil from soil for fluorometry (Fu *et al.* 2000) was tried on larger soil samples with variable results. Moisture content seems to play a role and in fact, extractions from soil slurries are particularly effective, possibly because of the separation of soil particles with the resulting increase in surface area available to the solvent. For field samples, it may be necessary to refine the protocol or to adopt the more detailed protocols from Zeng and Yu (1996) and Zeng *et al.* (1997).

All the extractions carried out in this project were done by shaking the sample in 1-2 volumes of *n*-hexane. If possible, this was done in the vessel used to carry out the experiment. Where necessary, this was followed by a brief centrifugation step to separate the organic and aqueous phases. A sample of the organic phase was then

applied to the GC column as described below with no further pre-treatment. In this way, the risk of post-incubation alteration of the LAB (by sorption, chemical transformation, etc.) was minimised. Details of the extraction are described in more detail later (Sections 5.4.5.4, 6.2.4 and 8.1.4).

Where an internal standard was needed to quantify the LAB, a measured quantity of $^{13}\text{C}_{12}$ LAB was added to the sample immediately prior to extraction.

3.2 Gas chromatography (GC)

Chromatography is the blanket term given to a number of techniques used to separate mixtures of substances based on their chemical and physical properties. Chromatographic techniques rely on the differential partitioning of analytes between a stationary phase and a mobile phase. In gas chromatography, the mobile phase is gaseous and the stationary phase may be a liquid or, more commonly a solid surface.

A chromatogram only provides two pieces of information – retention time and quantity (often expressed as peak height, peak area or half-height peak width). For more detailed analysis, the sample from the chromatograph is immediately routed to a complementary technique.

3.2.1 Choice of GC column

The critical component in a gas chromatogram is the column. The resolution of a column is dependent on a number of factors, related by Equation 3.1.

$$R = \frac{1}{4} \sqrt{\frac{L}{h}} \times \frac{k}{k+1} \times \frac{\alpha-1}{\alpha}$$

Equation 3.1 Resolution of a GC column

where:

R = Resolution

L = Length of column

h = Height equivalent to a theoretical plate

α = k_1/k_2 = selectivity

A number of factors affect the suitability of a column for a particular purpose:

- Type – glass/steel, packed/capillary etc.
 - The most commonly type of column in current use is a glass capillary. In this type, the internal wall of the capillary provides the stationary phase. This is coated to make it more or less polar depending on the nature of the material to be analysed.
- Internal diameter
 - Smaller i.d. → greater surface area/volume so better resolution but smaller capacity. Applying a very small sample volume can be

problematic but this can be overcome by using a split injection where only a proportion of the injected sample enters the column properly.

- Coating – will impact on separation chemistry
 - HP5 5% diphenyl-95% dimethyl polysiloxane is a non-polar coating which is suitable for separation of environmental samples and aromatic compounds.
- Length
 - Double length → double cost but only improve efficiency by 41% (according to Equation 3) so appreciable savings may be made with relatively little loss of resolution if a shorter column is used. The cheapest way of influencing resolution is by manipulating the temperature at which the analysis is carried out – effectively, improving separation by differentiating on basis of boiling point. Modern GCs allow the temperature to be held at a set point and/or varied linearly.

A 30 m, 0.32 mm i.d., 0.25 μm film thickness, HP-5 column was available at Napier and was used in early work. Later in the project, a new GC-MS was delivered and fitted with a 30 m, 0.25 mm i.d., 0.25 μm film thickness HP-5 column. This resulted in slightly improved resolution.

3.2.2 Quantification

The quantity of each homologue is expressed in terms of peak area. This allowed direct comparison of peaks within a single chromatogram.

In order to make comparisons between chromatograms, an internal standard was employed. A measured amount of 1C₁₂ LAB, a terminal isomer and so not present in the LAB mixture, was added to samples immediately prior to the extraction step. In this way, an additional peak representing a known amount of an LAB homologue would be present in the chromatogram and other peaks could be quantified in relation to it.

3.3 Detectors

3.3.1 Mass spectrometry (MS)

Mass spectrometry is a sensitive technique that gives information on molecular mass and molecular structure of an analyte.

Samples are volatilised (already done if the sample is coming from a GC) and ionised (Electron impact/Photoionisation/Spark-source ionisation/Fast atom bombardment) to produce gas-phase ions. Depending on the ionisation technique used, the analyte may become more or less fragmented so that in addition to the ionised analyte, there are other, smaller ions present. These may be unaltered components of the parent molecule, or they may undergo a conformational change to a more stable form.

The ionised sample is accelerated by an applied voltage and the fragments separated by magnetic field, time-of-flight (TOF) or Quadropole analyser (“stable path analyser”) according to mass/charge (m/z) ratios. The ions are detected by, e.g., an electron multiplier.

A mass spectrum is a plot of intensity against m/z . Assuming the sample has not been too badly fragmented, the highest m/z peak will be the parent ion. The fragmentation pattern provides clues to the identity of the parent ion. This analysis can be aided by automated searches of commercially produced libraries of standard mass spectra.

By combining the retention time information from the chromatograph with the mass spectrum, it is possible to deduce the identity of each eluted species.

3.3.2 Flame ionisation detection (FID)

This general-purpose detection technique is especially sensitive to hydrocarbons. The effluent from the GC is mixed with hydrogen and air and ignited. Combustion of hydrocarbons result in the production of ions, which are detected by an electrode situated close to the flame. The detector is sensitive to the mass of analyte, rather than the concentration (Su 2000).

Note that while FID is a sensitive technique and is less susceptible to contamination than MS, it does not give information about the identity of the analytes. It is best suited to routine analysis of predictable samples whose elution order has been well

characterised using GC-MS and may well be useful for the routine analysis of samples to monitor removal of LAB. Note that both MS and FID are destructive techniques and therefore they cannot be used in series.

3.3.3 Thermal conductivity detection (TCD)

TCD is a non-destructive technique, and thus one that can be used alone, or in series with other, destructive or non-destructive methods such as MS or FID. The technique works by responding to variations in thermal conductivity from the baseline value of the carrier gas. It may be of use in detecting methane CH₄ in the headspace of anaerobic cultures. A TCD instrument was not available and so was not used in this project.

3.3.4 Electron capture detection (ECD)

An ECD device was available for use with the newer GC obtained by Napier University. ECD is best suited to the detection of pesticides and halogenated organic compounds. When used with LAB, no meaningful response was observed. Therefore, this was not used for routine analysis.

3.3.5 Compound-specific isotope analysis

Anaerobic toluene degradation has been shown to result in small (~2‰) enrichment of the stable ¹³C isotope (Ahad *et al.* 2000) – if the same is true of other compounds, then this may provide a means to monitor natural attenuation of LAB. A similar enrichment of ²H was seen during methanogenic degradation of toluene (Ward *et al.* 2000).

The enrichment of an isotope in a sample is expressed as, e.g., a $\delta^{13}\text{C}$ value, calculated as shown in Equation 3.2.

$$\delta^{13}\text{C} = \frac{R_{\text{sample}} - R_{\text{std}}}{R_{\text{std}}} \times 1000$$

Equation 3.2. $\delta^{13}\text{C}$ value

where:

$R_{\text{sample}} = {}^{13}\text{C}/{}^{12}\text{C}$ in sample

$R_{\text{std}} = {}^{13}\text{C}/{}^{12}\text{C}$ in standard

It had been expected that the Scottish Universities Reactor Research Centre (SURRC), a CLARRC partner, would carry out this analysis. Unfortunately, funding did not allow this avenue to be explored.

The main analytical tool used in the project was therefore GC-MS. It proved to be a robust method of generating high-quality qualitative and quantitative data on the composition of unused cable oil and oil extracted from aqueous culture, and contaminated soils and slurries.

4 GC-MS analysis of cable oil

4.1 Analytical instruments

Two GC-MS systems were available during the project. The method used to analyse LAB samples was based closely on that developed by Rosie Hyuhn at Reading University (R. Hyuhn, pers.comm.).

Initially, separation was carried out on a Hewlett-Packard (Agilent) HP5890 Series II gas chromatograph fitted with an HP7673 autoinjector. The samples (~10 μl in 1.5 ml *n*-hexane) were applied as 1 μl splitless injections at 250°C to an HP5 (5% polysiloxane), 0.32 mm i.d., 0.25 μm film thickness, 30 m capillary column with He as the carrier gas, flowing at 1 ml min^{-1} . The column was held at 50°C for 2 min, increased at 10°C min^{-1} , held at 100°C for 2 min, increased at 2.5°C min^{-1} , held at 150°C for 2 min, increased at 5°C min^{-1} , and finally held at 300°C for 1 min. The GC effluent was fed to an HP5972 mass-selective detector at 280°C with *m/z* range of 35-500 scanned at ~1 s^{-1} .

Later, samples were analysed as above but using an Agilent 6890 GC with a 7683 injector and a 5973 MSD. The column was identical apart from the internal diameter (0.25 mm) and samples were applied as a 1 μl 1:10 split to avoid overloading the column. The oven program was unchanged.

4.2 Gas chromatography of unused cable oil

A typical gas chromatogram of fresh cable oil is shown in Figure 4.1. The abscissa is time so components to the left of the chromatogram are eluted first. A total of 19 LAB peaks are resolved, in four groups. The first group of isomers to be eluted are those with an alkane chain of length 10 ($10C_m$ homologues), followed by 11, 12 and 13. Within each group, the internal isomers are eluted earliest. The peaks are labelled accordingly. The terminal isomers are not found in the commercial mixture being studied, but $1C_{12}$ LAB was used as an internal standard to allow peaks to be quantified and this homologue is eluted in the interval between $3C_{13}$ LAB and $2C_{13}$ LAB. It did not prove possible to fully resolve the two most external isomers of $13C_m$ LAB, but examination of mass spectra from either end of the peak designated $7+6C_{13}$ LAB confirms that this is a compound peak.

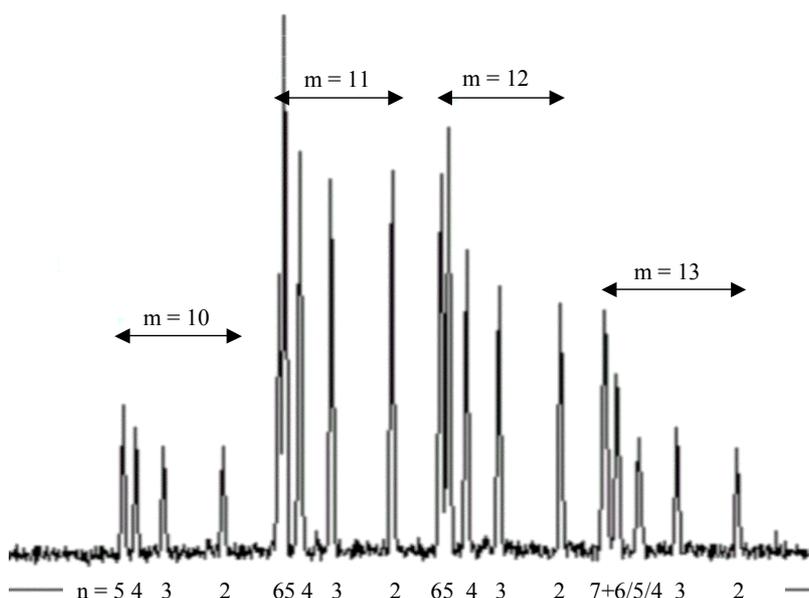


Figure 4.1 Typical gas chromatogram of cable oil showing resolution of individual homologues of form nC_m where n = length of alkyl chain and m = position of phenyl group. The $1C_{12}$ LAB internal standard (not shown) elutes between $3C_{13}$ LAB and $2C_{13}$ LAB.

4.3 Identification of GC peaks

The identities of each peak were positively identified by a combination of a search against two commercially available MS libraries (Wiley138 and NIST2000) and by direct examination of the mass spectrum of each peak.

The mass spectrum produced by a chemical species gives a number of clues to its identity. The major fragments produced by linear alkylbenzenes are shown in Figure 4.2. Mass fragments are generally referred to by the ratio of mass to charge, m/z . In the case of electron impact ionisation, as used in the instruments used in this project, ions almost always carry only a single electron so the m/z value is synonymous with the relative molecular mass.

The largest fragment seen in the mass spectrum of an LAB peak is usually the parent ion, which has an m/z equal to the molecular weight of the parent molecule (m/z 218, 232, 246, 260). In the case of LAB, this is not generally the most abundant, or base peak as monoaromatic molecules are susceptible to fragmentation at the bond α , and, where the alkane chain is greater than C_2 , β to the phenyl group (Kemp 1987). The resulting aromatic fragments are unstable and so undergo a variety of spontaneous reconfigurations. A rupture of the bond α to the benzene ring will result in a phenyl cation (m/z 77), though this is a minor component of LAB mass spectra, being more a feature of BTEX compounds.

The structure of LAB means that there are two bonds β to the phenyl group, and the parent ion may be fragmented at either or both of these bonds. If both bonds are

ruptured, then the fragment arising from the resulting toluene ion will be apparent as a tropylium ion at m/z 91 (Kemp 1987).

Fragments seen at m/z 105, 119, 133, 147 and 161, are presumably the result of only one β bond being ruptured. The m/z values for these fragments differ by 14 mass units (i.e., CH_2), but are one less than would be expected if the fragments did not undergo further rearrangement. The literature does not seem to include much in the way of explanation for the sizes of these fragments, though they are major components of the mass spectra of LABs. Depending on the length of the alkane chain, it is probable that these fragments represent intramolecular cyclisation products (Huynh *et al.* 2000) or alkenes. Figure 4.2 includes likely structures.

While not seen in the commercial mixture of LABs in common use, the terminal isomers of LAB are notable in that rupture of the only bond β to the ring results in a McLafferty rearrangement to form a methylenecyclohexadienyl radical ion (m/z 92). This is useful in confirming the identity of the 1C_{12} LAB internal standard.

The R groups that are lost are all *n*-alkanes and are seen at m/z 57, 71, 85 and 99, with the corresponding alkenes at m/z 55, 69, 83 and 97. Again, these differ from each other by simple multiples of 14.

This confirms the identity of individual peaks in the chromatogram with a high degree of certainty and the elution order can be used to identify peaks in

chromatograms obtained using other detectors so long as the chromatographic conditions are similar.

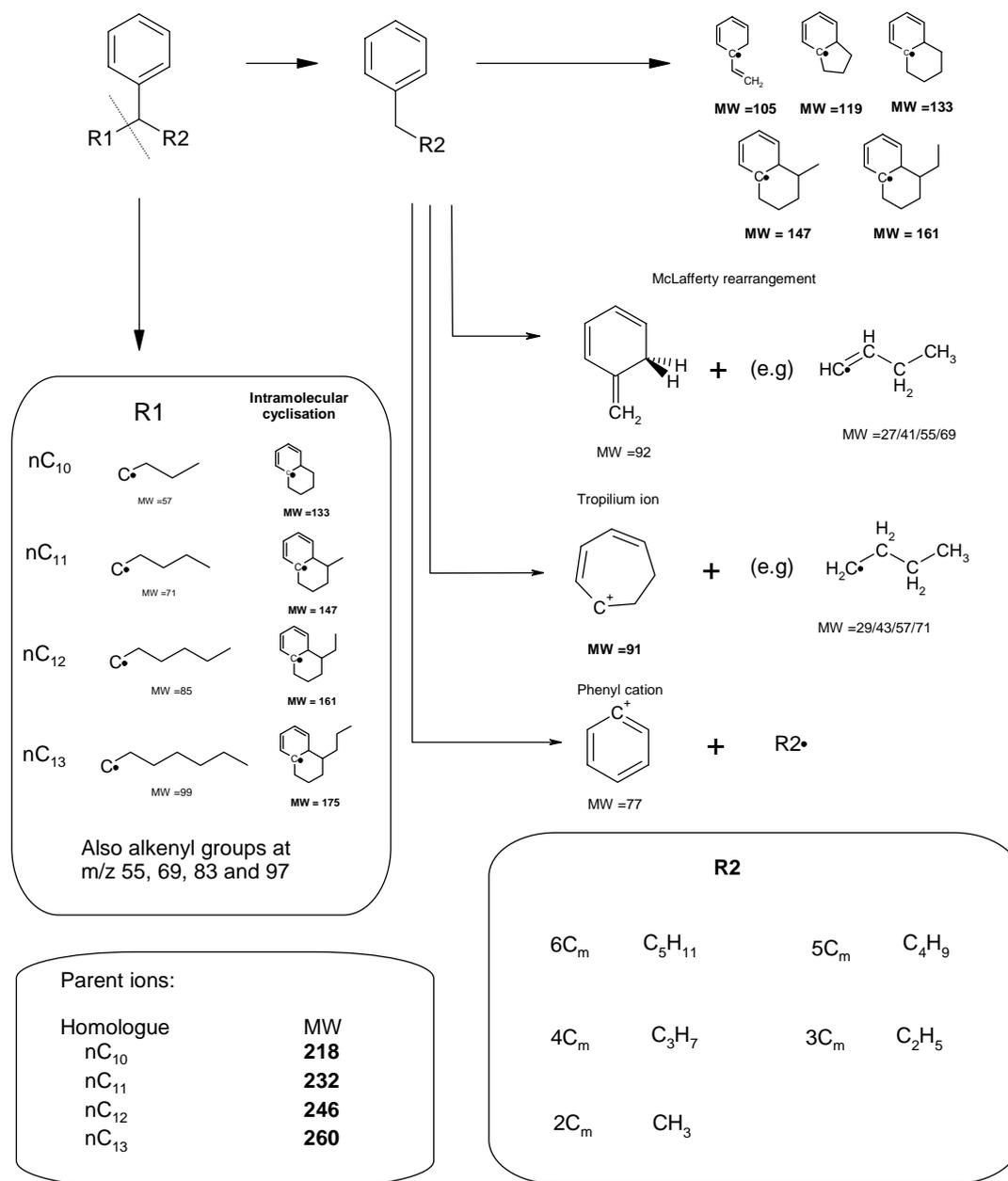


Figure 4.2 Summary of mass spectral fragments from LAB, indicating probable sources. Molecular weights in BOLD are diagnostic. Structures for MW = 105, 119, 133, 147, 161 and 175 are conjectural.

5 Toxicology

Environmental risk assessment in the UK is founded on the concept of a pollution linkage enshrined in Part II the Environment Protection Act (HMSO 1990) , the Environment Act (HMSO 1995) and most recently the Contaminated Land Regulations (England) (HMSO 2000).

Accordingly, for LAB to be considered a pollutant in soil, a pollution linkage must be identified. There may be a source such as a leaking cable or a reservoir of residual oil, and a possible pathway via groundwater, but without a susceptible target, the linkage is incomplete. The existence of a target implies an adverse effect and so it is necessary to demonstrate and if possible quantify this effect in order to arrive at a target value for remediation. Toxicity is probably the most obvious effect a chemical will have on living organisms.

5.1 Bioavailability of linear alkylbenzenes

In aquatic environments, LAB is readily accumulated by mussels (Widdows *et al.* 2002; Tsutsumi *et al.* 2002), though it is not known whether it is toxic to these organisms (Donkin *et al.* 1991). LAB has also been extracted from muscle tissue in fish (Venkatesan *et al.* 2002). In soil, the tendency of LAB to sorb to soil particles will reduce the availability of the material to organisms.

5.2 Toxicity of linear alkylbenzenes

As a high volume ($> 10^6$ t y^{-1}) product, the commercial mixture of C₁₀₋₁₃ alkyl derivatives of benzene has been evaluated in accordance with both the European Council regulation 793/93 of 23 March 1993 on the evaluation and control of the risks of existing substances (European Council 1993) and the voluntary USEPA High Production Volume Challenge Program (Heinze 2001a).

Unsurprisingly, given the major route taken by LAB to the environment (it has been estimated that some 2 kt y^{-1} of LAB is discharged into the sewage system in Europe (Hansen *et al.* 2000)), most published data relate to aquatic organisms (Gledhill *et al.* 1991). Fish have been observed in water with a visible layer of LAB on the surface with no observable ill effects. However, LAB is acutely toxic to *Daphnia magna* and is classified “N; R50 Very toxic to aquatic organisms”. Published toxicity data are summarised in Table 5.1.

Limited research has been done into the toxicity of LAB in soil. Unpublished data (P. Lee, pers. comm.) indicate that LAB does not cause any fatalities in earthworms exposed for 14 d to concentrations up to 1% dry weight soil (10 g kg^{-1}) in either sterile or non-sterile soil (OECD 1984).

For the purposes of labelling and classification of substances in Europe, Directive 67/548/EEC (European Council 1967), the “Dangerous Substances Directive”, and its subsequent amendments (European Commission 1993) defined environmental risk phrases (Table 5.2).

Table 5.1 Summary of available toxicity data for C₁₀₋₁₃ LAB. Sources cited in Heinze (2001b)

Organism	Value
Aquatic toxicity	
<i>Danio rerio</i> (FW zebra fish, 14 d static daily renewal)	LC50 > 0.0055 mg/l [1]
<i>Lepomis macrochirus</i> (FW fish 96 h static)	LC50 > 1000 mg/l [2]
<i>Leuciscus idus</i> (FW fish 48 h static)	LC50 > 1000 mg/l [3]
<i>Danio rerio</i> (FW zebrafish 21 d flow-through)	LC50 > 0.079 mg/l [4]
<i>Daphnia magna</i> (FW crustacean 48 h static)	NOEC > 0.013 mg/l [1] EC50 > 0.013 mg/l [1]
<i>Daphnia magna</i> (FW crustacean, 48 h) OECD Guideline 202, part 1 “ <i>Daphnia</i> sp. Acute Immobilisation Test”	EC50 > 0.04 mg/l [5] EC50 > 0.1 mg/l [4]
<i>Selenastrum capricornutum</i> (algae, 96 h) USEPA method 600/9-78-018 The <i>Selenastrum capricornutum</i> Printz algal assay	EC50 > 1000 mg/l [2]
<i>Scenedesmus subspicatus</i> (algae, 72 h)	EC50 > 0.1 mg/l [6]
Acute Oral Toxicity	
Rat (single oral dose, OECD Guideline 401)	LD50 > 5000 mg/kg body weight [7]
Rat (single oral dose)	LD50 > 10000 mg/kg body weight [8]
Acute Inhalation Toxicity	
Rat	LC50 > 1.82 mg/l [9] LC50 > 71 mg/l [10]

Organism	Value
Acute Dermal Toxicity	
Rat	LD50 > 2000 mg/kg body weight [11]
Repeated Dose Toxicity	
Rat (Sprague-Dawley strain, repeated dose toxicity) Inhalation 6 h/d, 5 d/week for 70 d	NOAEL = 102 ppm [12]
Rat (4 weeks, oral, daily in diet)	LOAEL = 125 mg/kg body weight [13]
Genetic Toxicity (<i>in Vitro</i>)	
<i>Salmonella typhimurium</i>	Negative [14] Negative [15]
Chinese Hamster Ovary (CHO) cells	Negative [15]
Genetic Toxicity (<i>in Vivo</i>)	
Rat (Sprague-Dawley strain) Bone marrow chromosome aberration assay	Negative [15]
Toxicity to Reproduction	
Rat (35 week, two generation study)	NOAEL Parental = 50 mg/kg body weight [15] NOAEL F1 = 50 mg/kg body weight [15]

Source:

- 1 - Calcinai, Cavalli, *et al.* (2001)
- 2 - Gledhill, Saeger, *et al.* (1991)
- 3 - Huls (1987)
- 4 - Fernandez, Alonso, *et al.* (2000)
- 5 - Verge, Bravo, *et al.* (1999)
- 6 - Moreno, Verge, *et al.* (2000)
- 7 - Huntingdon Research Centre (1984b)
- 8 - Younger Laboratories (1978)
- 9 - Monsanto Industrial Chemicals Company (1982)
- 10 - Unattributed, cited in Heinze (2001b)
- 11 - Huntingdon Research Centre (1984a)
- 12 - Monsanto Industrial Chemicals Company (1986)
- 13 - Monsanto, cited in Heinze (2001b)
- 14 - Bronzetti, Galli, *et al.* (1991)
- 15 - Robinson & Nair (1992)

To date, no criteria have been set for risk phrases not specifically affecting the aquatic environment (or the ozone layer), presumably due to the limited data that is available for terrestrial organisms. The European Centre for Ecotoxicology and Toxicology of Chemicals suggests that aquatic toxicity data may be of use in estimating soil toxicity (ECETOC 2002). This was on the basis that the primary source of exposure will be pore water and that generally bioavailability and thus toxicity of a given substance will be lower in soil than in water due to sorption, aging etc.

Table 5.2 Environmental Risk phrases (European Commission 1993)

Number	Description	Criteria
R50	Very toxic to aquatic organisms	$LC/EC50 \leq 1 \text{ mg l}^{-1}$ for fish, <i>Daphnia</i> or algae
R51	Toxic to aquatic organisms	$1 > LC/EC50 < 10 \text{ mg l}^{-1}$ for fish, <i>Daphnia</i> or algae
R52	Harmful to aquatic organisms	$10 > LC/EC50 < 100 \text{ mg l}^{-1}$ for fish, <i>Daphnia</i> or algae or evidence of danger to structure and/or function of aquatic ecosystems
R53	May cause long-term adverse effects in the aquatic environment	Not readily degradable in aquatic systems or $\log P_{ow} \geq 3.0$, unless $BCF \leq 100$ (special considerations for metals)
R54	Toxic to flora	Not defined
R55	Toxic to fauna	Not defined
R56	Toxic to soil organisms	Not defined
R57	Toxic to bees	Not defined
R58	May cause long-term adverse effects in the environment	Not defined
R59	Dangerous for the ozone layer	Substances covered by Council Regulation 594/91/EEC (European Council 1991)

5.3 Environmental toxicology using *Caenorhabditis elegans*

Nematodes perform an important role in the recycling of soil nutrients, and are present in great abundance in soils, where they live in pore water and feed on bacteria (Yeates & Bongers 1999). A number of species have been assessed for their usefulness as indicators of ecotoxicity, including *Cruzanema tripartitum* (Lau *et al.* 1997), *Caenorhabditis elegans*, *Panagrellus redivivus* and *Pristionchus pacificus* (Boyd & Williams 2003).

The commonly used free-living soil nematode *Caenorhabditis elegans* is very well characterised thanks to its use in studies of eukaryotic development (Lewis & Fleming 1995). There are both self-fertilizing hermaphrodite and male forms, with males being produced in response to environmental stress, presumably to increase the genetic variability of the population. It is easily cultured, and will produce large populations of genetically identical individuals in a short time.

Under conditions of low food availability or overcrowding, *C. elegans* is able to produce a non-feeding, almost dormant form known as a dauer larva. This is resistant to desiccation and extremes of temperature, and is effectively non-aging. The entire life cycle (Figure 5.1) takes 5.5 d at 15°C, 3.5 d at 20°C, and 2.5 d at 25°C. Above 25°C, the worms are sterile. Adults live for approximately two weeks but the incorporation of a dauer stage can extend the longevity of the worms markedly.

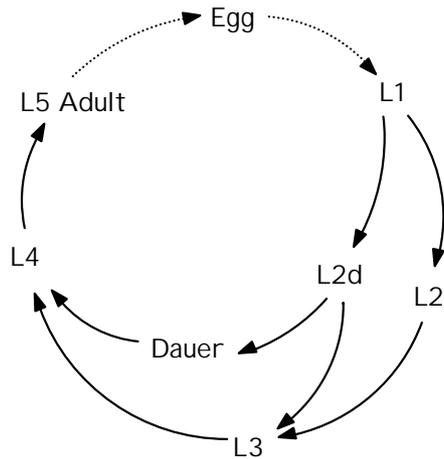


Figure 5.1 Life cycle of *Caenorhabditis elegans*. (L1 = First larval stage etc. Stages are marked by a moult of the epidermis and each stage differs from the previous one by size. Only L5 individuals are sexually mature).

C. elegans has been used for testing the toxicity of wastewaters (Williams & D. Dusenbery 1990; Cressman III & Williams 1997; Hitchcock *et al.* 1997) and for toxicity studies by exposing worms to potential toxins in cultures on agar plates (Kohra *et al.* 2002) and more recently in aquatic media (Ura *et al.* 2002). However, the lack of an effective means of recovering nematodes from soil without causing excessive stress to the animals meant that their use as a tool to investigate soil ecotoxicology was limited.

Two methods were commonly used for extracting nematodes from soil (Niblack & Hussey 1985). Sucrose flotation uses a dense sucrose solution to separate the worms from the matrix but the high osmotic potential renders many of the worms inactive. The Baermann funnel method, which relies on the tendency of soil nematodes to migrate downwards in saturated soil placed on a mesh support in a glass funnel and

collect in the liquid in the stem of the funnel, is a lengthy procedure, limited by the motility of the organisms, and it only recovers nematodes that are alive and moving.

Donkin and Dusenberry (1993) developed a differential density flotation method using colloidal silica that had all the advantages of sucrose flotation without the osmotic effects. The new method was used to derive concentration-response survival curves for copper in four soils. They went on to use the method to investigate the factors affecting the toxicity of zinc, cadmium, copper and lead in a variety of soils (1994).

The values of the LC50 (concentrations for which, statistically, 50% organisms tested would be expected to die) determined for *C. elegans* exposed to nitrate salts of cadmium, copper, zinc, lead and nickel in artificial soils for 24 h are similar to those determined for the earth worm *Eisenia fetida* (Peredney & Williams 2000). Other studies also point to *C. elegans* as an alternative to earthworm studies (Boyd *et al.* 2001), at least for heavy metal toxicity.

Some use has been made of *C. elegans* to assess hydrocarbon contamination on soils (Saterbak *et al.* 1999). More recently, *C. elegans* behaviour has proved to be good model for neurotoxicity studies (Williams *et al.* 2000; Anderson *et al.* 2004; Cole & Williams 2004).

Multiple endpoints have been identified for nematodes based on lethality, feeding, locomotion and reproduction (Dhawan *et al.* 1999; Anderson *et al.* 2001; Boyd *et al.*

2003). Effective monitoring of sub-lethal end points is most easily done using video analysis equipment and software (Dusenbery, D. 1996) and since this equipment was not available, and there was little time for method development, it was decided that mortality would be used.

The standard method E2172-01 (ASTM International 2001) was developed to evaluate the acute toxicity of substances in soil (Freeman *et al.* 1998; Freeman *et al.* 1999). It allows the study of the lethal or sub-lethal effects of chemical compounds on the nematodes. The method used here is based closely on this standard.

5.4 Materials and methods

5.4.1 Summary of experimental method

C. elegans were exposed to a range of concentrations of LAB in soil under carefully controlled conditions of moisture, pH and handling. The experimental protocol is summarised in Table 5.3.

Table 5.3 Timetable for soil toxicology test with *C. elegans*

Day	Operation
-7	<ul style="list-style-type: none"> • Prepare soil <ul style="list-style-type: none"> ○ add and mix soil ○ hydrate enough for at least 3 replicate treatments plus pH measurement • Place test containers in incubator and allow time to equilibrate
0	<ul style="list-style-type: none"> • Add 10 worms to each treatment using a flamed platinum wire
+1	<ul style="list-style-type: none"> • Rinse soil & worms into 50 ml centrifuge tube with Ludox • Centrifuge at 2000 rpm for 2 min • Leave for 15 min • Pour liquid into a 90-mm Petri dish • Transfer worms to NA plate with <i>E. coli</i> OP50 and score live/dead <ul style="list-style-type: none"> ○ Live = moving before or after gentle probing with platinum wire ○ Dead = no movement or not recovered.

5.4.2 Test organisms

A culture of *Caenorhabditis elegans* wild type strain N2 was obtained from Dr Bill Gregory at the Institute of Cell, Animal and Population Biology (ICAPB), The University of Edinburgh.

5.4.3 Preparation of media and solutions

5.4.3.1 Nutrient agar

C. elegans were cultured in sterile 90-mm disposable Petri dishes of nutrient agar inoculated with a lawn of *Escherichia coli* OP50, a uracil deficient strain also obtained from Dr Bill Gregory. The use of a strain with a nutritional deficiency ensured that bacterial cell numbers were low enough that dauer larvae were generated. Plates were inoculated and incubated at 37 °C overnight prior to use.

5.4.3.2 M9 buffer

M9 buffer (Table 5.4) was used to handle worms in bulk without the osmotic stress that would be imposed by using sterile water.

Table 5.4 M9 buffer (Brenner 1974)

Na ₂ HPO ₄	6.0 g
KH ₂ PO ₄	3.0 g
NaCl	5.0 g
MgSO ₄ .7H ₂ O	0.25 g
dH ₂ O	1000 ml

Dissolve solid components in the water and autoclave at 121 °C for 15 minutes

5.4.3.3 Alkaline hypochlorite solution

Alkaline hypochlorite solution for use in the preparation of synchronised cultures was prepared as shown in Table 5.5.

Table 5.5 Alkaline hypochlorite solution (to axenise 1 Petri dish)

Clorox bleach or equivalent (4-6% sodium hypochlorite)	2.0 ml
1 molar NaOH solution (1 g in 25 ml dH ₂ O)	5.0 ml
Store solutions separately and mix shortly before use	

5.4.3.4 Ficoll suspension

Ficoll 400 is used for the preparation of dauer larvae. As supplied, the density is too high and so it is diluted to 15% (w/v) in 0.1 molar NaCl (Table 5.6).

Table 5.6 Ficoll solution

Ficoll 400	15.0 g
0.1 molar NaCl solution	85.0 ml

5.4.3.5 Ludox suspension

In order to recover the test organisms from the soil matrix, a colloidal silica suspension (Ludox) was used. This was diluted to result in a colloid with a density greater than that of the worms, but less than the soil, without the osmotic pressure differential that would be caused by using saline or glucose solution of the same density. It was prepared by diluting 50 ml Ludox HS-40 colloidal silica with the same volume of distilled water. The solution was adjusted to pH 7 by the addition (c. 10 ten drops) of concentrated hydrochloric acid.

5.4.4 Manipulation of *C. elegans*

5.4.4.1 General maintenance

The dishes of worms were incubated at 20°C in a closed container lined with absorbent paper dampened with water to reduce evaporation. To maintain the stock culture nematodes were transferred to a fresh lawn of *E. coli* OP50. This was done by pipetting 1-2 ml M9 buffer onto the surface of the existing stock culture, shaking gently in order to suspend the worms in the buffer and pipetting the suspension onto the new plate. A fresh culture of nematodes was used for each test. Three cultures were kept at a time to ensure that sufficient individuals were available for all the tests.

5.4.4.2 Synchronised Cultures

Since *C. elegans* has markedly different resistance to environmental stress at different stages of its life cycle, the test animals were all taken from a synchronised culture of adult worms.

This method takes advantage of the fact that dauer larvae are effectively arrested at the L2 larval stage and so when they are revived they will all reach maturity simultaneously. Dauers were prepared from a nine-day culture. The worms were suspended in 3 ml M9 buffer from which 1 ml was removed and pipetted on top of 2 ml of Ficoll suspension (see Section 5.4.3.4) in a 15 ml centrifuge tube, taking care not to mix the two layers. The buffer floats on the Ficoll. The tube was left for 10 min, during which time the active adults migrated to the upper surface, while the relatively immotile dauer larvae sank into the lower layer. The top layer was

removed and the dauer larvae washed three times with distilled water by centrifuging (at 2000 rpm for 2 min and pouring off the supernatant. The dauer larvae thus obtained were placed on *E. coli* OP50 plates and incubated at 20°C for 48 h to mature.

After 48 h incubation, the adults were collected in 5 ml M9 buffer, which was then placed in a 15 ml centrifuge tube. They were washed twice with dH₂O and treated with 7 ml alkaline hypochlorite solution for 15 min. This solution kills the gravid adults and lyses the body wall to release the eggs contained inside. The tubes were vortexed briefly every 2 min during treatment with alkaline hypochlorite to ensure that the embryos did not die of oxygen deprivation. Eggs were harvested by centrifuging at 1000 rpm for 2 minutes and washing the pellet twice in 10 ml M9 buffer. The final pellet was resuspended in no more than 0.5 ml M9 buffer and the eggs transferred onto NA plates inoculated with *E. coli* OP50. These were incubated at 20°C for 4 d to obtain a synchronised culture of adult worms.

5.4.5 Test soils

5.4.5.1 Soil collection

Soil (Table 5.7) was collected for this investigation, as well as respirometric (Section 7.2.2.1) and soil column (Section 8.1.2) experiments, from the Bush Estate, Edinburgh by removing the turf and topsoil to a depth of approximately 20 cm. Subsoil was dug to a total depth of approximately 35 cm and transferred to clean plastic buckets. The topsoil and turf was replaced.

The subsoil was kept refrigerated in sealed polythene bags until required for use. It was also used in both the respirometric investigation (Section 7.2.2.1) and in soil column experiments (Section 8.1.2).

Table 5.7 Properties of soil used in toxicology, anaerobic respirometry and soil column experiments

Property	Value
pH	5.80
NH₄:N	20.3 µg ml ⁻¹
NO₃:N	42.2 µg ml ⁻¹
Dry matter	80.26 %
Total organic carbon (TOC)	5.8 %
Particle size distribution	
Sand (2 to 0.6 mm)	4.6 %
Sand (0.6 to 0.2 mm)	14.1 %
Sand (0.2 to 0.06 mm)	25.2 %
Coarse silt	22.9 %
Medium Silt	9.5 %
Fine Silt	6.8 %
Clay	16.5 %
Particle Density	2.62 g cm ⁻³
Bulk Density	1.56 g cm ⁻³
Gravimetric moisture at saturation	0.29 g g ⁻¹
Volumetric Moisture - at saturation	0.42 cm ³ cm ⁻³
Porosity	0.43 cm ³ cm ⁻³
Water Holding Capacity	0.29 cm ³ cm ⁻³

5.4.5.2 Determination of moisture content

It is important to know the moisture content of the soil used. Not only is it necessary if contaminant concentration is to be related to dry weight of soil, but also the water content of the soil has an influence on efficiency with which LAB can be mixed with the soil. If the moisture content exceeds ~15 %, the soil forms clumps when mixed and the oil is not evenly distributed. Moisture content of the soil was determined by accurately weighing 3-g soil samples and drying at 105°C for 24 h or to constant weight.

5.4.5.3 Addition and mixing of LAB

Each test was carried out with a range of concentrations of LAB in soil up to 2 % w/w. All measurements were relative to dry weight of soil. The greatest concentration was mixed first, with lower concentrations being produced by diluting contaminated soil with uncontaminated soil in appropriate proportions.

Soil was first adjusted to 15 % moisture content to allow effective mixing with minimum dust production. Enough soil to carry out two tests was prepared at a time.

All apparatus that was exposed to the soil was autoclaved. The soil and oil were mixed in a Kilner jar using a custom-made mixer fabricated from 5 mm diameter stainless steel rod (Plate 5.1). Moisture content was then adjusted to 40 %. The soils were placed in polythene bags and stored in the dark at 4°C until required.



Plate 5.1 Apparatus used to mix soil and LAB

5.4.5.4 Verifying homogeneity of test soils

The efficiency of the mixing method was confirmed by taking multiple, 1-g samples and extracting the LAB by shaking in 2 ml hexane. This was quantified by GC-MS using $1C_{12}$ LAB as an internal standard, as previously described.

5.4.6 Test chambers

5.4.6.1 Soil

Three replicates of 2.33 g (dry weight) of each of six concentrations of LAB in hydrated soil (plus uncontaminated control soil) were placed in individual 35×10 mm (diameter \times depth) polystyrene Petri dishes. The soil surface was smoothed with a stainless steel spatula to facilitate the introduction of the nematodes. Once prepared, the containers were placed in a sealed container lined with absorbent paper dampened with water, this was placed in a 20°C incubator for a period of 7 d to allow the soils to equilibrate.

5.4.6.2 Test organisms

Each test chamber was populated with ten nematodes from 3 to 4 d-old synchronised cultures. Several handling techniques were tried. A pick was made by flattening a 0.46 mm diameter platinum wire. Once flattened, the wire was cut at an acute angle and mounted in a metal handle. The nematodes were removed under the microscope by lifting under the body (Plate 5.2). The wire was flame sterilised and allowed to cool between each transfer. Care was taken not to injure the worms during transfer. To reduce the possibility of the seed culture becoming desiccated, 0.5 ml M9 buffer was pipetted onto the surface of the agar. As necessary, this was supplemented with

additional buffer but care was taken not to add too much buffer since it proved difficult to capture the worms if they were not constrained by the thickness of the liquid film to move in just two dimensions.

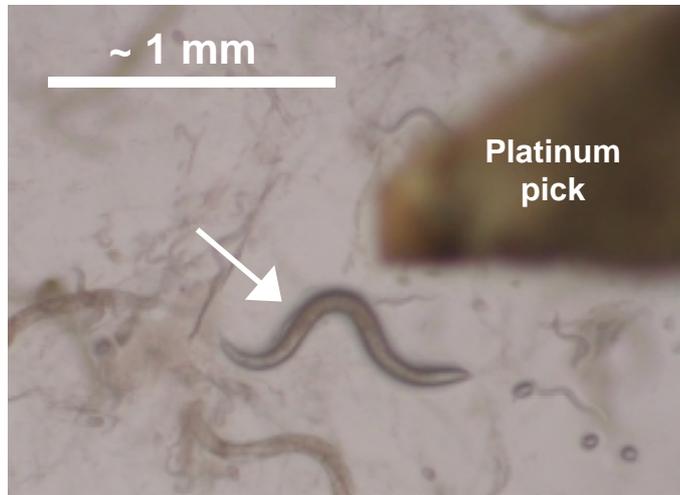


Plate 5.2 Handling of individual *Caenorhabditis elegans* (arrowed) using a platinum pick. Image shows an adult worm as well as larvae and eggs

For the first two tests, the method used was to select 10 nematodes from the plate one at a time and deposit them in a single well of a 96-well microtitre plate containing M9 buffer. The buffer and the nematodes were then removed using a 300 μ l pipette and deposited in the test container. This method proved unsatisfactory: the additional buffer increased the moisture content of the soil and it was impossible to verify that the nematodes were alive when they arrived in the test chamber. Examination of the pipette tips revealed that some of the nematodes remained in the tip and therefore not all the containers contained 10 organisms.

In subsequent tests, the soil was lit from above using a fibre optic light source and individual worms were placed directly on the soil surface under a dissecting

stereomicroscope (Plate 5.3). The nematodes were observed to ensure that they burrowed into the soil. Where there was any doubt regarding the health of a worm, it was removed from the soil, the pick was flamed and a new worm was transferred.

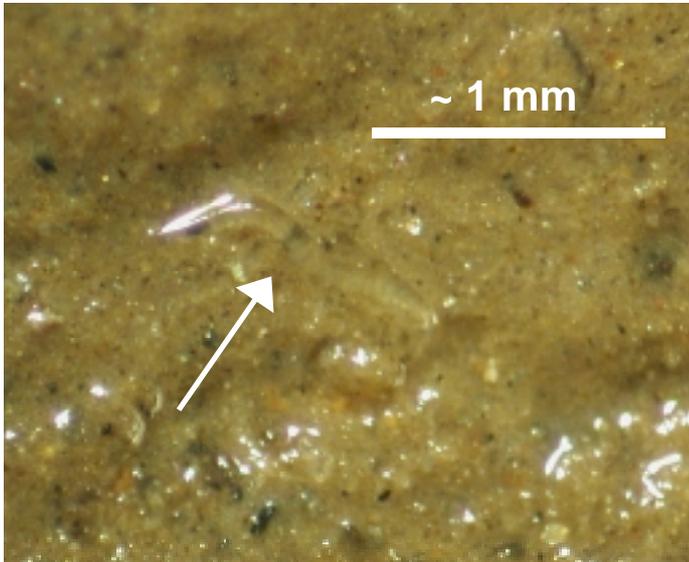


Plate 5.3 Adult *Caenorhabditis elegans* (arrowed) on soil

The test chambers were placed in a closed container lined with damp absorbent paper and incubated for 24 hours in the dark at 20°C.

5.4.7 Retrieval of test organisms

Test organisms were recovered by differential density flotation in colloidal silica (Ludox). The contents of each test chamber were transferred to a clean 50-ml centrifuge tube. The bulk of the soil was carefully transferred using a clean spatula. Care was taken to avoid damaging the worms. Soil adhering to the test chamber was rinsed with the Ludox suspension. Initially, 5 ml Ludox suspension was used, but it proved difficult to find all the test organisms in this volume in a reasonable time so

this was reduced to 2 ml in later tests. The tubes were capped and vortexed gently in order to suspend the soil. They were centrifuged at 2000 rpm for 2 min, then the tubes were removed and left to settle for 15 to 30 min in order to allow the nematodes to migrate to the liquid surface.

5.4.8 Scoring

The liquid was withdrawn using a 5-ml pipette and placed in a clean Petri dish with a grid ruled on the base to facilitate the search for test organisms. It was inspected under a stereomicroscope and any worms found removed using a 200- μ l pipette. If the worms were seen to be moving before removal, they were counted as living. If there was any doubt, they were deposited on a lawn of *E. coli* OP50 to rule out the possibility that their immobility was due to a lack of food. If after several minutes the worms did not react to gentle stimulation of the anterior end with a platinum wire they were counted as dead. Any worms not recovered are also counted as dead. For exposures of 24 h, the dead worms were not likely to have decomposed and so at least 80% of the test organisms were recovered.

5.4.9 Calculations

The mortality for each of the three replicates of each treatment was summed and the LC50 (*C. elegans*, 24 h) was calculated by linear interpolation (Norberg-King 1993), trimmed Spearman-Kärber (Hamilton *et al.* 1977; Hamilton *et al.* 1978) and Probit analysis (Finney 1964). All were calculated using software from the USEPA web site (Environmental Protection Agency 2003) as described below.

5.4.10 Verification of the LAB content of the soils

The LAB content of the soil remaining in the centrifuge tubes was verified by GC-MS of a hexane extraction with 1C₁₂ LAB internal standard. This was done both to verify the relative oil contents of the different concentrations and, by analysis of the isomeric composition, to ensure that no appreciable biodegradation had occurred during the equilibration period (rationale and method described in Chapter 6).

5.5 Results

5.5.1 Quality control

The experiment was carried out several times to develop the methods. Two of the experiments (Nos. 3 and 6) yielded data with 80 % recovery of test animals and with a clear increase of mortality with concentration. Mortality in the negative control treatments (0 % LAB) was higher than the 10 % recommended in the standard method (ASTM International 2001) but it was not possible to improve on this in the time available. The oil content and isomeric composition were analysed by GC-MS (Figure 5.2) to verify that the soil samples were homogeneous. LAB recovery was 71.3% (standard deviation = 6.99, n = 9).

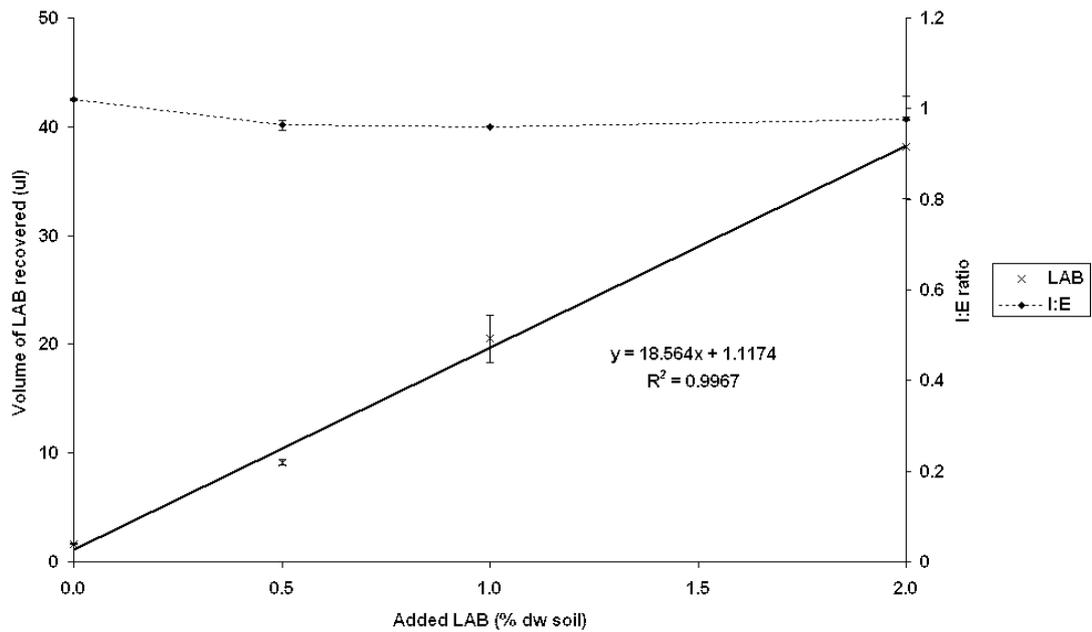


Figure 5.2 Verification of oil content and isomeric composition. Error bars = 1 SD, n = 3

5.5.2 Recovery and mortality

Two raw data from Experiments 3 and 6 are presented in Figure 5.3 and Figure 5.4.

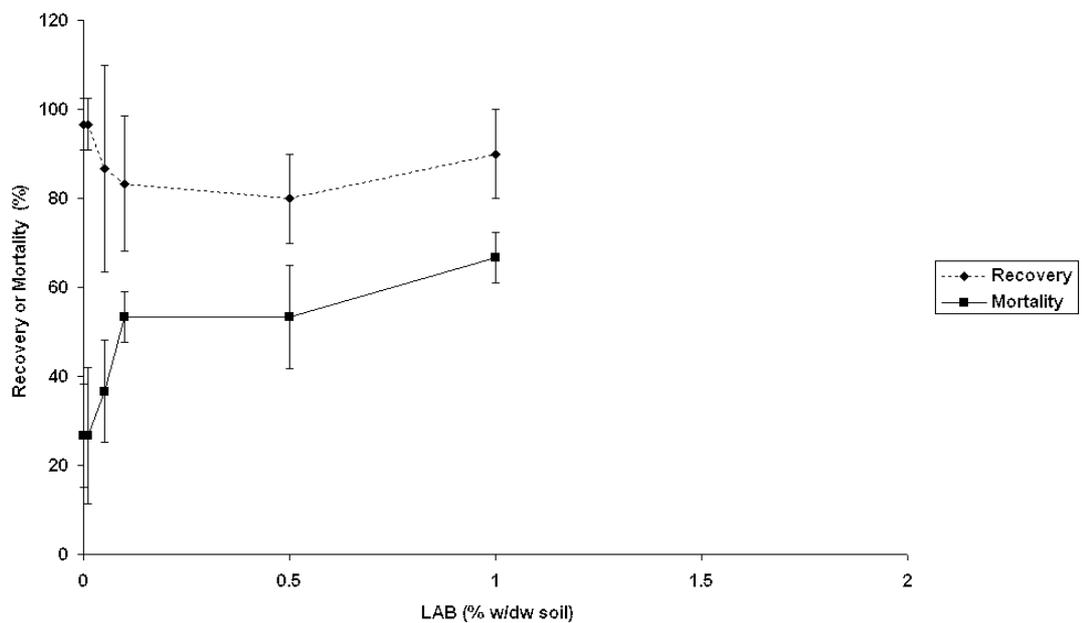


Figure 5.3 Experiment 3 - Plot of recovery and mortality vs. LAB % dry weight soil. Error bars = 1 SD, n=30

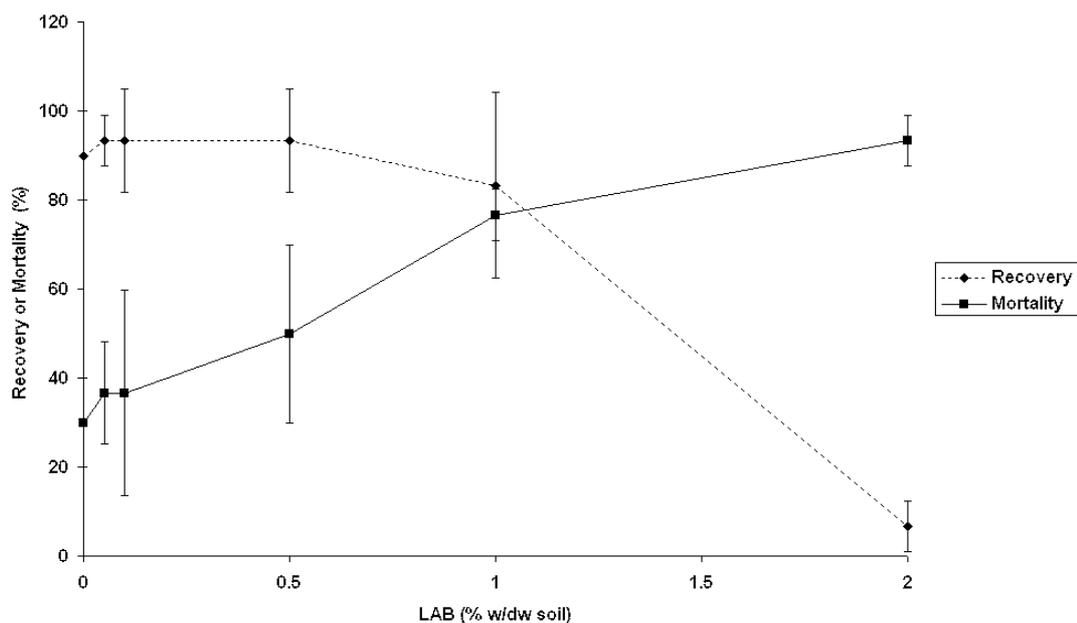


Figure 5.4 Experiment 6 - Plot of recovery and mortality against LAB % dry weight soil. Error bars = 1 SD, n = 30

5.5.3 LC50 by trimmed Spearman-Kärber method

This was calculated using the MS-DOS program “tsk.exe” obtained from the USEPA web site (Environmental Protection Agency 2003). The trimmed Spearman-Kärber method gives a single median concentration with 95% confidence limits.

Data from the three replicates were pooled to give mortality out of 30 for individual experiments and either 30 or 60 for pooled data from both experiments 3 and 6. The LC50 value calculated from the pooled data was 0.52% with a 95% confidence that the true value lay between 0.405 and 0.68%. These data are compared to the other methods in Section 5.5.6 and raw output from the program is reproduced in the Appendix.

5.5.4 LC50 by Probit method

Survival data from replicate treatments were pooled to give figures out of 30 (or 60) and input to the USEPA MS-DOS program “probit.exe” as per the user guide. See Appendix for raw data.

Probit analysis yields concentrations with a range of lethality. The response is plotted in Figure 5.5. This illustrates the inaccuracy seen at the extremities where the data does not cover a wide enough range (e.g. Experiment 3). The LC₅₀ data are plotted in Figure 5.6 for comparison. The LC₅₀ value calculated by this method is 0.846% with a 95% confidence that the true value is between 0.57% and 1.10%

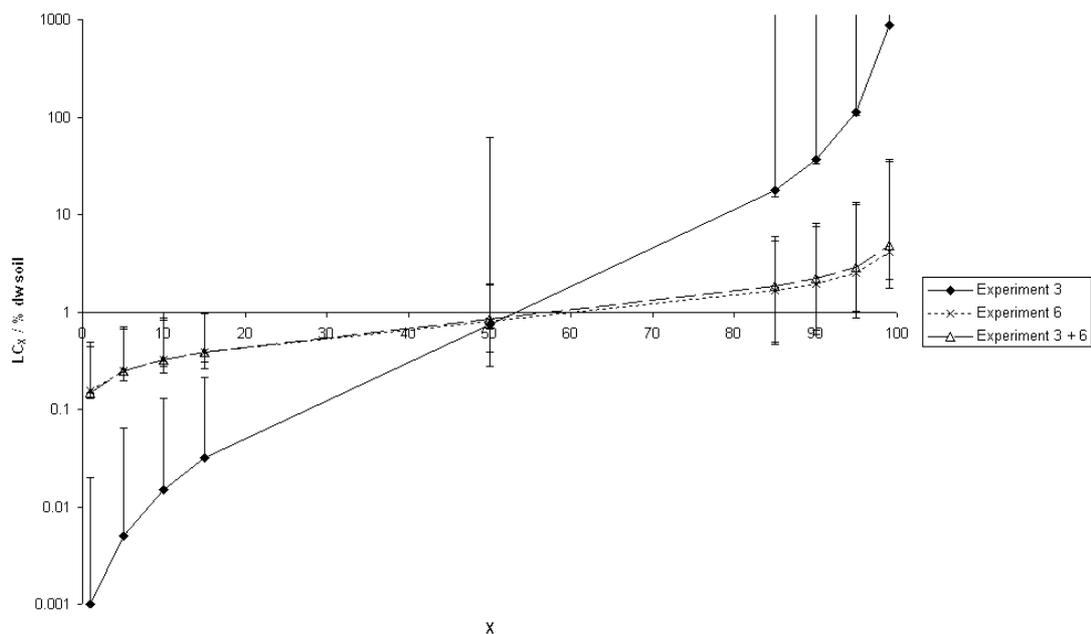


Figure 5.5 Concentration of LAB required to kill X % of a population of *C. elegans*. Calculated by Probit analysis. Error bars = 95 % confidence intervals, n = 180 (individual experiments) or 360 (pooled data). Upper 95 % confidence limits for mortalities above 80 % for experiment 3 are effectively infinite.

5.5.5 LC50 by linear interpolation

Survival data for individual replicates were input into the MS-DOS program “icpin.exe” from USEPA according to the user guide. This was done for individual experiments and for all data generated in Experiments 3 and 6. In no case was there more than six replicates and so “expanded confidence limits” were used to indicate the spread of the data, as detailed in the user manual (Norberg-King 1993).

Like the trimmed Spearman-Kärber method, this program yields a single median value. The pooled data from Experiments 3 and 6 give an LC50 value of 0.78% with lower and upper 95% confidence limits of 0.50% and 0.96% , respectively. All the data are plotted in Figure 5.6 and the program output is reproduced in the Appendix.

5.5.6 Comparison of LC50 by different methods

Figure 5.6 shows the concentration of LAB in soil required to kill 50% of a population of *C. elegans*, as calculated by a variety of commonly used methods. There were no significant differences between the different methods or between experiments. The upper 95% confidence interval for the probit analysis was rather high (61%) and is not shown in full in order to keep the y-axis at a meaningful scale. The linear interpolation program was unable to generate 95% confidence intervals for experiment 3.

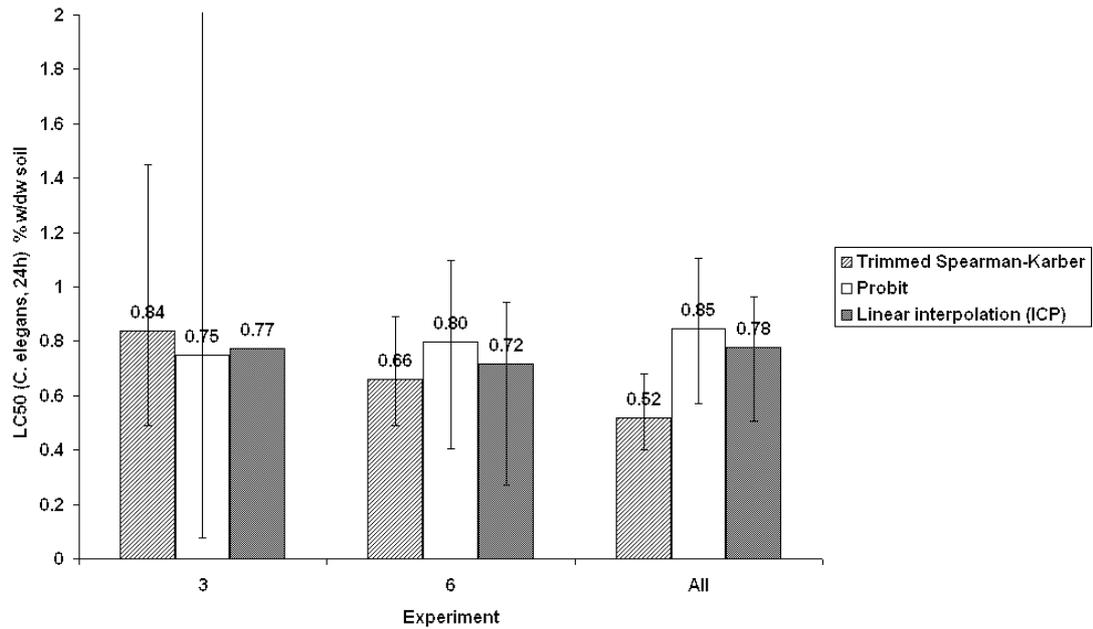


Figure 5.6 Comparison of LC50 (24h, *C. elegans* in soil) calculated by different methods. Data from experiments 3, 6 and pooled data. Error bars = 95% confidence intervals, n = 180 (for individual experiments) or 360 (pooled data).

5.6 Discussion

As an endpoint for a toxicity assay, death is of limited usefulness. It is relatively easy to identify but it does not give any information about the long-term effects of sub-lethal concentrations on the soil community. Nevertheless, LC50 remains the most commonly used measure of environmental toxicity.

C. elegans provides an increasingly popular model for toxicology in soil. Its small size and hence large surface area to volume ratio means that it is sensitive to contaminants over a much shorter timescale than, for instance, earthworms. However, this small size does have implications for handling the animals and this was evident in the high mortality seen in control populations, especially in early

experiments. It is likely that further practice and method development would reduce this to a more appropriate level.

That there was no significant difference between the three methods used to calculate the median lethal concentration suggests that any one could have been used. However, the methods do have specific limitations that will make them more or less suitable.

Linear interpolation is the oldest method, but it assumes a linear relationship whereas the actual relationship is generally sigmoid. It is most accurate in the middle of the curve but can be inaccurate if there is not a wide enough range of responses in the data set. This was obvious in experiment 3 where it was not possible to generate meaningful 95% confidence intervals.

Probit analysis assumes a sigmoid relationship between dose and effect and yields a range of effective concentrations. Accuracy declines rapidly with limited data, illustrated by the large 95% confidence intervals at the upper extremity of the plot from experiment 3, however accuracy is good in the middle of the curve.

The trimmed Spearman-Kärber method only gives a median (LC50) value but is the most accurate method if relatively few data are available. The only assumption made about the dose-response curve is that distribution of tolerance to the contaminant is symmetrical around the median value for the population. It can generate a value with

as few as two data points between 0 and 100% mortality and is relatively insensitive to anomalous results.

Since there was no significant difference between the LC50 calculated from the two successful experiments, it was felt that it would be appropriate to pool the data to obtain a more accurate estimate.

5.7 Conclusions

The most conservative estimate, as well as the narrowest confidence interval, is given by pooling all the available data and applying the trimmed Spearman-Kärber method. Using this method, the LC50 for LAB in soil is calculated to be 0.52% by dry weight of soil (5200 mg kg⁻¹), with 95% confidence that the actual value lies between 0.40% and 0.68% (4000-6800 mg kg⁻¹). While this is high compared to the ranges given for aquatic toxicity (Table 5.2), the greater heterogeneity of soil means that bioavailability will be much lower. It might be argued that the Dutch level indicating serious contamination of soil at 1 g kg⁻¹ (Pronk 2000) is a little high.

6 Aqueous culture

Linear alkylbenzenes (LAB) are common environmental contaminants associated with a number of industrial and domestic activities. Although natural environments receiving LAB may exhibit a range of redox conditions, until now only aerobic biodegradation of these compounds has been conclusively demonstrated, although other alkylbenzenes, notably toluene and ethylbenzene, have been seen to be degraded with a variety of terminal electron acceptors.

Where LAB contamination occurs it is important to identify degradation and measure its extent in order to make decisions on whether monitored natural attenuation (intrinsic bioremediation) is sufficient to eliminate the pollution, or whether active remediation techniques are required.

6.1 Introduction

Linear alkylbenzenes (LAB) have been produced commercially since the early 1960s for use in a variety of industries. The synthetic production pathway results in a mixture of isomers with a variety of alkyl chain lengths, dependent on the feedstock, with the phenyl group in any but the terminal position (Swisher *et al.* 1961). Conventionally, these isomers are described using the form nC_m LAB, where n = position of the benzene ring and m = number of carbon atoms in the longest aliphatic chain. For instance, using this convention, (1-methyl, undecyl)-benzene (also known as 2-phenyldodecane or dodecyl-2-benzene) is $2C_{12}$ LAB.

Mixtures with an alkyl chain length of C₁₀₋₁₃ (see Figure 6.1 for relative abundance of individual homologues) are used to insulate buried electrical transmission cables.

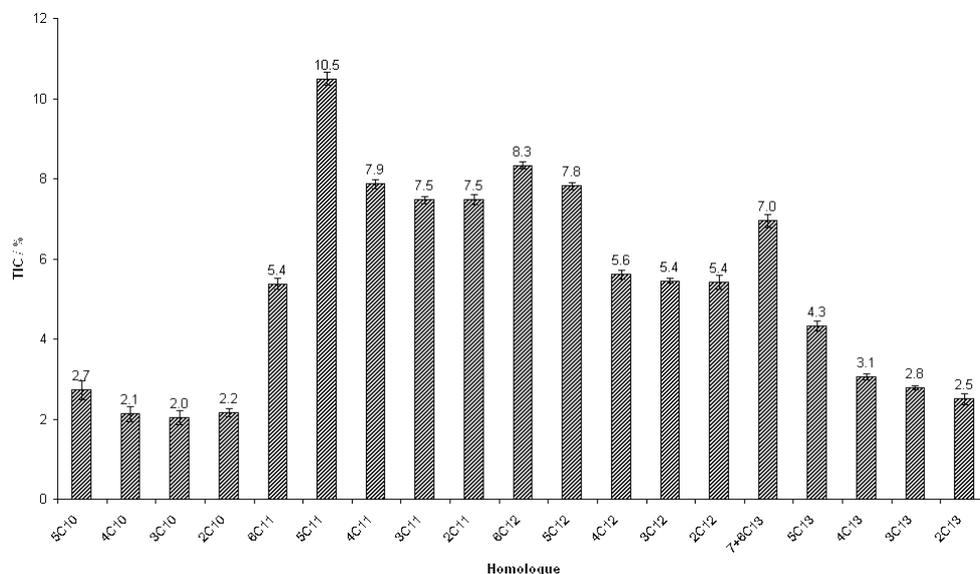


Figure 6.1 Relative abundance of individual homologues of LAB found by GC-MS of typical undegraded cable oil (author's own data, error bars = 1 standard deviation, n = 33).

Leakage of this cable oil may occur due to deterioration of cable components or through mechanical damage. When a leak is detected, contaminated soil is excavated to effect a repair to the cable, but oil that has migrated away from the immediate vicinity of the cable may be inaccessible, e.g. due to surface infrastructure or other buried services. Monitored natural attenuation is an attractive, and perhaps the only financially viable, strategy in such circumstances.

Similar mixtures of C_{10-13/14} LAB are also used in the manufacture of linear alkylbenzenesulphonate (LAS) detergents. Trace amounts remain in the detergent and thus are found in aquatic sediments where LAS contamination occurs. For the

same reason, LAB is also seen in sewage sludge used to amend soils (Mangas *et al.* 1998). LAB was used for a period in the early 1990s as a major component of drilling oil (Steber & Herold 1995; M-I. *LLC* 1998), and has been identified as occurring naturally in the *n*-hexane fraction of some crude oils (Ellis *et al.* 1996; Dutta & Harayama 2001).

It has been shown that internal (phenyl position > 4) isomers of LAB are more resistant to microbial degradation than external (phenyl position ≤ 4) ones (Bayona *et al.* 1986). This preferential degradation has been demonstrated both in the field and using defined bacterial cultures including a *Pseudomonas* sp. (Bayona *et al.* 1986) and *Nocardia amarae* (now *Gordonia amarae*) (Bhatia & Singh 1996).

Aerobic degradation mechanisms suggested by Bhatia and Singh (1996) all start with an ω-oxidation of a terminal methyl group, followed by β-oxidation of the alkyl chain. This was in agreement with the pathways seen elsewhere (Smith & Ratledge 1989; Smith 1990) for the degradation of terminal LAB isomers, where initial attack was at the methyl terminus of longer chains, but on the phenyl group if the chain was shorter.

The relative amounts of different homologues of LAB in environmental samples can be used as an indicator of biodegradation. A commonly used predictor is the ratio of internal (I) to external (E) isomers of nC₁₂ LAB (Takada & Ishiwatari 1990; 1991), calculated as I:E = (6C₁₂ LAB + 5C₁₂ LAB)/(4C₁₂ LAB + 3C₁₂ LAB + 2C₁₂ LAB). It has been assumed that significant degradation only occurs under aerobic conditions

(Holt & Bernstein 1992). However, indications that anaerobic degradation of LAB may occur (Eganhouse *et al.* 2000; Johnson *et al.* 2001) have prompted investigations of cable oil biodegradation where the LAB is exposed to a variety of terminal electron acceptors (TEA).

There are advantages to restricting the measure of isomeric composition to a single chain length:

- (i) the number of external isomers is fixed at three for all homologues, but as alkyl chain length increases, so does the number of possible internal isomers, so different chain lengths cannot be compared directly; and
- (ii) C₁₂ LAB isomers are present as major components in all currently available commercial LAB mixtures (C₁₀₋₁₄ and C₁₀₋₁₃) and have been used in other work (Takada & Ishiwatari 1990), allowing comparisons to be made.

There is a potential disadvantage to using a ratio, in that if all the external isomers are depleted, the ratio will tend to infinity. However, the inclusion of 4C₁₂ as an external isomer means that the C₁₂ I:E ratio in fresh LAB is close to unity and large values of the I:E ratio will only be approached at very high degrees of degradation. In addition, the use of a logarithmic scale both reduces the effect of this limitation and helps to linearise the resulting plot, making the regression a satisfactory predictor of biodegradation. Data from aqueous cultures of soil micro-organisms containing

LAB cable oil as the sole carbon and energy source with a variety of electron acceptors is presented, and the linear regressions are compared using statistical methods.

6.2 Materials and methods

6.2.1 Batch cultures

Universal bottles containing 20 ml of Bushnell-Haas (B-H) broth (1.0 g KH_2PO_4 , 1.0 g K_2HPO_4 , 1.0 g NH_4NO_3 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g FeCl_3 (as 5 ml of 10 g l^{-1} stock solution), 0.02 g CaCl_2 , 1000 ml dH_2O (Atlas 1993)) plus a variety of terminal electron acceptors were autoclaved. 100 μl filter-sterilised LAB cable oil was added as the sole carbon source. Cultures were inoculated with 100 μl of a suspension of organisms cultured from cable oil-contaminated soil (Johnson *et al.* 2001).

In addition, a freeze-dried culture of the Gram-negative *Geobacter metallireducens* strain G-15 isolated from river sediments (Lovley *et al.* 1993), was obtained from the German Collection of Microorganisms and Cell Cultures (Cat. No. 7210) and cultured in Bijou bottles containing *Geobacter* medium 579 (Table 6.1) with and without LAB. *Geobacter* has been shown to be able to anaerobically oxidise benzoate, toluene, phenol, and p-cresol (Lovley *et al.* 1989; Lovley & Lonergan 1990) using iron, manganese (Lovley & Phillips 1988) and uranium (Lovley *et al.* 1991) as TEAs. It has been shown to produce magnetite (Lovley *et al.* 1987) and is considered to be especially significant in the degradation of hydrocarbons in soil due to its ability to mobilise insoluble Fe(III) (Childers *et al.* 2002).

6.2.2 Inoculum

The original soil inoculum had been enriched in Bushnell-Haas broth for several months with LAB as the sole carbon source and a variety of electron acceptors. Appreciable growth only occurred at the LAB:water interface with nitrate as the TEA and so the organisms were cultured anaerobically for seven d in Casein/Peptone/Starch/Nitrate (CPSN) broth (1 ml glycerol, 2 drops 0.01% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.5 g peptone, 0.5 g casein hydrolysate, 0.5 g soluble starch, 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g K_2PO_4 , 1000 ml dH_2O , pH 7). The appearance of the enrichments after incubation is shown in Plate 6.1



Plate 6.1 CPSN enrichments from ANA (left) and MET (right) cultures. Blue colour and gas in Durham tube and at liquid surface indicates nitrate reduction

This was then centrifuged to separate bacterial cells. The medium was poured off and replaced with B-H broth. Cells were re-suspended by vortexing. The cells were washed three times in this way to avoid introducing LAB or additional nutrients into

the experimental cultures. The resulting washed cell suspension was used to inoculate the experimental bottles.

G. metallireducens was rehydrated and cultured anaerobically in *Geobacter* medium (Table 6.1) for approximately four months before being used to inoculate the experimental systems.

6.2.3 Experimental systems

6.2.3.1 Enrichment cultures

Aerobic (AER) and control (CON) bottles were incubated in a shaker/incubator at 25°C. Other cultures containing either no additional TEA (MET), nitrate (NIT), sulphate (SUL) or both nitrate and sulphate (ANA) were loosely capped and incubated at 25°C under an 85% N₂/10% CO₂/5% H₂ atmosphere in a National Appliance Co. anaerobic glove box (Model #3650). Added TEAs were calculated to be stoichiometrically 3-4 times more than that required for complete oxidation of the hydrocarbon. Sodium molybdate ($1.0 \times 10^{-2} \text{ mol l}^{-1}$) was added to the NIT cultures to inhibit sulphate-reduction (Newport & Nedwell 1988). Similarly, $1.0 \times 10^{-2} \text{ mol l}^{-1}$ sodium chlorate was added to inhibit nitrate reduction (Hynes & Knowles 1983) in the SUL cultures. The NIT and SUL cultures contained $2.5 \times 10^{-2} \text{ mol l}^{-1}$ 2-bromoethanesulfonic acid (BES) to inhibit methanogenesis (Löffler *et al.* 1997). The CON bottles contained both additional nitrate and sulphate, but no inhibitors.

Table 6.1 *Geobacter* medium 579 (German Collection of Microorganisms and Cell Cultures)

Fe(III) citrate	13.7 g
NaHCO ₃	2.5 g
NH ₄ Cl	1.5 g
NaH ₂ PO ₄	0.6 g
KCl	0.1 g
Na-acetate	2.5 g
Vitamin solution (see below)	10 ml
Trace element solution (see below)	10 ml
Na ₂ WO ₄ .2H ₂ O	0.25 mg
dH ₂ O	980 ml

Adjust pH to between 6.7 and 7.0. Prepare medium anaerobically under 80 % N₂ + 20 % CO₂ gas mixture. First dissolve ferric citrate in water and adjust to pH 6.0, then add other ingredients. Use 3 to 10 % inoculum.

Vitamin solution:

Biotin	2 mg
Folic acid	2 mg
Pyridoxine-HCl	10 mg
Thiamine-HCl.2H ₂ O	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
D-Ca-pantothenate	5 mg
Vitamin B12	0.1 mg
P-Aminobenzoic acid	5 mg
Lipoic acid (= DL-6,8-thioitic acid)	5 mg
dH ₂ O	1000 ml

Trace element solution:

Nitrilotriacetic acid	1.5 g
MgSO ₄ .7H ₂ O	3 g
MgSO ₄ .2H ₂ O	0.5 g
NaCl	1 g
FeSO ₄ .7H ₂ O	0.1 g
CoSO ₄ .7H ₂ O	0.18 g
CaCl ₂ .2H ₂ O	0.1 g
ZnSO ₄ .2H ₂ O	0.18 g
CuSO ₄ .2H ₂ O	0.01 g
KAl(SO ₄) ₂ .12H ₂ O	0.02 g
Orthoboric acid H ₃ BO ₃	0.01 g
Na ₂ MoO ₄ .2H ₂ O	0.01 g
NiCl ₂ .6H ₂ O	0.025 g
Na ₂ SeO ₃ .5H ₂ O	0.3 mg
dH ₂ O	1000 ml

First dissolve nitrilotriacetic acid and adjust pH to 6.5 with KOH then add minerals. Adjust final pH to 7.0 with KOH.

6.2.3.2 *Geobacter* cultures

Bijou bottles were set up with 1 ml of either complete *Geobacter* medium or with the Fe(III) citrate replaced by a molar equivalent of either Fe(III) chloride or KNO₃. The potassium nitrate was used to investigate whether *Geobacter* could also use nitrate as an electron acceptor. Each medium was then contaminated with 1 µl cable oil and/or inoculated with 100 µl of *Geobacter* culture. They were then incubated as described above.

6.2.4 LAB extraction

Three vials from each treatment were harvested and destructively analyzed at ten points over a period of 97 d. The contents of each universal bottle were poured into a 100 ml conical flask and the bottle rinsed twice with 10 ml *n*-hexane, which was added to the flask, along with a further 20 ml *n*-hexane. Immediately prior to the extraction step, 5 µl of pure 1C₁₂ LAB (1- phenyldodecane, Sigma Aldrich Catalogue Number 44178), a terminal isomer (and therefore absent from the synthetic mixture), was added to serve as an internal GC standard to allow the peak areas to be quantified. The conical flasks were sealed with catering cling-film to prevent spillage and evaporation of the solvent and the LAB was extracted by shaking vigorously for 20 min. A ~1.5 ml aliquot of the organic fraction was pipetted into a GC vial for analysis by GC-MS.

In the case of the *Geobacter* cultures, which were carried out as a separate investigation, extraction was carried out in the Bijou bottles using 0.1 µl of 1C₁₂ LAB standard and 2 ml hexane. After 20 min shaking, the bottles were centrifuged

briefly at 3000 rpm to separate the phases and 1 ml was taken from the organic fraction for analysis.

6.2.5 GC-MS Analysis

Separation was carried out on a Hewlett-Packard (Agilent) HP5890 Series II gas chromatograph fitted with an HP7673 autoinjector. The samples were applied as 1 μ l splitless injections at 250°C to an HP5 (5% polysiloxane), 0.32 mm i.d., 0.25 μ m film thickness, 30 m capillary column with He as the carrier gas at a flow rate of 1 ml min^{-1} . The column was held at 50°C for 2 min, increased at 10°C min^{-1} , held at 100°C for 2 min, increased at 2.5°C min^{-1} , held at 150°C for 2 min, increased at 5°C min^{-1} , and finally held at 300°C for 1 min. The GC effluent was fed to an HP5972 mass-selective detector at 280°C with m/z range of 35-500 scanned at $\sim 1 \text{ s}^{-1}$. *Geobacter* cultures were analysed as above but using an Agilent 6890 GC with a 7683 injector and a 5973 MSD. The column was identical apart from the internal diameter (0.25 mm). Samples were applied as a 1 μ l 1:10 split to avoid overloading the column. The oven program was unchanged.

Peaks in the total ion current (TIC) chromatograms of fresh LAB and control bottle extracts were identified as being identical by searching against the Wiley138 or NIST2000 mass spectrum library, and by examining the mass spectra directly. Peaks in the experimental bottles were identified by comparison to the control bottle chromatograms, with particular attention to retention times. Peak areas were compared to the 1C₁₂ LAB internal standard and summed to calculate the total volume of LAB recovered.

In this work, there were no interfering peaks and so total ion current provides a good measure of isomeric abundance, however in environmental samples containing other hydrocarbons it might be necessary to use single ion chromatograms (m/z 91 is indicative of $\geq 3C_m$ isomers, with m/z 105 diagnostic of $2C_m$ isomers. Identity can be confirmed by examining the parent ion).

Data from the experimental bottles were compared to the control bottles to discount removal of LAB via physical and chemical routes (sorption, volatilization, etc.). Loss from the control (CON) bottles, which were open to the atmosphere, was higher than anticipated, rendering them unsuitable for comparing to the sealed, anaerobic treatments. Anaerobic bottles (MET) containing no additional TEA, which were included to investigate whether methanogenic biodegradation occurred, showed no appreciable biodegradation and so were used as anaerobic controls.

6.2.5.1 Calculations

Peaks in the gas chromatogram for each sample were integrated using HP Chemstation software and their areas calculated as percentages of the sum of all the peaks. The volume of LAB recovered was calculated by summing the TIC peak areas due to LAB isomers and comparing to the $5 \mu\text{l}$ $1C_{12}$ standard (Equation 6.1).

$$[nC_m]_{\text{volume}} = \frac{[1C_{12}]_{\text{volume}} \times [nC_m]_{\text{peakarea}}}{[1C_{12}]_{\text{peakarea}}}$$

Equation 6.1 Volume represented by each GC peak

The ratios of internal (I) to external (E) isomers of nC₁₂ LAB were also calculated (Equation 6.2).

$$nC_{12}I : E = \frac{6C_{12} + 5C_{12}}{4C_{12} + 3C_{12} + 2C_{12}}$$

Equation 6.2 Calculation of nC₁₂ I:E ratio

6.2.6 Nitrate/nitrite analysis

Enrichment cultures were also analysed for nitrate and nitrite content. Following hexane extraction of LAB, samples of the aqueous fraction were diluted ($\times 10^{-2}$) and filtered through a Whatman GF/C glass microfibre filter, before further dilution for analysis. Triplicate 5 ml aliquots of each of three dilutions (10^{-2} , 10^{-3} and 10^{-4}) were assayed for total oxidised inorganic nitrogen (TON = NO₃ + NO₂) and nitrite. TON was measured by a modified hydrazine reduction method (Franson, Eaton, Clesceri, and Greenberg 1995) in which nitrate was reduced to nitrite, which was measured by diazotization with sulphanilamide and coupling with *n*-(1-naphthyl)-ethylenediamine dihydrochloride (NEDD). The resulting azo dye was measured colorimetrically at 535 nm using a Gilford Instrument Stasar II with a 1 cm path length flow through cell, and compared to appropriate standards. Nitrite was determined in the same way, but with the omission of the reduction step.

6.3 Results and discussion

6.3.1 Extraction efficiency

A variety of analytical methods has been used to analyze LAB degradation (Takada & Ishiwatari 1985; Eganhouse 1986; Hartmann *et al.* 2000). GC-MS has proved particularly sensitive (Hartmann *et al.* 2000) and the efficiency of the method is not heavily dependent on the details of extraction and GC methods. For soil, shaking in hexane has been shown to be up to 90% efficient, in terms of LAB extraction, over a range of soil types and moisture contents (Schwab *et al.* 1999; Fu *et al.* 2000). In any event, because a ratio is being measured, rather than an absolute amount, the extraction efficiency is not critical, so long as the extraction method is not selective for different isomers. Extraction efficiency, being the volume of LAB recovered from the anaerobic control (MET) bottles divided by the volume of LAB added at the start of the experiment, was 74 % (n = 33, SD = 12.3 %).

6.3.2 Enrichment cultures

Percentage recovery of LAB, and the corresponding nC₁₂ LAB I:E ratio from a range of enrichment cultures are shown in Figure 6.2 to Figure 6.7.

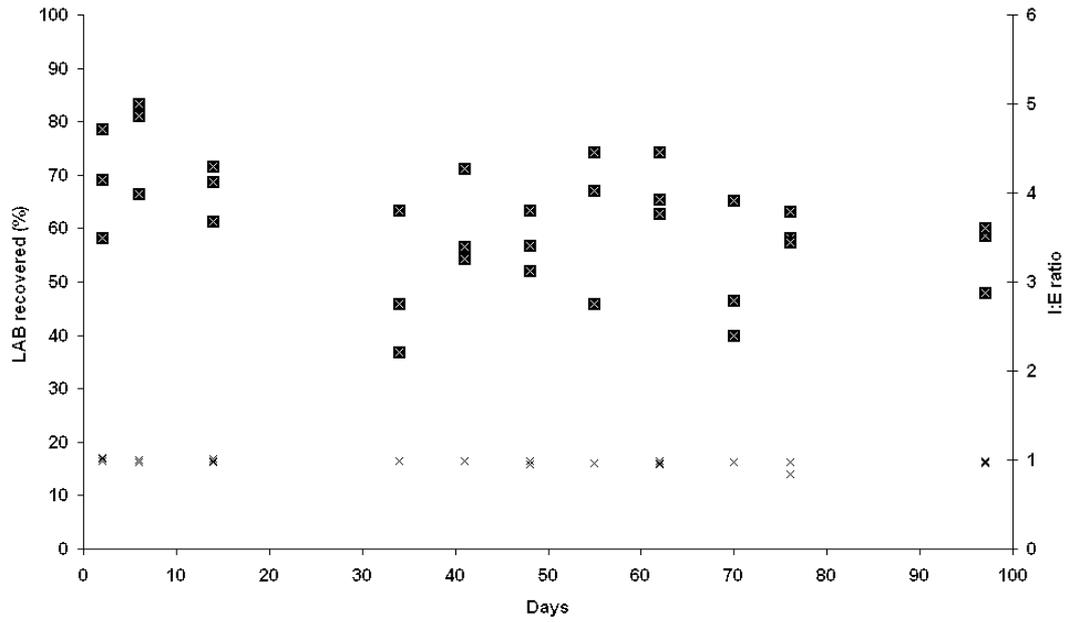


Figure 6.2 Amount of LAB recovered from individual CON cultures (filled markers) and I:E ratios (open markers) plotted against time.

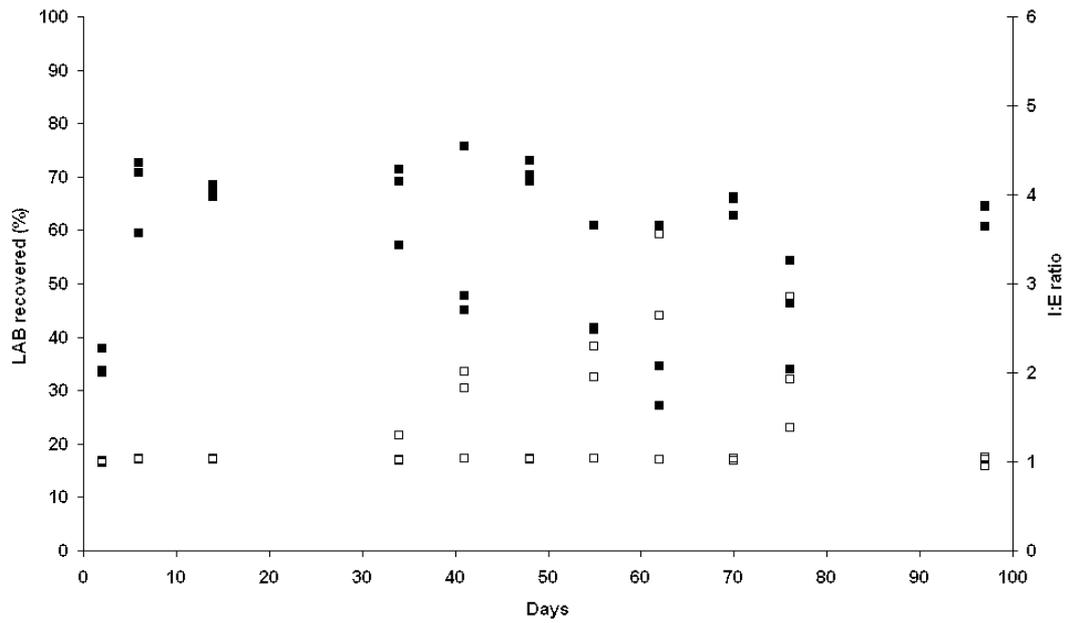


Figure 6.3 Amount of LAB recovered from individual ANA cultures (filled markers) and I:E ratios (open markers) plotted against time.

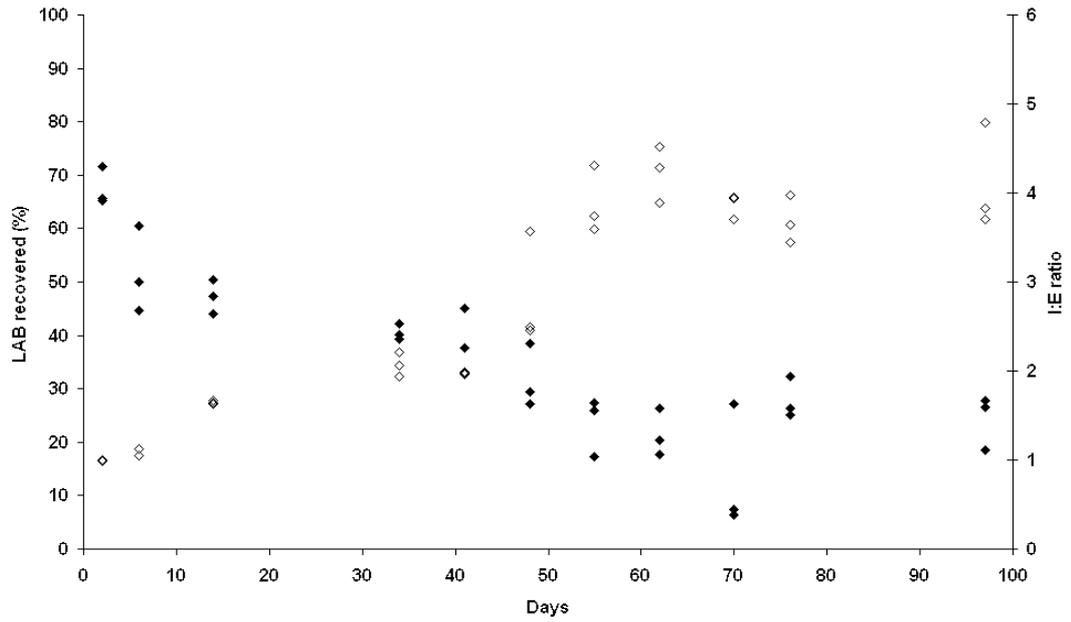


Figure 6.4 Amount of LAB recovered from individual AER cultures (filled markers) and I:E ratios (open markers) plotted against time.

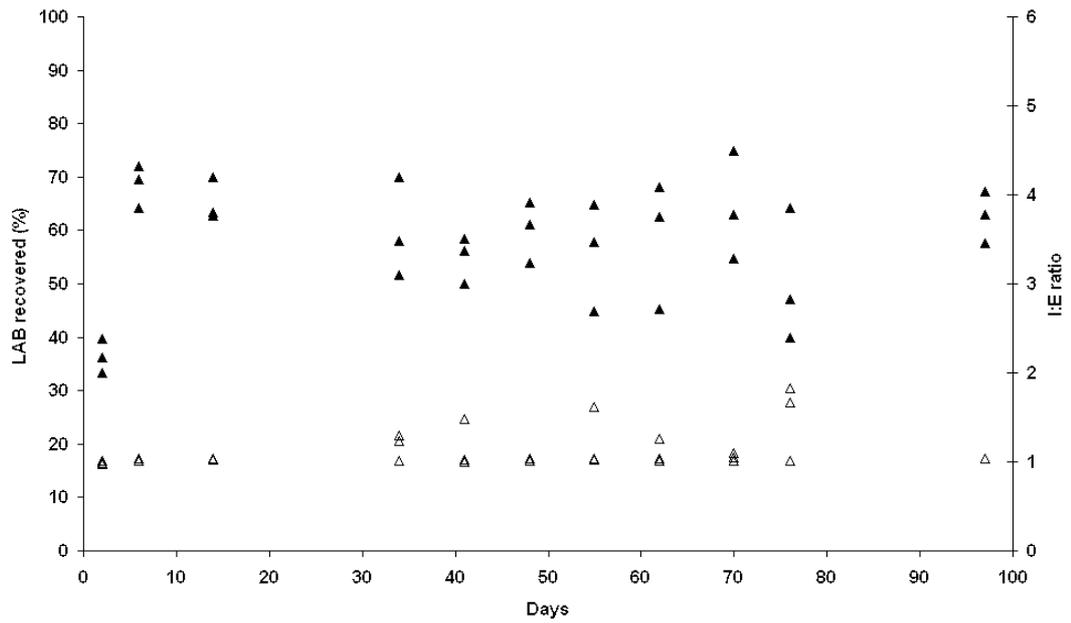


Figure 6.5 Amount of LAB recovered from individual NIT cultures (filled markers) and I:E ratios (open markers) plotted against time.

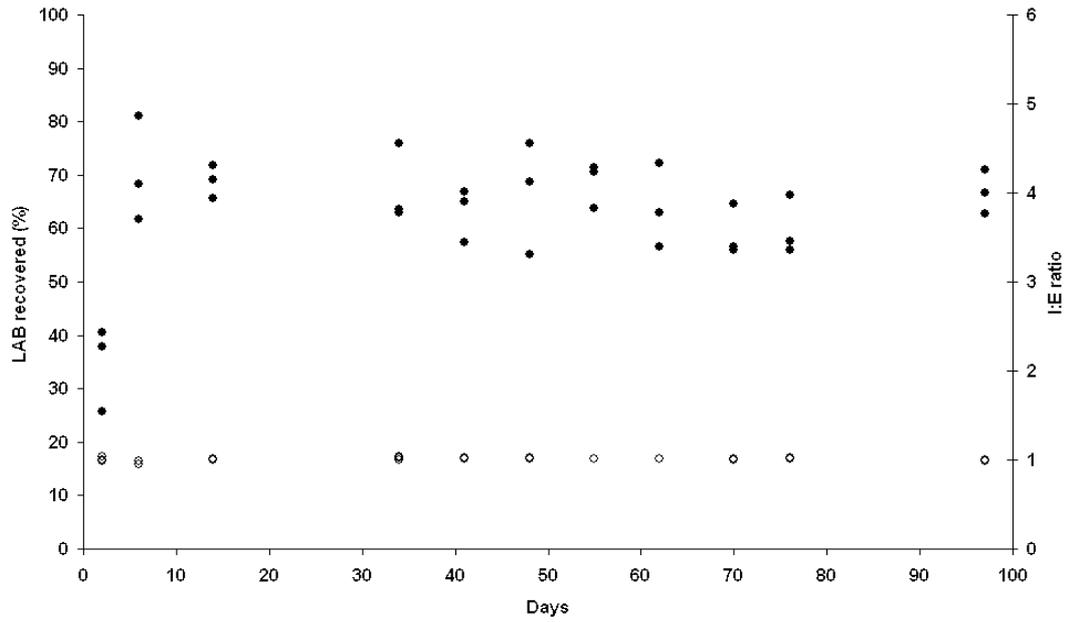


Figure 6.6 Amount of LAB recovered from individual SUL cultures (filled markers) and I:E ratios (open markers) plotted against time

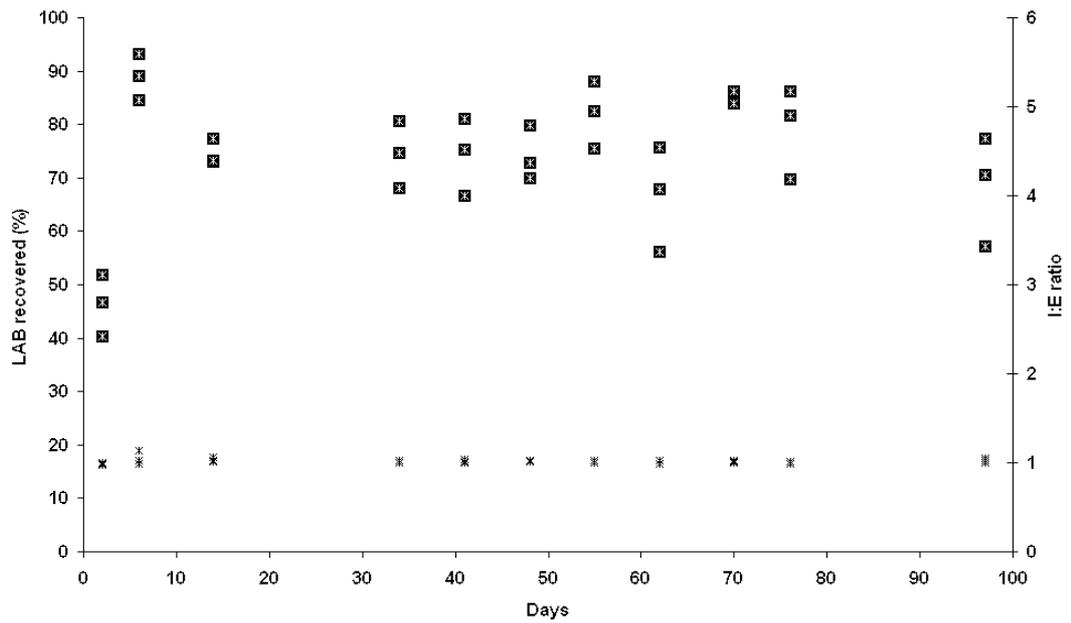


Figure 6.7 Amount of LAB recovered from individual MET cultures (filled markers) and I:E ratios (open markers) plotted against time.

Many of the anaerobic treatments failed to show any degradation at all. Microbial growth was not quantified, but where it occurred it was visible as a pellicle at the LAB:water interface. No growth was observed in any of the bottles containing sulphate as the sole additional TEA. This confirms that the activity in the ANA bottles was due to nitrate-reduction, rather than sulphate-reduction and is supported by the depletion of total oxidisable nitrogen compared to the CON bottle in both NIT and ANA cultures (Figure 6.8), but not AER and SUL (Figure 6.9). This was to be expected since the original enrichments also failed to display measurable activity under these conditions and the inoculum was prepared using nitrate-reducing enrichments.

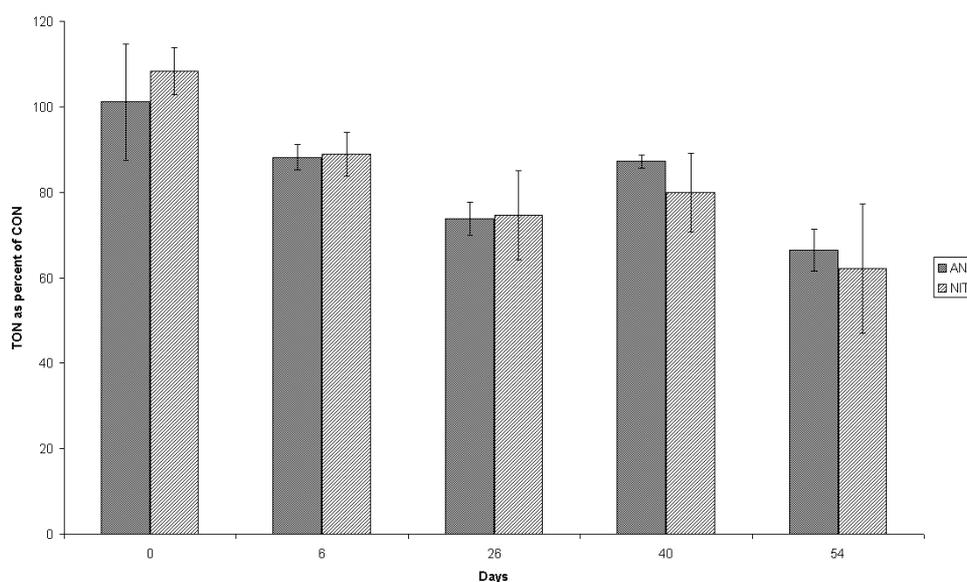


Figure 6.8 Total oxidised inorganic nitrogen relative to CON (error bars = 1 standard deviation, n = 3).

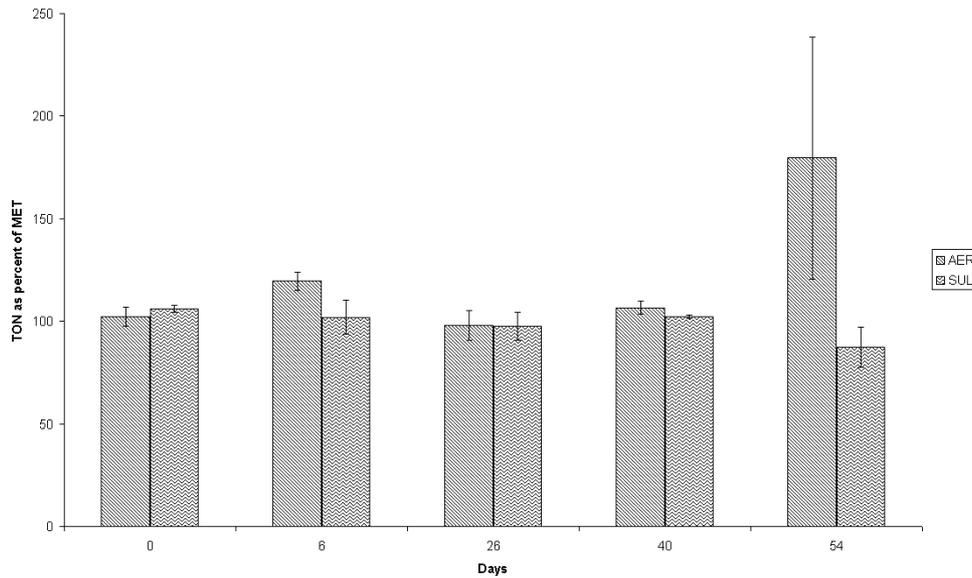


Figure 6.9 Total oxidised inorganic nitrogen relative to MET (error bars = 1 standard deviation, n = 3).

There was no significant evolution of nitrite in any of the treatments (Figure 6.10).

The apparent elevation of nitrite in the NIT treatments is an artefact due to interference from molybdate (Plate 6.2).

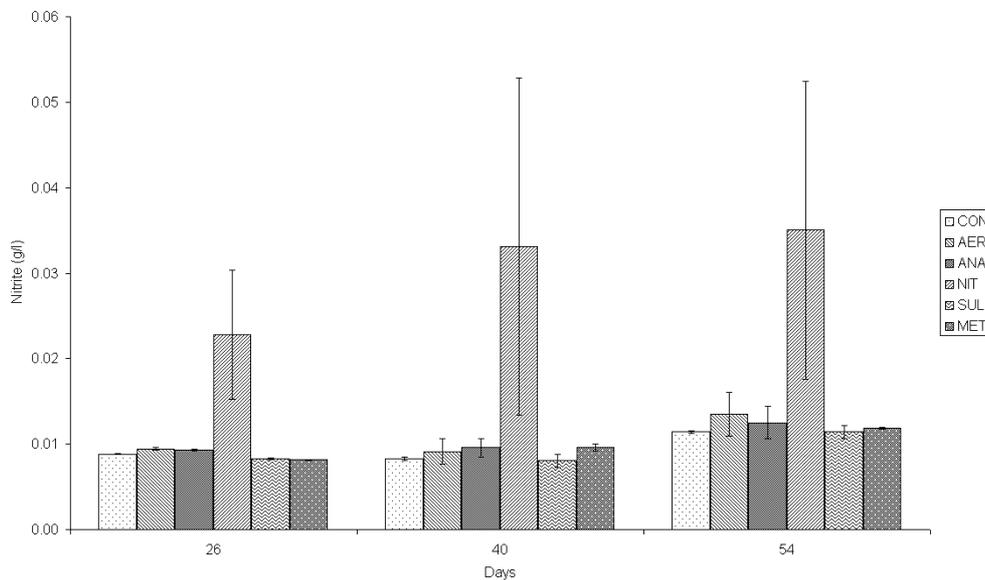


Figure 6.10 Nitrite evolution (error bars = 1 standard deviation, n = 3) N.B. Apparent elevation of nitrite in the NIT treatments is an artefact due to interference from molybdate



Plate 6.2 Nitrite test on various dilutions of NaNO_2 and NaMO_4 to show interference from molybdate

Rapid and extensive biodegradation was seen under aerobic conditions. Anaerobic biodegradation was associated with dissimilatory nitrate reduction, with no degradation seen under sulphate-reducing or methanogenic conditions. The ratio of internal to external C_{12} LAB isomers was calculated and the \log_{10} of this value regressed against percentage biodegradation of LAB cable oil for each of the experimental conditions. Since the intention was to detect any relationship between isomeric composition and degree of degradation, data points from anaerobic bottles with less than 10% degradation or a \log_{10} (I:E) of less than 0.02 were discarded. Data from Takada and Ishiwatari (1990) were similarly analyzed. (Table 6.2).

Table 6.2 Linear regression of % biodegradation of LAB vs. \log_{10} internal:external (I:E) nC_{12} LAB isomer ratio with different terminal electron acceptors

Terminal electron acceptor	Slope	Intercept	r^2 (%)
Aerobic (O ₂)	73.0	17.4	61.0
Multiple TEAs (anaerobic – nitrate and sulphate)	92.1	13.0	97.6
Nitrate (plus molybdate to inhibit sulphate reduction)	126.9	13.4	79.0
Aerobic (O ₂) data from Takada and Ishiwatari (1990)	81.0	15.0	96.0
Composite regression using all data	77.9	16.4	84.3

The linear fit obtained using all the data was found to be significant ($p < 0.001$). The regression lines for aerobic and anaerobic biodegradation were checked to see whether they were statistically different by comparing them to this regression as described by Draper (Draper and Smith 1983), and the null hypothesis - that the slopes and intercepts of the individual regressions were identical to the composite line - was accepted in every case ($p < 0.05$). All the available degradation data were plotted (Figure 6.11) to yield a regression equation that may be applied to field data where the redox history is unknown.

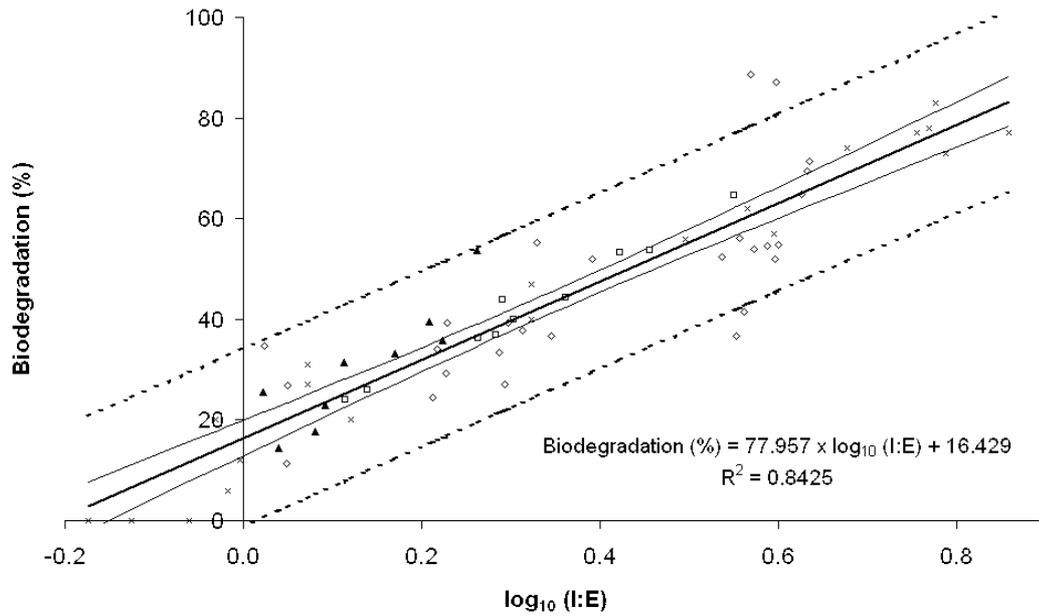


Figure 6.11 Plot of % biodegradation of LAB vs. log₁₀ internal:external (I:E) nC12 LAB isomer ratio with linear regression (heavy) line, 95% confidence (light) and 95 % prediction intervals (broken lines). Data points are from individual bottles: aerobic (◇), anaerobic containing multiple TEAs (□) or nitrate alone (▲) and aerobic data from the literature (Takada & Ishiwatari 1990) (×)

6.3.3 Isolates from enrichment cultures

Since it appeared that biodegradation was associated with nitrate reduction, the ANA and MET cultures were further grown on CPSN agar and colonies isolated. The isolated colonies were used to inoculate Bushnell-Haas medium with LAB and incubated in the same way as the enrichment cultures. These were left for more than a year before being harvested shortly before the end of the project.

Figure 6.12 shows the volume of LAB recovered from the isolates after 386 d incubation. For comparison, NIT, ANA and MET enrichments were harvested and analysed at the same time. The enrichments had been incubated for 589 d.

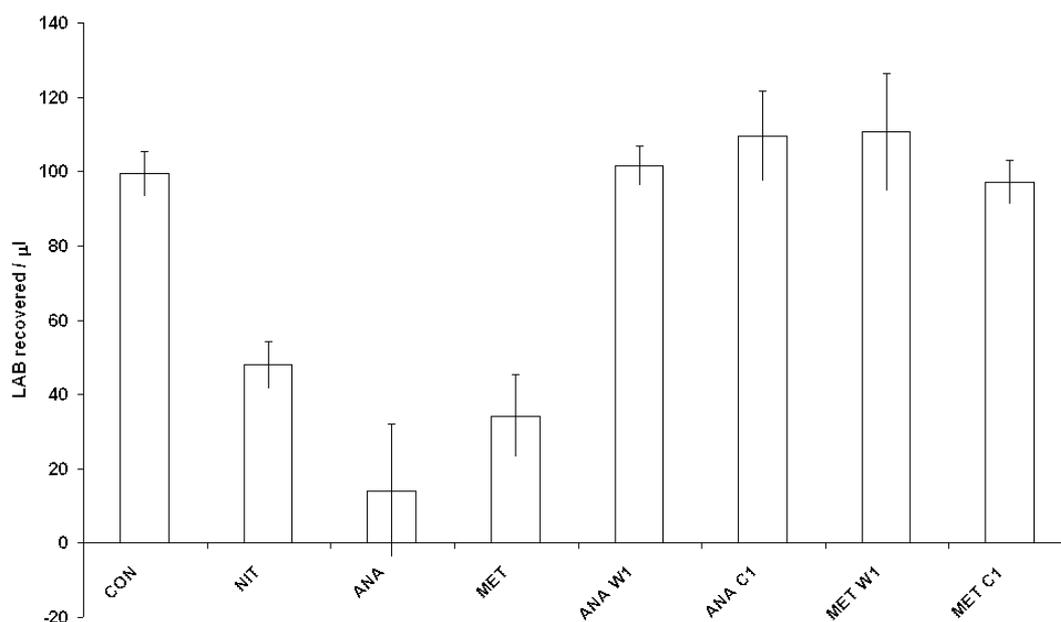


Figure 6.12 LAB recovered from enrichment cultures (NIT, ANA, MET) after 589 d and isolates (CON, ANA W1, ANA C1, MET W1, MET C1) after 386 d. Error bars = 1 SD, n= 3

It is clear that there was no significant degradation of LAB in the isolates, while there was extensive degradation in the enrichments from which the organisms were isolated. This strongly suggests either that the isolated organisms were not involved in the degradation of the LAB, or that the biodegradation was due to a consortium of organisms working in concert rather than a single species.

To confirm that the reduction in the amount of LAB recovered was due to biotransformation, Figure 6.13 shows the $n\text{C}_{12}$ LAB I:E ratio for the same samples.

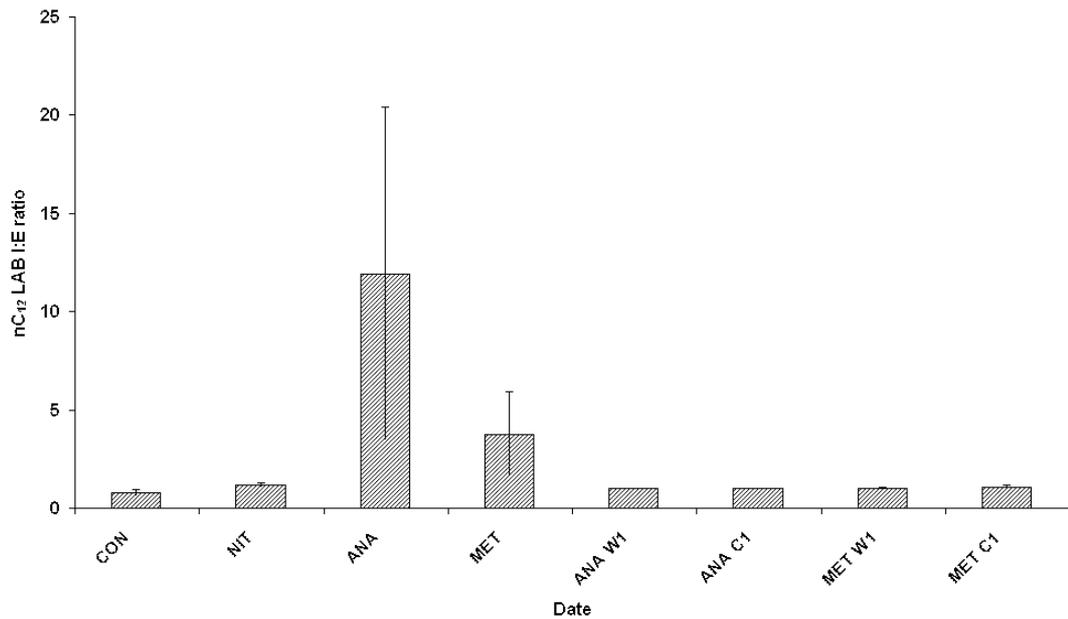


Figure 6.13 nC12 LAB I:E ratio of cable oil recovered from enrichment cultures (NIT, ANA, MET) after 589 d and isolates (CON, ANA W1, ANA C1, MET W1, MET C1) after 386 d. Error bars = 1 SD, n= 3

As previously demonstrated, there was an inverse relationship between the amount of LAB recovered and the isomeric composition, expressed as I:E ratio. The regression for this data set is $\% \text{ degradation} = 82.3 \times (\log \text{ I:E}) + 6.1$, $r^2 = 0.76$, which is close to the relationship already found.

As had been seen in the earlier enrichment cultures, the greatest biodegradation was seen in the ANA enrichments. Unlike previously, there was also significant degradation (up to approximately 60%) in the MET culture. Time did not allow this to be investigated further but it is possible that this was due to nitrate-reduction using the nitrate in the Bushnell-Haas medium.

6.3.4 Pure *G. metallireducens* cultures

Data from *Geobacter* cultures (Figure 6.14, Figure 6.15) show a reduction in the recovery of LAB from all treatments including the uninoculated control. It is likely that this represents a combination of sorption of the oil to glass surfaces and to precipitated iron salts in the medium; and volatilisation of the LAB into the headspace.

There is no significant change in the I:E ratio in any of the treatments (Figure 6.16, Figure 6.17), suggesting that no biological transformation has occurred.

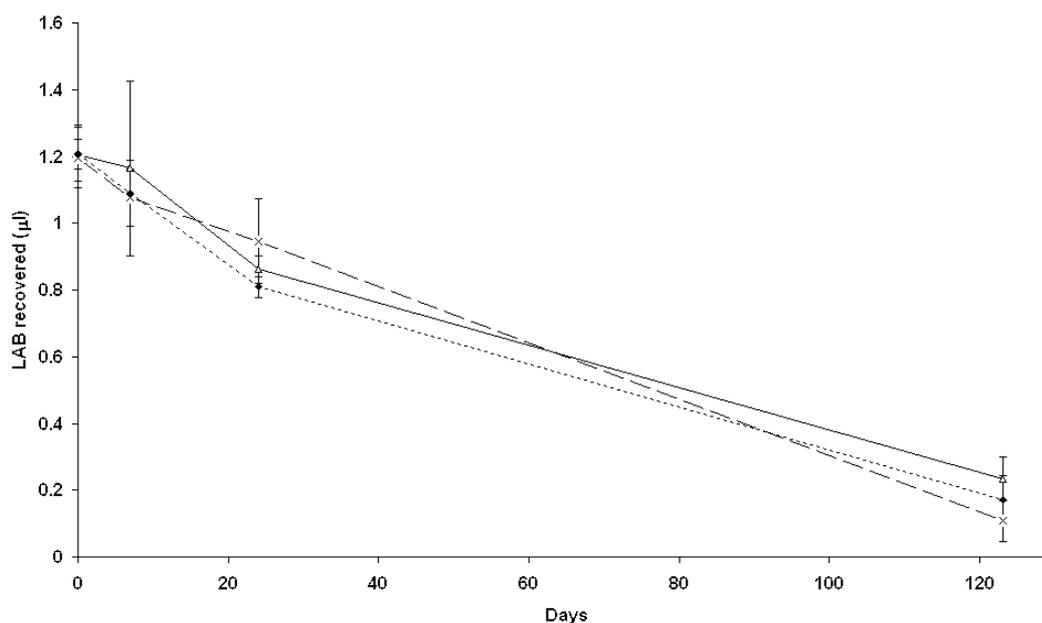


Figure 6.14 Variation of LAB recovered from uninoculated cultures. Treatments differed in terminal electron acceptors: Fe citrate (open triangles), FeCl (filled diamonds) or KNO₃ (x). Error bars = 1 SD, n = 3.

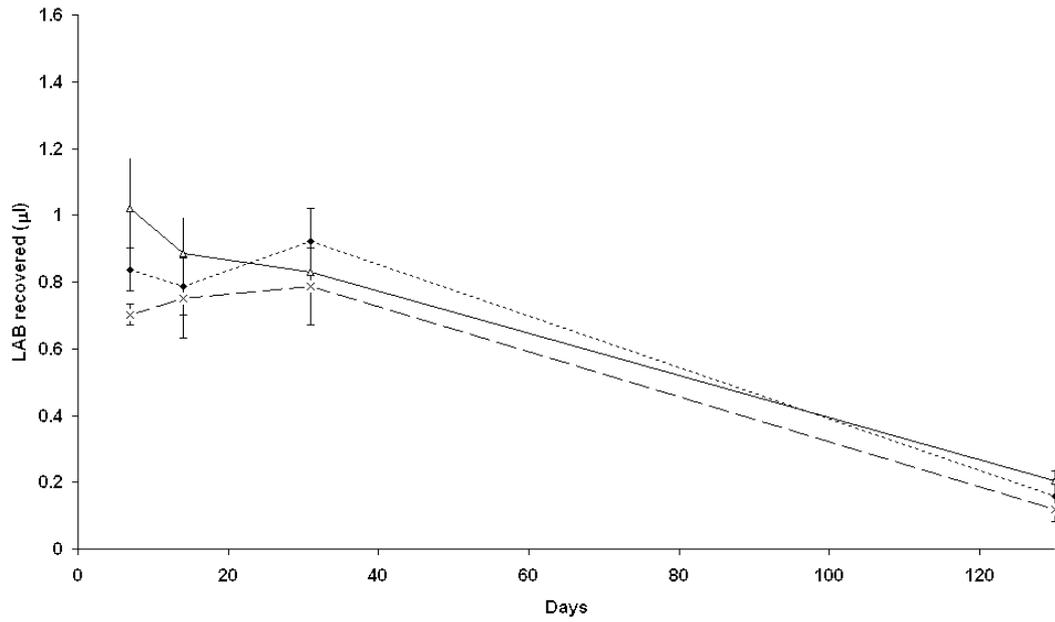


Figure 6.15 Variation of LAB recovered from cultures inoculated with *Geobacter metallireducens*. Treatments differed in terminal electron acceptors: Fe citrate (open triangles), FeCl (filled diamonds) or KNO₃ (x). Error bars = 1 SD, n = 3

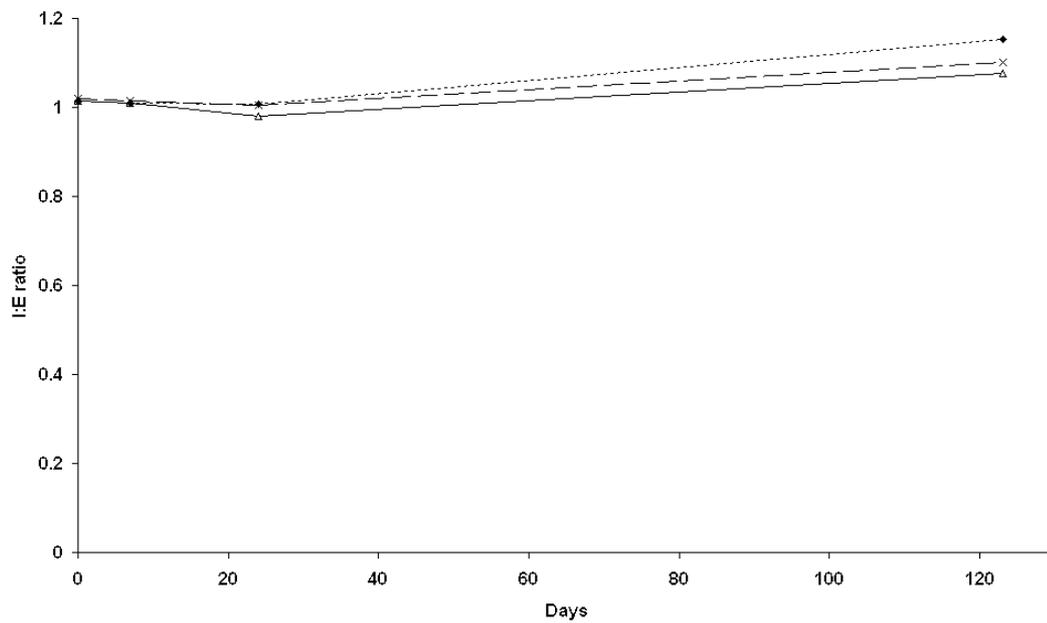


Figure 6.16 Variation of I:E ratio in uninoculated cultures. Treatments differed in terminal electron acceptors: Fe citrate (open triangles), FeCl (filled diamonds) or KNO₃ (x). Error bars = 1 SD, n = 3

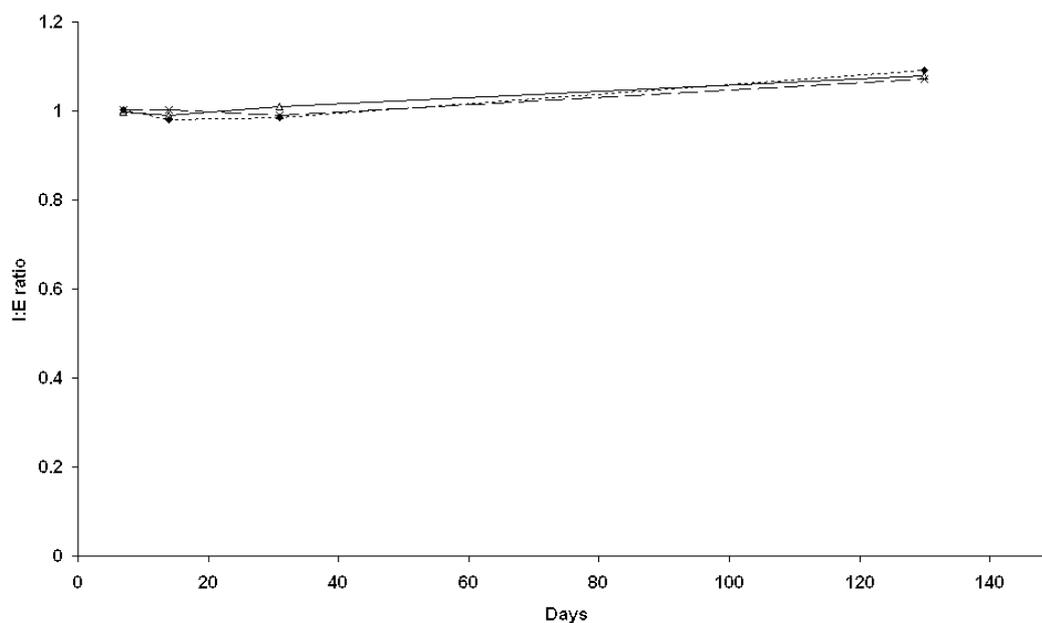


Figure 6.17 Variation of I:E ratio in cultures inoculated with *Geobacter metallireducens*. Treatments differed in terminal electron acceptors: Fe citrate (open triangles), FeCl (filled diamonds) or KNO₃ (x). Error bars = 1 SD, n = 3.

6.4 Conclusions

LAB is biodegraded in liquid enrichment culture under both aerobic and nitrate-reducing conditions. The fact that the aerobic degradation was so rapid using anaerobically cultured inoculum suggests that facultative anaerobes may play a significant role. Differential degradation of internal and external isomers follows similar patterns under a range of conditions, while physical and chemical removal does not significantly favor any isomer.

The ratio of C₁₂ isomers of LAB in the *n*-hexane fraction may provide a robust indicator of the degree of biological degradation in soils that have been contaminated with LAB, even where the redox history of the site is unknown. Statistical analysis of these results and published data reveals that the C₁₂ LAB isomeric ratio varies with

biodegradation independently of the terminal electron acceptor used. Biodegradation (B) can be estimated from the ratio of internal (I) to external (E) isomers of C₁₂ LAB by the equation $B (\%) = 78.0 \times \log_{10} (C_{12} \text{ I:E ratio}) + 16.4$. This relationship can be used to determine the degree of biodegradation of LAB in a range of environments including sites where the redox history is not known, making it a powerful yet simple tool for monitoring LAB biodegradation in the environment.

The data from *Geobacter* cultures indicates that while it is able to grow in the presence of LAB and an additional carbon/energy source with Fe(III), there is no evidence of significant biodegradation after 130 d.

7 Anaerobic ^{14}C LAB respirometry

7.1 Introduction

Most naturally occurring carbon (98.89%) has an atomic weight of 12. Two other isotopes - with the same number of protons and electrons, but different numbers of neutrons - exist. The stable isotope ^{13}C (with one additional neutron) makes up most of the remaining 1.11% but there is also an unstable “radioisotope” in which there are two additional neutrons and so a mass of 14. ^{14}C decays, with a half-life of 5730 ± 40 y, to ^{14}N . Each decay is accompanied by the emission of a beta particle (electron) which can be detected using a scintillation counter. By incorporating an unstable isotope in a molecule, it is possible to use the radioactive decay to track the fate of the labelled atom.

Respirometry by trapping radiolabelled material (Figure 7.1) in NaOH was complemented by running soil flask cultures without radiolabelled material in parallel to allow comparative GC-MS.

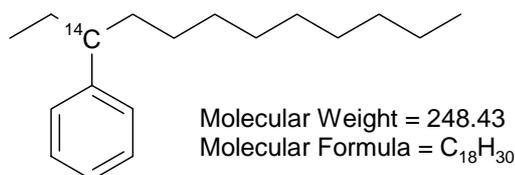


Figure 7.1 Radiolabelled 3^{14}C_{12} LAB to show location of labelled atom

7.2 Materials and methods

7.2.1 Respirometer design

Anaerobic respirometers were adapted from the design developed by Reid *et al.* (2001). The respirometers consisted of a sealed glass bottle containing soil slurry (Plate 7.1). Mineralization of a radiolabelled LAB homologue was determined by measuring the activity in 2 ml of two molar NaOH contained in a scintillation vial suspended above the slurry. Activity was also measured in the soil slurry by sample oxidation and scintillation counting. The second experiment included a small polyurethane (PU) bung in the mouth of the NaOH vial to trap volatilised LAB and products other than CO₂.

7.2.2 Preparation of test soils

7.2.2.1 Test soil

Loam soil was collected from the Bush Estate near Edinburgh as described previously (Section 5.4.5.1).

7.2.2.2 Determination of moisture content

Moisture was determined gravimetrically by weighing samples of soil and drying at 105°C for 24 hours or to constant weight. This information was used to calculate the amount of Bushnell-Haas to add to reach a final soil:moisture ratio of 1:2.

7.2.2.3 Sterilization

Soil for killed control respirometers was sterilised by autoclaving 500 g batches at 121°C for 1 h on three consecutive d.

7.2.2.4 Contamination

Unlabelled LAB cable oil was added using a pipette and mixed using a stainless steel impeller. Efficiency of mixing was confirmed by extraction of LAB from random sub samples by shaking in hexane with a $1C_{12}$ LAB internal standard and analysis by GC-MS.

7.2.3 Inoculum

Where an inoculum was used, it was a mixture of organisms previously isolated from contaminated soil (Section 6.2.2). The organisms were freeze-dried, revived in CPSN medium and washed three times in Bushnell Haas broth before being added to the respirometers.

7.2.4 Assembly

7.2.4.1 Hardware

Each respirometer consisted of a 250 ml Schott/Duran bottle with a screw cap and PTFE (Teflon) liner. A hole was drilled through the centre of the cap and liner to accept a short length of threaded stainless steel rod. This was secured with a stainless steel washer and wing nut on either side. A smaller (1.5 mm) hole was also drilled in the cap, but not the liner, to allow gas to be added/removed and radiolabelled LAB to be added via a hollow needle piercing the PTFE septum. A stainless steel crocodile clip was pushed on to the end of the threaded rod and used to suspend the vial of NaOH above the soil slurry. When assembling the respirometers, care was taken to ensure that the vial was not positioned directly below the smaller hole in the cap so that injected material did not go straight into the vial.



Plate 7.1 Soil respirometer under preparation. The bubbles on the surface of the soil slurry are a result of the vacuum being applied via the needle piercing the Teflon liner through a hole in the cap. Also visible are the stainless steel rod and crocodile clip

7.2.4.2 Soil and moisture

Soils were weighed into the respirometer bottles and water was added as sterile Bushnell-Haas broth, either with or without additional nitrate to form a 1:2 soil:water slurry. This ratio has been shown to be optimal for biodegradation of hydrocarbons in soil (Doick & Semple 2003).

Total nitrate included nitrate in Bushnell Haas medium plus additional nitrate where indicated. Added nitrate was calculated as being ~4 times that required for mineralization of the LAB.

The first experiment (Table 7.1) investigated the fate of LAB in soils contaminated with two concentrations of cable oil (0.1 and 1.0% w/dry weight soil), saturated with Bushnell-Haas medium with or without additional nitrate.

Table 7.1 Summary of respirometer contents for first experiment

Treatment	Soil / g dry weight	Cable oil / ml	BH medium / ml	Total nitrate / mmol l⁻¹
Control	20	0.213	35	12
0.1 % LAB	20	0.027	35	12
1 % LAB	20	0.213	35	12
0.1% LAB + nitrate	20	0.027	35	32
1% LAB + nitrate	20	0.213	35	32

The second experiment (Table 7.2) looked at a single concentration of LAB (0.1%) with Bushnell-Haas medium, with and without oxygen release compound (ORC[®]).

Table 7.2 Summary of respirometer contents for second experiment

Treatment	Soil / g dry weight	Cable oil / ml	BH medium / ml	ORC[®] /g
Control	20	0.027	35	None
0.1 % LAB	20	0.027	35	None
0.1% LAB + ORC [®]	20	0.027	35	1.0

ORC[®] is a commercial product from Regenesi Bioremediation Products consisting of magnesium peroxide intercalated with magnesium phosphate which has been successfully used to accelerate the bioremediation of a variety of compounds (Koenigsberg and Norris 1999). When hydrated, the magnesium peroxide releases oxygen according to the scheme $MgO_2 + H_2O \rightarrow \frac{1}{2}O_2 + Mg(OH)_2$. By intercalating

the active component in magnesium phosphate, the manufacturers claim that the oxygen is released over a period of 6-9 months. A sample of ORC[®] was obtained from Dr Anita Lewis at r³ Environmental Technology Ltd for use in this experiment. Advice was sought from the technical support department of Regenesys regarding the correct amount of ORC[®] to use. They advised that a rule-of-thumb was to use 3 g ORC[®]/g HC (Jeremy@regenesys.com, pers. comm.) and this was adopted for the respirometry study.

7.2.4.3 Purging

The slurry was degassed by piercing the PTFE septum with an 18 gauge needle attached to a three-way valve and alternate application of a vacuum while swirling bottle until no more gas released from the slurry and purging with O₂-free N₂ gas. This was repeated three times to ensure that the respirometer was anaerobic.

7.2.5 Addition of radiolabelled LAB

A primary standard of ¹⁴C radiolabelled 3C₁₂ LAB, provided by Philip Lee at Lancaster University, was diluted with toluene in the proportions 90 µl of primary standard + 110 µl toluene. The activity of 3 × 5 µl aliquots was checked by scintillation counting to ensure that the standard was well mixed, after which 5 µl was injected through the PTFE septum of each respirometer using a 10 µl Hamilton syringe. Care was taken to ensure that the labelled standard was not injected into the foam bung or vial. To prevent gaseous exchange with the external atmosphere, the hole in the respirometer cap was plugged with general-purpose silicone sealant.

7.2.6 Incubation

The respirometers were incubated at room temperature, in the dark, on a rotary shaker in a fume cupboard. They were harvested either all together at the end of the incubation period (first experiment), or single respirometers from each treatment were harvested at approximately three-week intervals (second experiment). For the second experiment, identical treatments without labelled LAB were also prepared in brown glass bottles sealed with suba-seal stoppers and incubated at 24°C on a shaker-incubator. These were harvested at intervals and the LAB extracted and nC₁₂ LAB I:E isomeric ratio determined as previously described.

7.2.7 Analysis

7.2.7.1 Harvesting and scintillation counting

The respirometers were harvested in a fume cupboard and activity in each compartment was measured by scintillation. For the first experiment, the unopened respirometers were all transported to Lancaster University and harvested there. The second experiment was harvested at Edinburgh University and soil samples frozen at -20°C for later analysis at Lancaster.

Soil samples were obtained by gently agitating the respirometer flask and immediately pouring either directly into the combustion thimble or into a small sample vial for freezing and storage. If frozen, the slurry was completely thawed in a refrigerated container, then agitated vigorously prior to sampling and oxidation.

Where a PU bung was used, it was removed from the mouth of the vial, taking care not to contaminate it with NaOH, and placed in a clean scintillation vial. 7 ml of Ultima Gold scintillation cocktail was added and the vial was capped and shaken.

The vial of NaOH was removed from the respirometer and the outside washed clean of any splashes of soil slurry by holding under a slow-running tap. The vial was capped and wiped with a paper tissue soaked in acetone. To each vial containing NaOH was added 5 ml Ultima Gold scintillation cocktail, the vial was tightly capped and shaken vigorously to mix the two phases.

7.2.7.2 Sample oxidation

To obtain a mass balance it was necessary to determine how much activity remained in the soil slurry. This was done by combusting the carbon in a sample of soil slurry and trapping the combustion gases using a Packard Model 307 Sample Oxidiser. A sample oxidiser was not available in Edinburgh but Dr Kirk Semple allowed access to the instrument in the Environmental Science Department, Lancaster University.

A weighed ($1.25 \text{ g} \pm 0.25 \text{ g}$) sample of the slurry was placed in a cellulose thimble and a cellulose cap placed on top. To ensure that the sample was combusted, 200 μl of CombustAid was added immediately prior to oxidation.

The thimble was placed in the sample oxidiser's platinum basket, which was rapidly heated to 1200°C in an oxygen-rich atmosphere, it was held at this temperature for 3 min, completely combusting the organic material. The combustion gases were

trapped in 20 ml of scintillation cocktail (10 ml Carbosorb + 10 ml Permafluor) and the activity was determined as above.

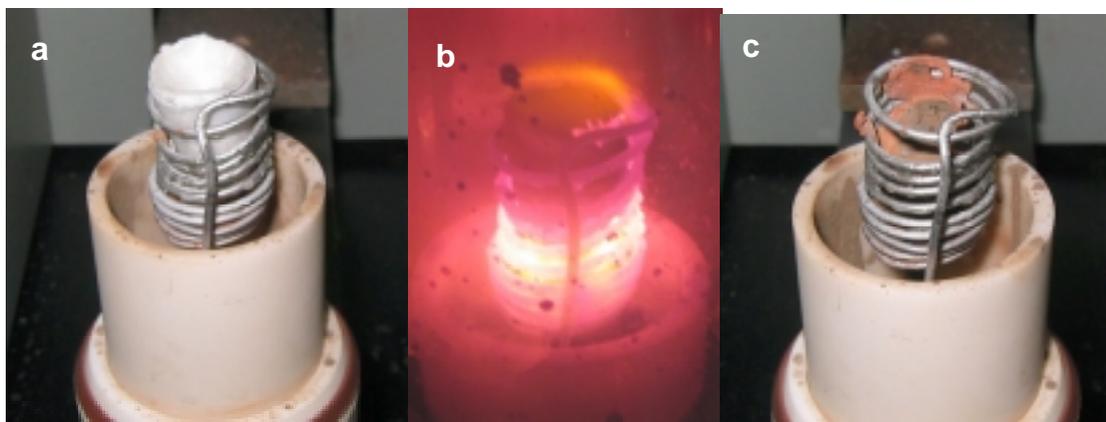


Plate 7.2 Stages in sample oxidation (a) paper thimble in platinum basket prior to oxidation, (b) during oxidation, (c) non-combustible material remaining after combustion

7.2.8 Calculations

The total quantity of both labelled and unlabelled $3C_{12}$ LAB represented by the activity measured by scintillation can be calculated according to Equation 7.1. Note that this does not necessarily reflect the amount of other homologues since, as has already been demonstrated, these may be degraded at different rates.

Equation 7.1 Volume of $3C_{12}$ represented in scintillant

$$[3C_{12}]_{NaOH} = \frac{[^{14}3C_{12}]_{in} \times [3C_{12}]_{in}}{[^{14}3C_{12}]_{NaOH}}$$

7.3 Results and discussion

7.3.1 Activity added to respirometers

The activity of the secondary standard added to the respirometers was measured and found to be $16816 \text{ dpm } \mu\text{l}^{-1}$ (SD = 215.9, n = 3). The activity in each respirometer was therefore 84082 dpm.

7.3.2 First respirometry experiment

The activity recovered from the NaOH (representing labelled CO_2 from mineralization of labelled LAB and the soluble portion of volatilised LAB) and soil slurry (undegraded or partially degraded LAB), as well as the balance of unrecovered activity is shown in Figure 7.2, expressed as μl of labelled LAB.

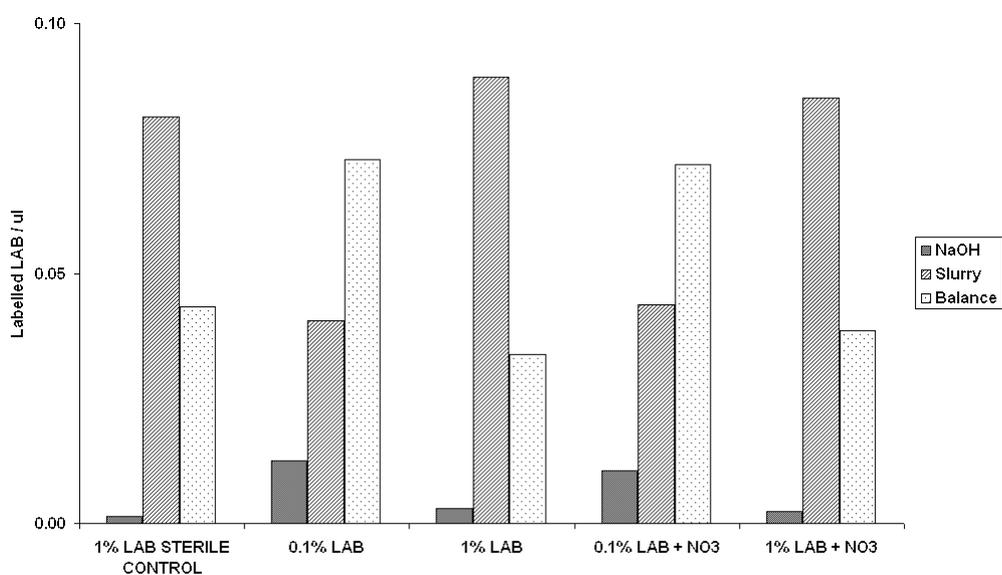


Figure 7.2 Fate of radiolabelled 3C_{12} LAB in first respirometry experiment. Treatments are described in Table 7.1

7.3.2.1 Effect of LAB concentration

At first sight, the data seem to indicate an order of magnitude difference between LAB concentrations in the labelled carbon recovered in the NaOH. The total volume of labelled and unlabelled $3C_{12}$ LAB represented by the activity recovered in the NaOH was calculated according to Equation 7.1 and is shown in Figure 7.3. The data are shown with and without two outlying data points.

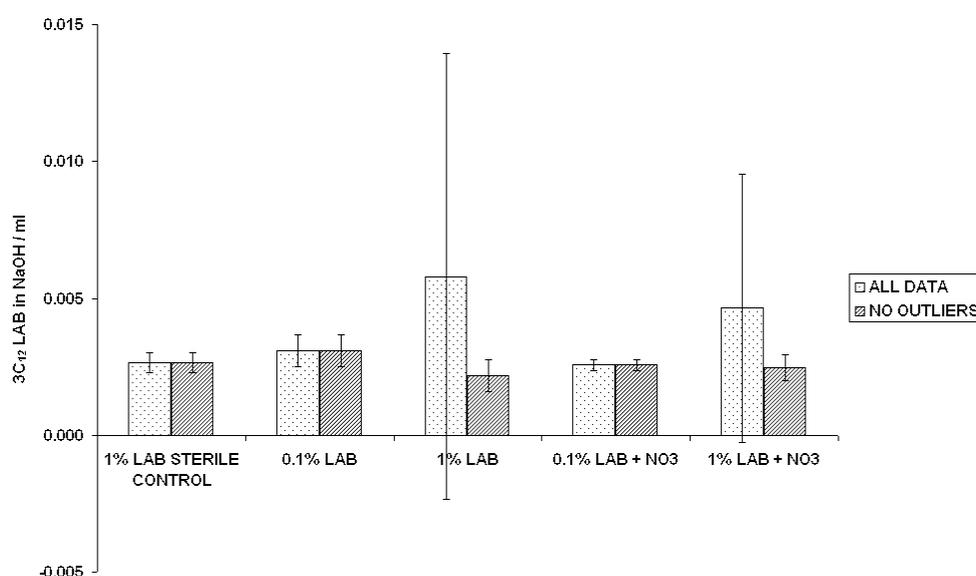


Figure 7.3 Volume of total $3C_{12}$ LAB represented by activity in NaOH in first respirometry experiment. Data presented with and without two anomalous points

It is clear that although the percentage of the labelled $3C_{12}$ LAB recovered from the two differs by a factor of ten, the mass of $3C_{12}$ LAB (sum of labelled and unlabelled) recovered in the NaOH trap is equal in all treatments. The difference is due to a simple dilution effect.

7.3.2.2 Effect of nitrate concentration

Calculations indicated that the nitrate already in the BH medium was stoichiometrically twice as much as is needed for complete mineralization of the LAB – hence there was no difference between these and additional nitrate treatment.

7.3.2.3 Balance of activity

Only 0.15 to 1.2 % of observed activity in NaOH of killed control can be explained in terms of volatility using published Henry's Law and aqueous solubility data, and the amount of activity that cannot be accounted for is rather high. Possible explanations for this are:

- Published figures are not accurate
- Control was not sterile
- Physical/chemical breakdown of LAB
- Adhesion to apparatus
- Losses of unlabelled oil during pugging
- Inhomogeneity of the slurry sample that was oxidised - the slurry was very wet (1:2 soil/water) and, even though it was agitated before sampling, it may have sedimented before the sample was removed

7.3.2.4 Limitations of first respirometry experiment

The first respirometric experiment was flawed in several ways:

- For reasons of space there was no killed control for the lower (0.1%) oil concentration.

- Only a single harvesting means that there is no temporal data. Can compare with existing aqueous culture data but would be good to address this if possible.
- No analysis of headspace gases was made.

In order to address some of these problems, a second experiment was carried out. Space limitations meant that no more than 25 respirometers could be set up at a time so single respirometers of each treatment were harvested at intervals rather than all together at the end of the experiment to give a time series, albeit without replication. Since there was no evidence of anaerobic biodegradation from the first experiment, the second experiment also incorporated treatments containing a commercially available oxygen release compound (ORC[®]). A polyurethane (PU) bung in the neck of the NaOH vial in each respirometer was used to trap volatile material.

7.3.3 Second respirometry experiment

7.3.3.1 Scintillation data

Scintillation data from the second experiment (Table 7.2) is presented in Figure 7.4 - Figure 7.7. Note that the y-axis scale differs between experimental compartments, but is the same for each treatment to allow ease of comparison. Each data point represents a single respirometer.

In all cases, a relatively steady state was achieved in the first three weeks, with no significant changes occurring after this time. There were distinct differences between the treatments and these are summarised in Figure 7.8.

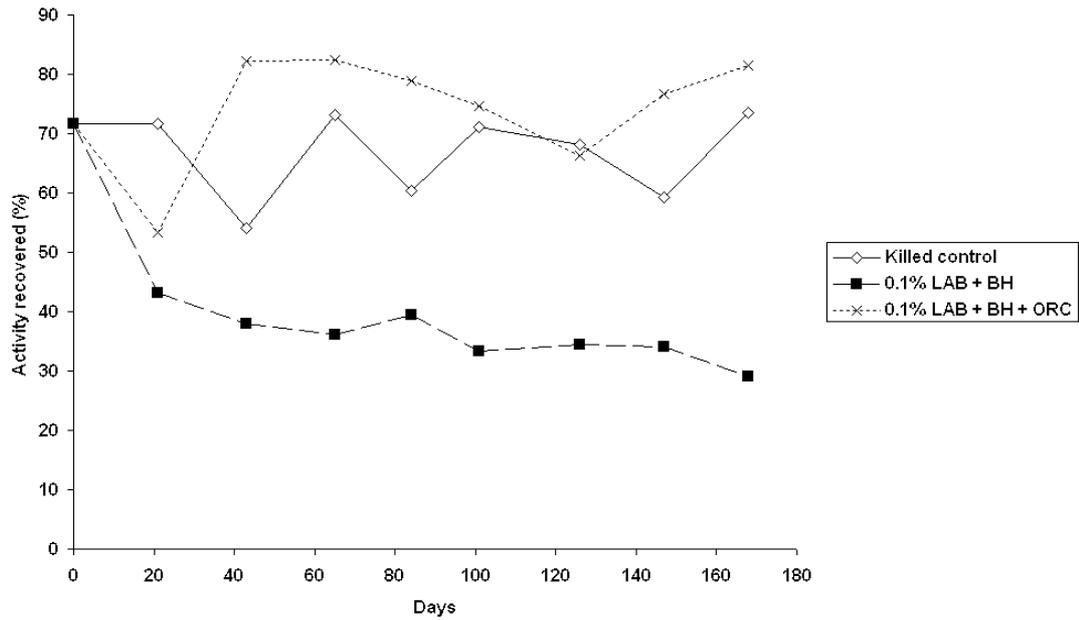


Figure 7.4 Activity detected in soil slurry under different conditions as a percentage of the total activity added. This represents undegraded or only partially degraded LAB remaining in the soil slurry.

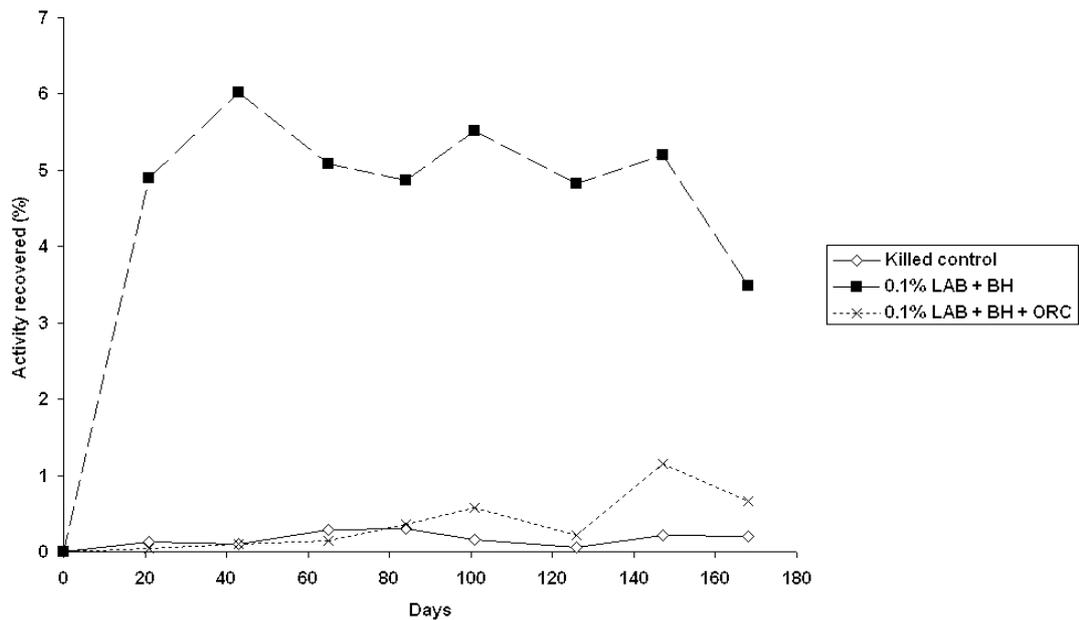


Figure 7.5 Activity detected in NaOH under different conditions as a percentage of the total activity added. This represents CO₂ from mineralised LAB and the soluble fraction of volatilised LAB and breakdown products.

Plate 7.3 shows the polyurethane bungs taken from the respirometers in the second experiment. The bungs have been removed from the respirometers and placed in a scintillation vial with a 5 ml of Ultima Gold scintillation cocktail (this was topped up to 7 ml prior to counting. The bungs represent a time series covering 168 d.

The most striking feature is the pigmentation seen in the bungs from the anaerobic respirometers. Interestingly, this does not seem to correlate with the activity seen in the bungs (Figure 7.6).

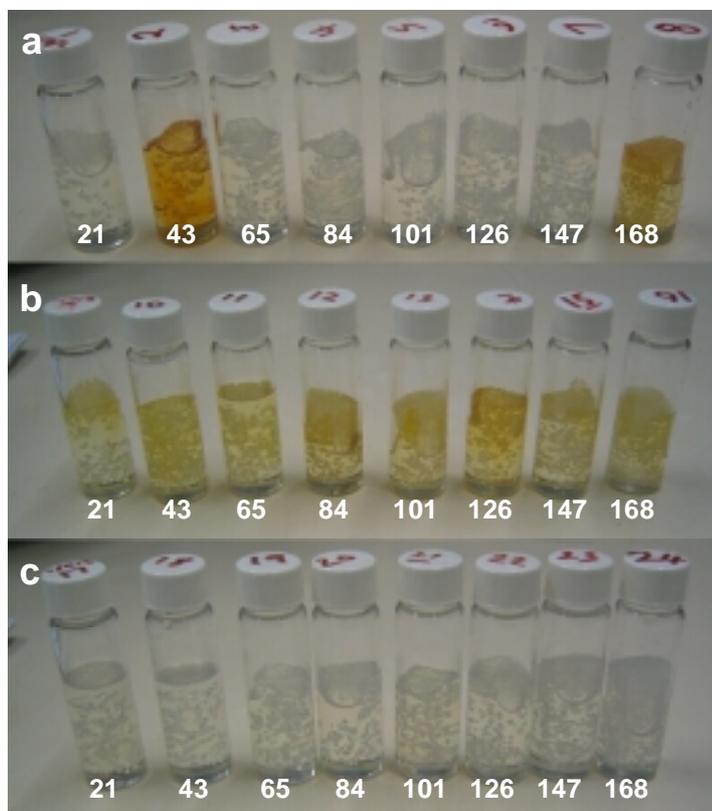


Plate 7.3 Appearance of PU bungs from respirometers (a) killed control, (b) anaerobic and (c) ORC. Numerals indicate time in d since start of experiment

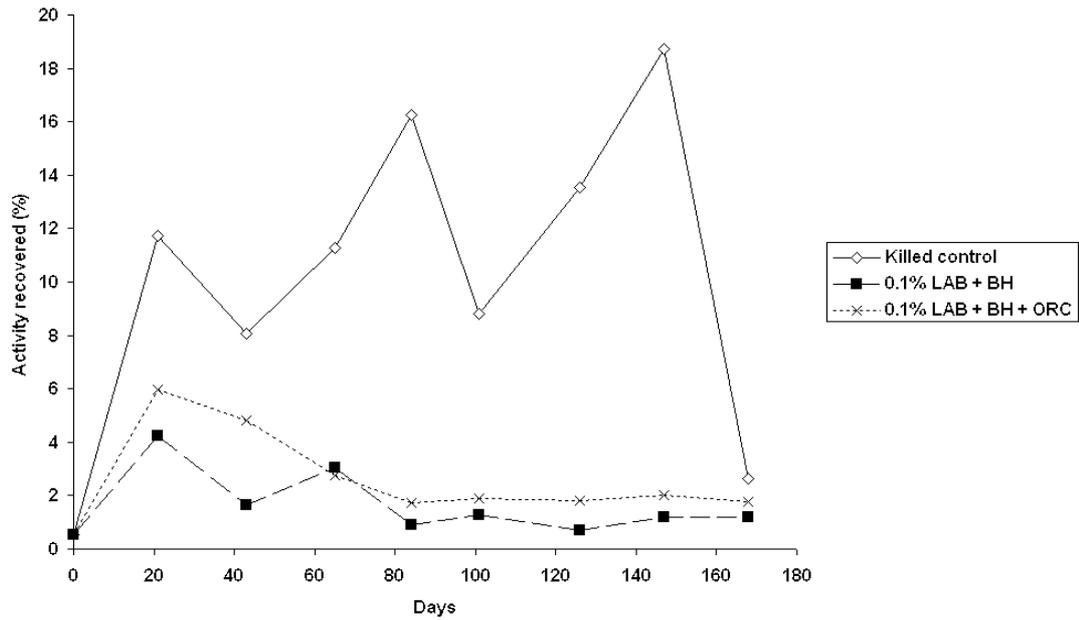


Figure 7.6 Activity detected in a polyurethane bung under different conditions as a percentage of the total activity added. This was included to detect volatile LAB and breakdown products

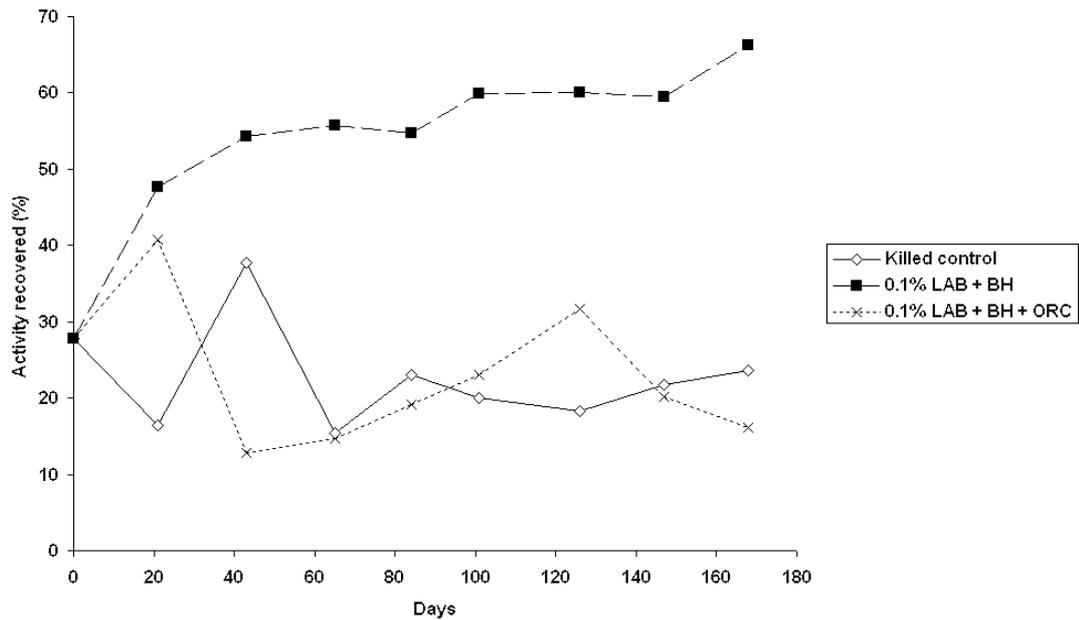


Figure 7.7 Activity not detected under different conditions as a percentage of the total activity added. Calculated by summing the activity detected from soil, NaOH and bungs, and subtracting from the total activity added to each respirometer

A proportion of the activity was not accounted for in all of the respirometers. In the control and ORC[®] systems this was more or less constant at about 22% over the course of the experiment. This can be explained by sorption to glassware etc. and losses through volatilization during the degassing operation. To account for these losses, the activity added to each respirometer was multiplied by 78 % (i.e. subtracting 22 %). The mean activity from different compartments (not including day 0) following this correction are plotted in Figure 7.8.

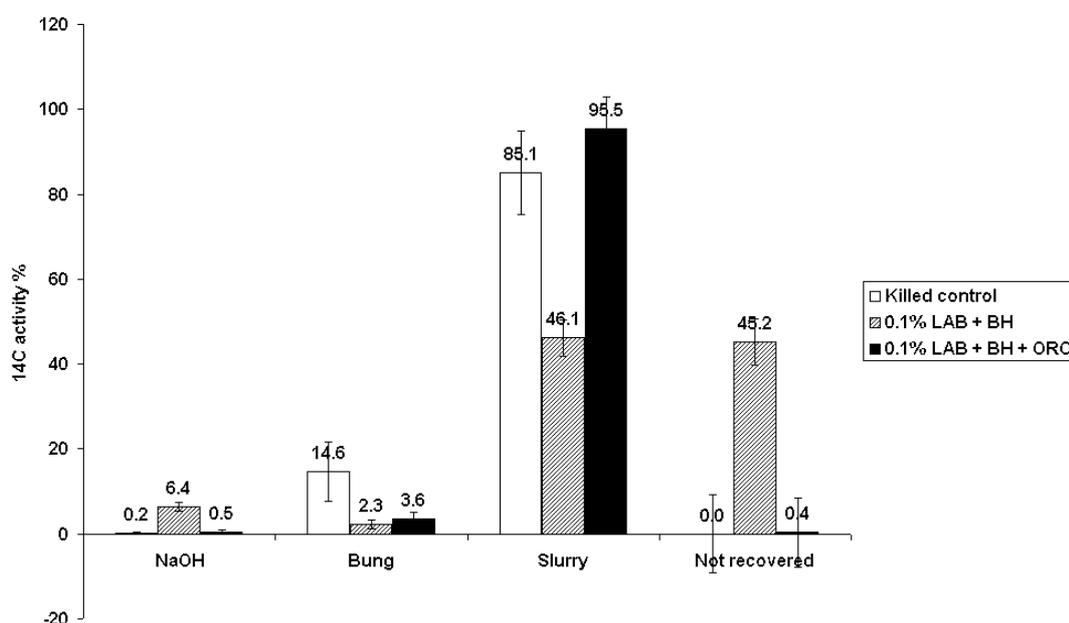


Figure 7.8 Summary of ¹⁴C activity found in different compartments of the second respirometry experiment. Data corrected for systematic losses seen in control respirometers due to sorption to glassware and loss during degassing of respirometers. Error bars = 1 SD, n = 8

To confirm that volatilization does occur in the temperature range experienced by the respirometers and soil slurry flask, additional flasks containing sterile B-H medium were contaminated with 100 µl LAB cable oil. Half the flasks were sealed and the

remainder closed with foam bungs so that they were open to the atmosphere. The flasks were incubated on a shaker incubator at 24°C and triplicates were harvested at intervals. The LAB was quantified by hexane extraction and GC-MS with a 1C₁₂ LAB. The results are shown in Figure 7.9. The limited data set indicates that there is some loss of LAB through volatilization and it is reasonable to assume that this will be more pronounced when the oil is exposed to vacuum, as occurred during the preparation of the soil slurry respirometers and flasks.

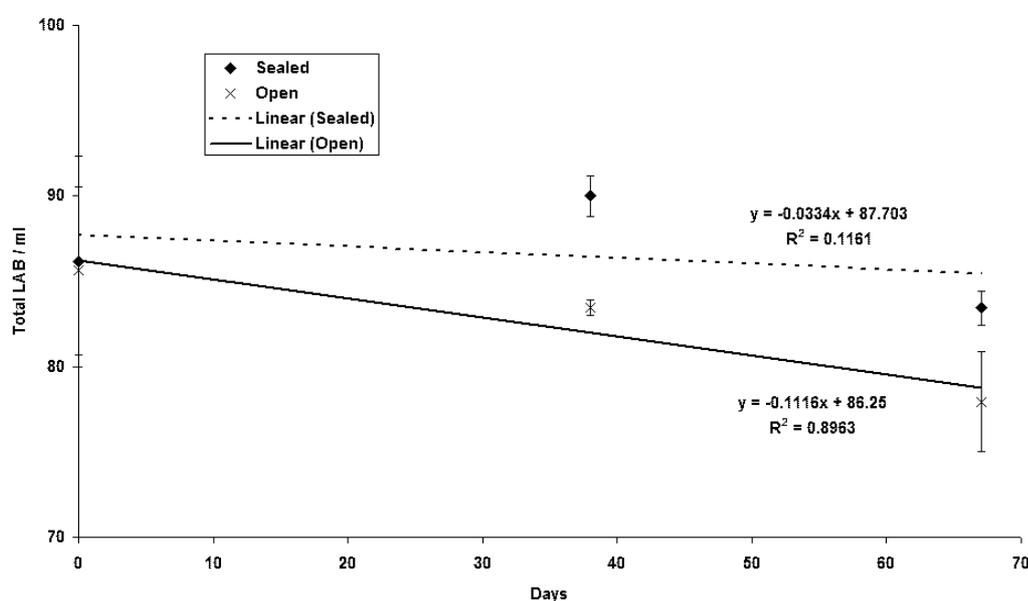


Figure 7.9 Changes in volume of LAB recovered from sealed and unsealed flasks over time

The activity recovered from the soil slurry in the ORC[®] respirometers was not significantly different from that in the control respirometers at about 90 % of the corrected activity added. However, that seen in the anaerobic respirometer was almost a factor of two lower, suggesting that at least some of the LAB had been transformed in the absence of oxygen to gaseous or volatile products. It is not clear

why the ORC[®] treatment did not show the pronounced biodegradation that might have been expected if oxygen was being released. Possible explanations might be that the magnesium phosphate, included in the composition to reduce the rate at which the magnesium peroxide decomposed, also provided an enhanced surface area for sorption of LAB, thus reducing its bioavailability, alternatively the ORC[®] may have had a toxic effect at the concentration used. Time did not allow for this to be investigated.

The activity seen in the NaOH of the control respirometer was constant and never exceeded 0.4 % of the total activity. This probably represents volatilised LAB in solution and sorbed to the surface of the vial. In contrast, the ORC treated respirometers reached 1.5 % and the anaerobic respirometers reached 7.7 %. The NaOH fraction in the anaerobic respirometers was at least an order of magnitude higher and while this may represent mineralised LAB, it is more likely that it is a result of the much higher proportion of activity not recovered in these treatments being volatile material in solution and sorbed to the surface of the vial.

The activity in the bungs represents high molecular weight volatile materials such as LAB. In the control respirometer this accounted for as much as 18.7 % of the total activity whereas in the anaerobic treatments the highest activity seen was 4.2 %, and in the ORC[®] respirometers it reached 6.0 %. In both these treatments, the highest value was seen in the first harvest of respirometers and declined to a steady minimum value after approximately 90 d. This may have been due to microbial breakdown of LAB to gaseous products on the bung surface. The lower volatility

seen in ORC[®] amended respirometers may have been due to the increased surface area for sorption due to the added magnesium phosphate. This is supported by the higher proportion recovered from the soil slurry compared to the killed control without ORC[®].

The balance of activity – that is, the difference between the activity added and that recovered from the respirometers, was approximately 45 % in the anaerobic treatments. Since systematic errors were accounted for by the correction made to the initial value, this is a significant finding and is presumably due to volatile or gaseous products other than CO₂. Possible candidates are low molecular weight hydrocarbons including BTEX, alkanes, CH₄ and fatty acids. It has already been noted that transformation to the equivalent fatty acid has been seen as a primary biodegradation step in a variety of hydrocarbons (Alvarez 2003).

7.3.3.2 Isomeric composition

Permission was not granted to analyse radiolabelled samples using GC-MS and so parallel treatments without radiolabelled material were also prepared to allow the nC₁₂ I:E isomeric ratio to be monitored over the same period. They contained 20 g dry weight of soil rather than the 25 g in the respirometers but the proportions of all components was identical. The data from these treatments is shown in (Figure 7.10). Each data point represents a single soil slurry flask.

There was a slight upward trend in nC₁₂ LAB I:E isomeric ratio in LAB recovered from the soil slurries from both the anaerobic and ORC[®] treatments, but this was not large enough to be significant in the time over which the experiment was conducted.

The volume of LAB recovered from the soil slurry flasks was highly variable, probably because of the small volume used to contaminate the soil, and the presence of soil particles onto which the oil became sorbed.

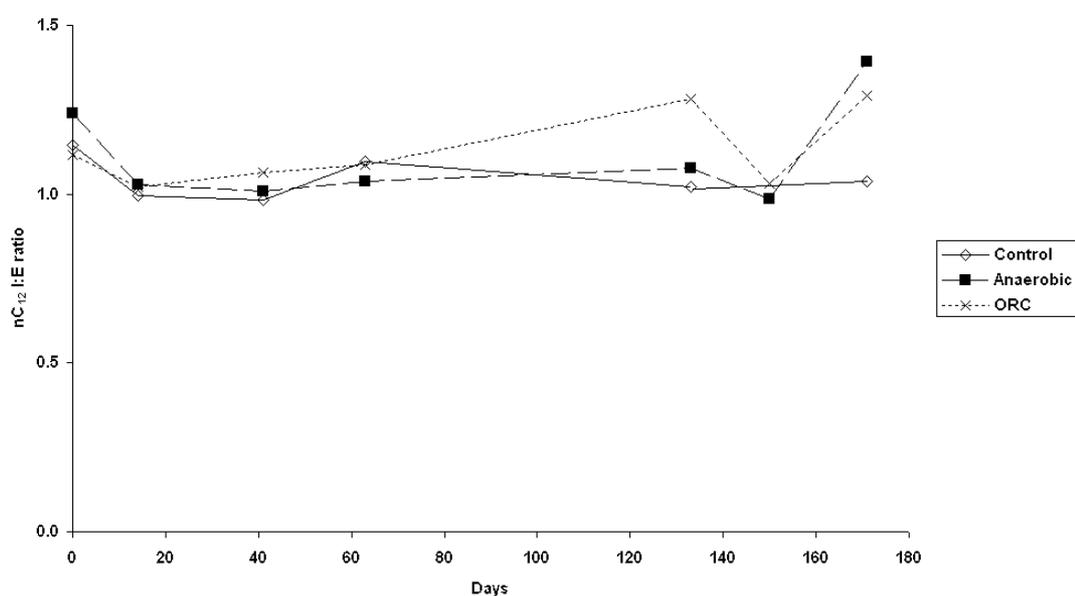


Figure 7.10 nC₁₂ I:E ratio of LAB extracted from soil slurry under different conditions.

Since the nC₁₂ LAB I:E isomeric composition did not vary greatly over time, the data was averaged over the course of the experiment and are shown in Figure 7.11.

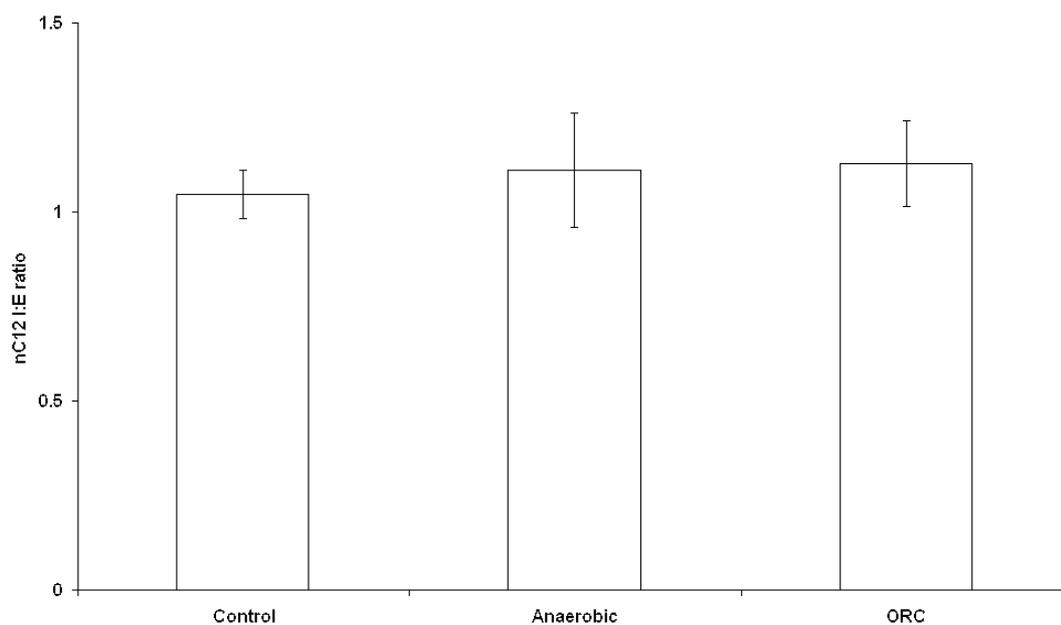


Figure 7.11 Mean nC12 LAB I:E ratios seen in soil slurry under different conditions. Error bars = 1 SD, n = 6 for Control or 7 for other treatments)

By revisiting the data generated in aqueous cultures analysed by GC-MS (see p 17 *et seq.*) it is possible to generate a graph linking the degree of degradation a single homologue, in this case 3C₁₂ LAB, to total degradation (Figure 7.12).

Of course, this does not account for additional, labeled 3C₁₂ LAB. It is not obvious what effect the addition of this homologue will have on degradation of unlabelled 3C₁₂ and other homologues. On the one hand there may be toxic effects from the additional LAB and on the other, this homologue will overrepresented in the respirometers. The assumption is that both labeled and unlabelled 3C₁₂ LAB are degraded to the same degree. Since the labeled LAB is added based on activity, rather than volume (the LAB is dissolved in toluene) it is not possible to say exactly how much of the labeled material is present.

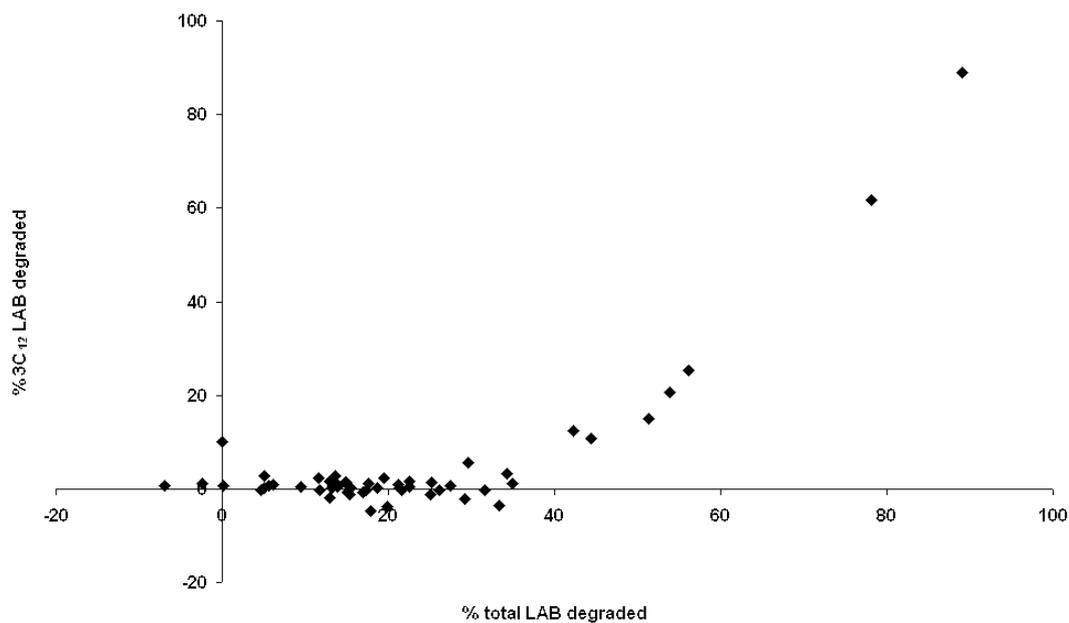


Figure 7.12 Relationship between degree of degradation of 3C₁₂ LAB and total LAB degradation seen in aqueous cultures under a range of conditions

Notwithstanding the addition of labeled LAB, no significant change in 3C₁₂ LAB is seen until approximately 40% of the total LAB has undergone primary degradation. It is likely that addition of the labeled homologue will reduce this to a lower value. Ignoring this, the primary degradation of approximately 45 % of the 3C₁₂ LAB seen in the anaerobic respirometer potentially equates to about 70 % primary degradation of total LAB. However this is not supported by the nC₁₂ I:E ratio data. The toluene used as a carrier for labeled LAB may also have acted as an additional carbon source in the respirometers, further affecting the results. No investigation has been made into whether toluene is more readily degraded than LAB, or whether there are any cometabolic processes.

7.3.3.3 Limitations of the second respirometry experiment

There remain a number of limitations to the experimental design used for respirometry:

- The most significant shortcoming of the second experiment was the lack of replication. Unfortunately, the space available only allowed a maximum of 25 respirometers to be incubated. However, the results were remarkably consistent over the period of the experiment and so the lack of replication over time was mitigated by the long time series.
- In retrospect it would have been preferable if the non-radiolabelled soil flasks had been prepared in exactly the same manner as the respirometers. Specifically, the inclusion of PU bungs in these treatments would have allowed confirmation of the volatility of the undegraded oil. Aerobic flasks were prepared with a PU bung and some of these were analyzed but degradation occurred rapidly and the remaining aerobic slurries dried out over the course of the experiment.
- The greatest change seems to have occurred in the first three weeks. Sampling more frequently during this time would have yielded data on short-term processes.
- Sterile control respirometer containing ORC[®] would have made for a more complete data set. However, as has been explained, space was limited
- Analysis of LAB from contaminated soils with and without exposure to vacuum would confirm whether this was a significant cause of oil loss.

- Increased pressure from gas production may have an inhibitory effect. The respirometers could have incorporated a pressure relief mechanism, preferably with volatile and CO₂ traps to maintain the mass balance.
- It is possible that exposure to vacuum affected the bacterial community.
- It may be useful to carry out parallel experiments using radiolabelled glucose to see if poor biodegradation is due to preferential use of other carbon sources.

7.4 Conclusions

There was a small, though not statistically significant, change in isomeric composition over 168 d. This is consistent with limited primary biodegradation of the LAB. The anaerobic, and to a lesser extent ORC[®] treatments evolved gaseous or volatile radiolabelled products, consistent with microbial breakdown.

There is therefore evidence for primary degradation of LAB in anaerobic soil slurry. However, in soil slurries amended with ORC[®] there seemed to be greatly reduced degradation, along with pronounced immobilization of LAB. This raises the question of whether reduced recovery of contaminants from field sites that have been treated with ORC[®] is in fact due to increased sorption rather than enhanced bioremediation, or whether the concentration of ORC[®] used was inappropriate.

8 Soil column experiments

A variety of designs for soil column experiments was considered, with the intent of approximating the conditions that would be expected in contaminated subsoil.

A Winogradsky column simply consists of a vertical column, filled with soil and open to the atmosphere at the top end. If a carbon source such as cable oil is mixed with the soil, and a range of electron acceptors are available, a natural E_H gradient will develop. A number of these columns may be set up and harvested at intervals by freezing, removing from the column and slicing into vertical intervals. Analysis of the cable oil content and microbiology of each slice will yield temporal and spatial information about degradation in a representative soil column.

Alternatively, the sequential soil column system described by Nay *et al.* (1999a; 1999b) could have been employed. This would have the advantage that the redox zones are clearly defined.

Resources did not allow for the construction of a sequential system and it was important to gather data on anaerobic conditions so it was decided to set up sealed, saturated soil columns, without seeking to impose particular redox conditions, apart from the exclusion of oxygen, so far as was possible. Strictly, this would result in a microcosm, rather than a true column experiment, but the nomenclature has been retained.

8.1 Materials and methods

8.1.1 Column design

The soil was packed in acrylic tubes sealed top and bottom with silicone rubber bungs (Plate 8.1). Overall dimensions: 500 mm × 60 mm diameter (external, includes bungs)/300 mm × 50 mm (internal). The bungs were bored with a single hole and fitted with a short length of borosilicate glass tubing. This in turn was connected to a short (~5 cm) length of silicone tubing, which was sealed with a tubing clamp. The bottoms of the tubes were first sealed with a bung. On top of the bung were placed a glass fibre filter and approximately 2 cm (compacted) quartz wool to prevent soil particles clogging the glass tube.

Treatments were:

- (i) sterile soil saturated with Bushnell-Haas medium (columns 1-3)
- (ii) non-sterile soil saturated with dH₂O (4-6),
- (iii) non-sterile soil saturated with B-H medium (7-9)
- (iv) non-sterile soil saturated with B-H + 1 mol l⁻¹ NaNO₃ (10-12).



Plate 8.1 Soil columns prior to incubation

8.1.2 Soils

Soil from the Bush Estate (as described previously) was passed through a 2 mm sieve. Its moisture content was ascertained by drying at 105°C for 24 h or to constant weight and combusted at 475°C to constant weight to find total organic carbon (TOC). Soil for the sterile control columns was autoclaved at 121°C for 1 h on three consecutive days.

8.1.2.1 Contamination

Soil was air dried if necessary to 15-18% moisture content and contaminated by adding 1% (w/dry weight soil) LAB and mixing thoroughly with a stainless steel impeller. The efficiency of mixing was checked by taking random sub samples, extracting the LAB by shaking in hexane and quantifying the oil in the organic phase by GC-MS as described previously. The final concentration of oil in the soil was checked in the same way as the TOC was found, by drying and combusting samples.

8.1.2.2 Packing

Soil was added from above to almost fill the tube (400 g dry weight) and the top bung fitted.

8.1.2.3 Saturation

The soil was saturated with either sterile dH₂O, Bushnell-Haas (B-H) medium or B-H + NaNO₃ by slowly filling from below. The column was completely filled with liquid before sealing so that there was no free liquid surface in contact with the air. In

this way it was hoped that no redox gradient would form over the height of the column.

8.1.2.4 Incubation

Once filled, the tubing clamps at top and bottom were tightened and the columns were incubated vertically in the dark at room temperature for five months.

8.1.3 Sampling

In order to sample soil from the saturated soil columns in such a way as to preserve the vertical distribution of LAB cable oil the columns were frozen and sectioned using a band saw.

8.1.3.1 Freezing

Columns were placed vertically in a PVC pipette jar, which in turn was placed inside an expanded polystyrene sleeve to provide insulation (Plate 8.2). Gaps between the cylindrical jar and the square cross-section sleeve were filled with “packing peanuts”. Liquid nitrogen was poured into the jar to completely cover the column. Once frozen, the columns were removed to cold storage.

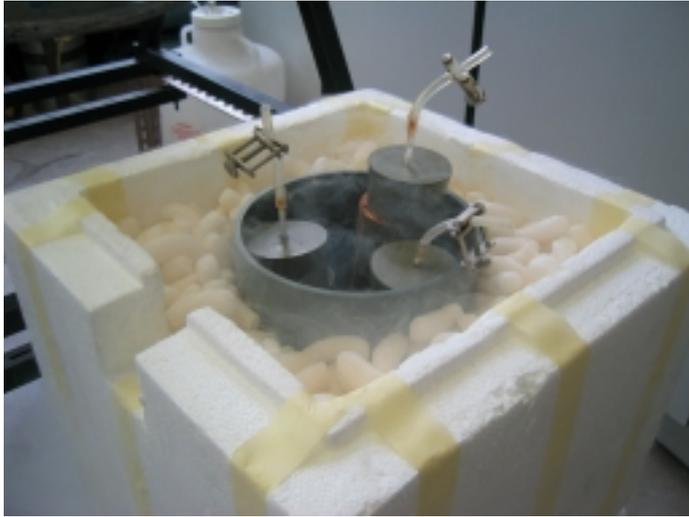


Plate 8.2 Freezing soil columns

8.1.3.2 Storage

Columns were stored horizontally in the dark at -20°C until they could be sampled.

8.1.3.3 Sectioning

The frozen column, still in its acrylic case was cut into 50 mm sections using a band saw (Plate 8.3). The soil surface was used as a datum and sections were numbered from the soil surface down such that section 1 represented 0-50 mm, section 2, 50-100 mm, etc.



Plate 8.3 Sectioning frozen columns

8.1.3.4 Homogenisation

To ensure that the soil in each section was homogeneous, the sections were placed in clear polyethylene bags and allowed to thaw. The saturated soil was pushed out of the acrylic tube, which was removed and the soil homogenised by repeatedly squeezing the bag by hand in a stomaching motion. A mechanical stomacher was available but it was decided to mix by hand to reduce the probability that the bag would be damaged by sharp inclusions in the soil.

8.1.3.5 Sampling

Sub samples of soil were weighed directly into glass sample vials to allow LAB extraction and analysis to be done. Samples were also removed for aerobic incubation, analysis of 16s rDNA and measurement of moisture content, respectively. The remaining soil samples were refrozen as soon as possible.

8.1.3.6 Aerobic incubation

Weighed samples (~1 g dry weight) were placed in vials with two volumes of sterile B-H medium and incubated aerobically at 27°C in a shaker-incubator for seven d.

8.1.4 Extraction

LAB was extracted from weighed samples (~1 g dry weight) of soil by shaking for 20 min in 2 ml hexane. Aliquots of $1C_{12}$ LAB were added to each soil sample immediately prior to extraction to act as an internal standard. The sample vials were centrifuged briefly at 2000 rpm to separate the soil and water from the organic (upper) phase and 1 ml was taken from the hexane fraction for analysis by GC-MS.

8.1.5 Analysis

LAB was quantified and the nC_{12} LAB I:E ratio was calculated as previously described.

8.2 Results and discussion

8.2.1 Appearance

The columns are shown before and after incubation for 253 d in Plate 8.4. The brownish discolouration of the liquid overlaying the soil in autoclaved control columns was humic material, released due to too rapid filling with liquid from below. This was remedied in subsequent columns by filling more slowly.

In all but the control columns, there was evidence of microbial activity. Horizontal fissures in the soil columns indicated that gas was produced. It is unlikely that these fissures were entirely the result of compaction of the soil due to the effect of gravity because in some columns, an appreciable volume of soil was displaced upwards. The increased pressure in these columns has resulted in much of the liquid being expelled, despite the efforts made to seal the tubes.

The evolution of gas seen in the soil columns mirrors that seen in anaerobic respirometers (see page 131 *et seq.*).

Despite the expulsion of liquid from some columns, there was no visual indication that a vertical redox gradient had formed in any of the columns. Therefore it would seem that there was no significant ingress of oxygen. Some columns had a red colouration at the soil surface, which was probably due to oxidation of reduced iron species by the residual oxygen in the water used to saturate the columns, but this did not penetrate beyond the top few millimetres. The overall lighter colour of columns after incubation was due to the use of a different camera with a flash, and does not represent a real change in soil colour.

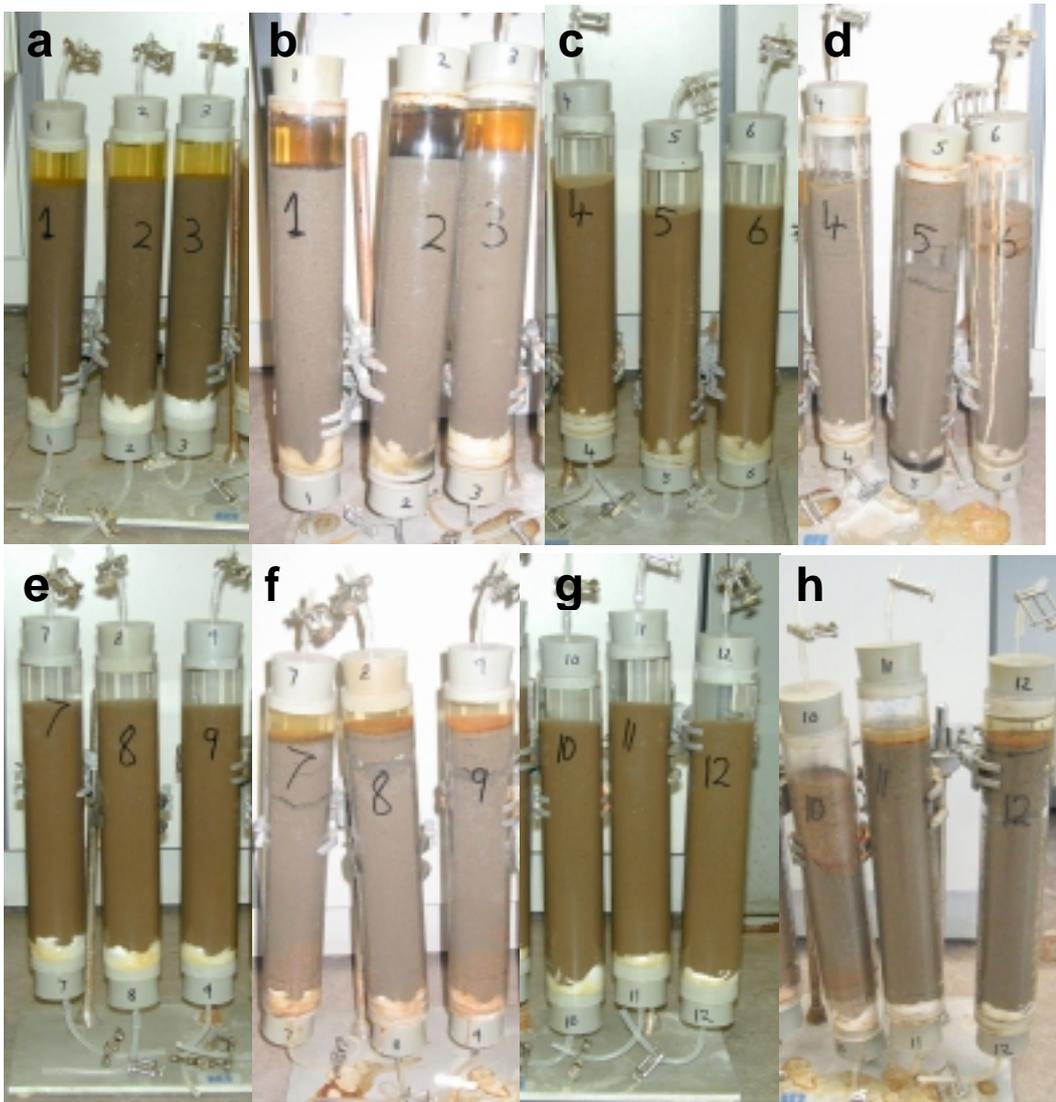


Plate 8.4 Appearance of soil columns before and after 253 d incubation: autoclaved soil before (a) and after (b), saturated with distilled water (c & d), Bushnell-Haas (e & f) and B-H plus nitrate (g & h)

Microbial growth may or may not be due to the presence of LAB in the soil since there was a certain amount of organic material already present (5.8 %, SD = 0.24 %, n = 3). The oil content in the contaminated soil was found to be 0.62%, SD = 0.03 %, n = 3). This was lower than the 1 % added because of sorption to glass and metal surfaces during preparation.

8.2.2 LAB content

The LAB content was obtained for alternate samples to identify whether (a) LAB had migrated vertically within the soil column and (b) whether there had been any degradation over the incubation period. LAB is expressed as a percentage of the total LAB recovered. This is less than 100% because a proportion of the LAB is irreversibly sorbed onto soil particles, apparatus etc.

LAB recovery after 253 d (Figure 8.1) and a further seven d of aerobic incubation (Figure 8.2) did not show any significant differences between treatments (Figure 8.3).

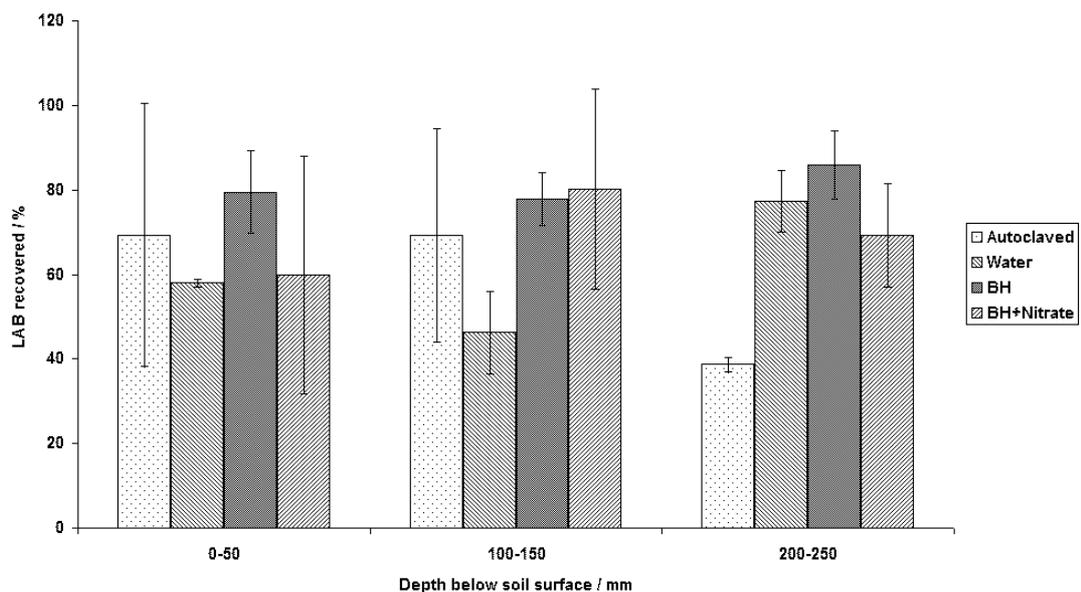


Figure 8.1 LAB vs. depth after 253d (error bars = 1 SD, n = 3)

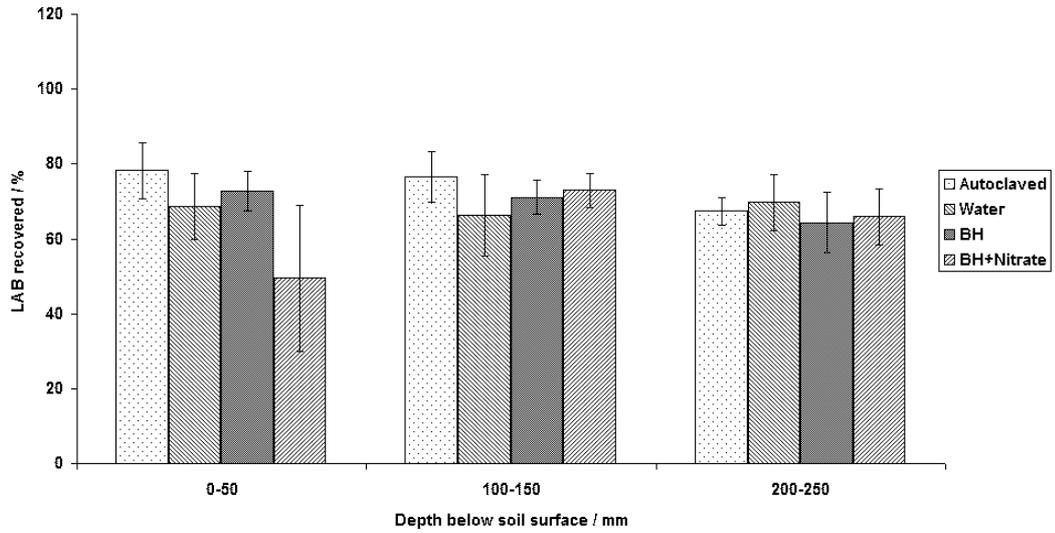


Figure 8.2 LAB recovery vs. after 253 d anaerobic + 7 d aerobic (error bars = 1 SD, n = 3)

There was no significant change in LAB recovery with depth, so the mean recovery from the three samples taken from each treatment was calculated (Figure 8.3).

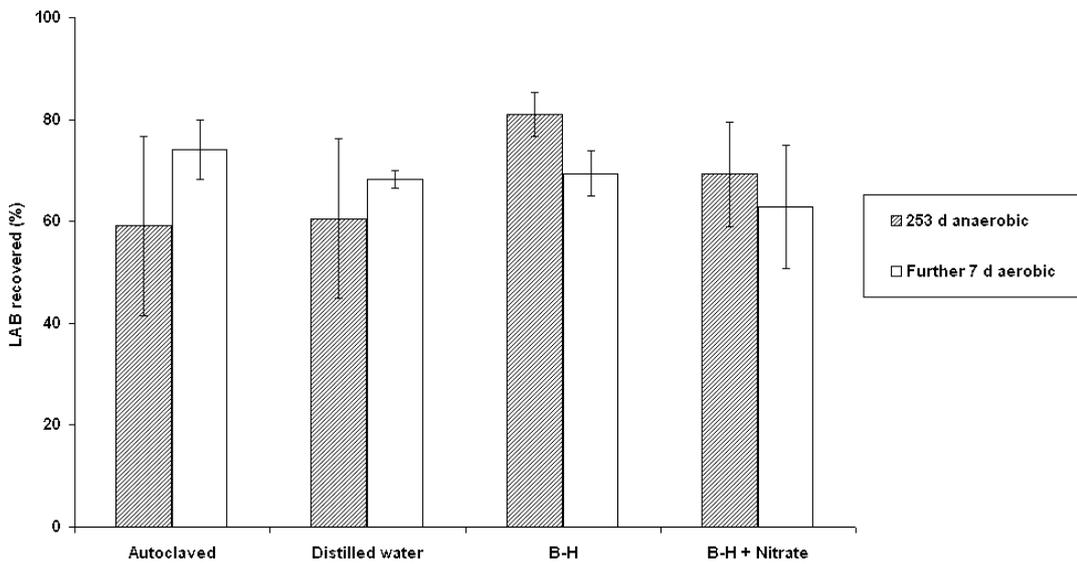


Figure 8.3 Mean LAB recovery (error bars = 1 SD, n = 3)

The ratio of internal to external isomers of nC₁₂ LAB is often used as an indicator of microbial degradation. There was no difference in the ratio relative to the control, after 253 d anaerobic incubation (Figure 8.4) or a further seven d aerobic incubation (Figure 8.5).

However, there was a significant difference between the anaerobic and anaerobic treatment (Figure 8.6). That this difference is seen both in control and experimental treatments suggest that either the autoclaving of soil did not effectively sterilise the soil, or the soil was reinoculated with cable oil-degrading organisms when it was sectioned and sampled. The latter is likely since it was not possible to sterilise the saw blade between columns.

The difference between the aerobic and anaerobic treatment is most clearly illustrated by a plot of 1C₁₂ LAB I:E ratio against LAB recovery. There is no relationship between the two measures after anaerobic incubation (Figure 8.7) whereas after another seven d of aerobic incubation there is a clear inverse relationship (Figure 8.8).

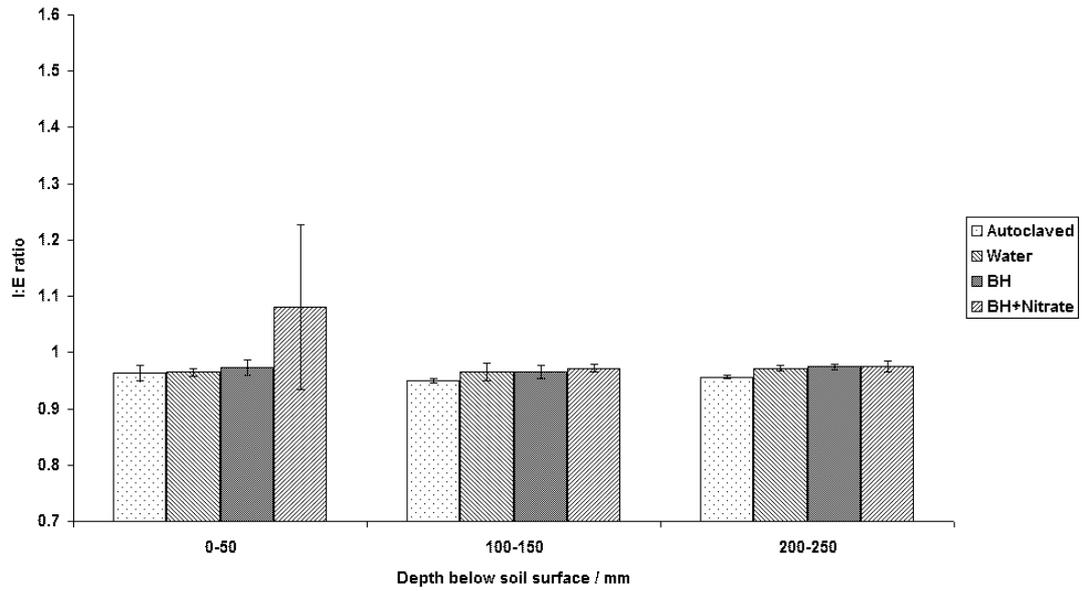


Figure 8.4 nC_{12} I:E ratio vs. depth after 253 d anaerobic (error bars = 1 SD, n = 3)

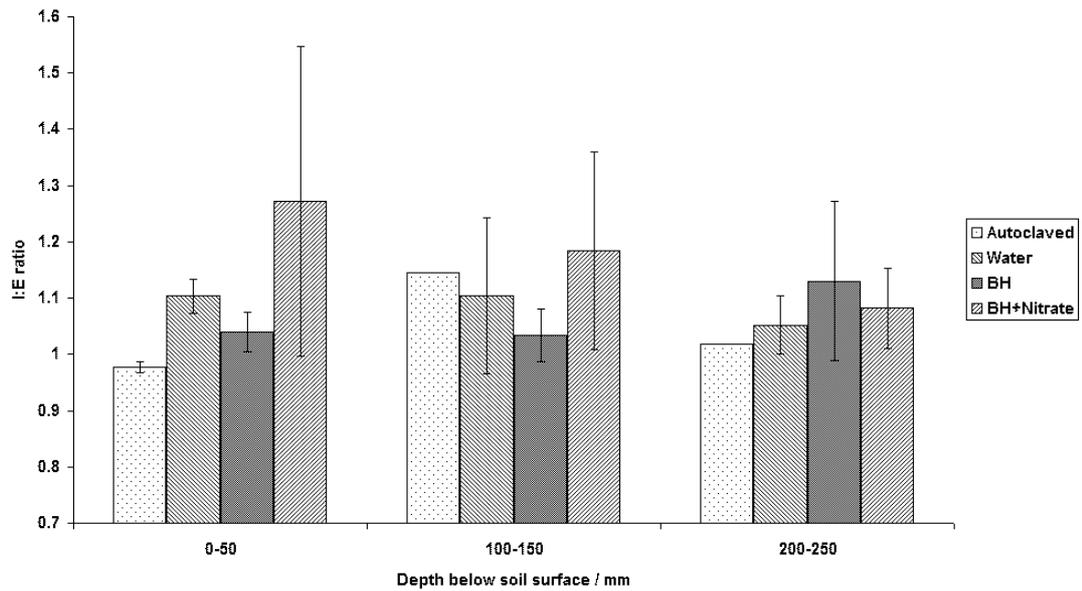


Figure 8.5 nC_{12} I:E ratio vs. depth after 253 d anaerobic + 7 d aerobic (error bars = 1 SD, n = 3)

As seen with the LAB recovery, there was no variation in nC_{12} ratio with depth in a single treatment so the mean value for each column was calculated (Figure 8.6).

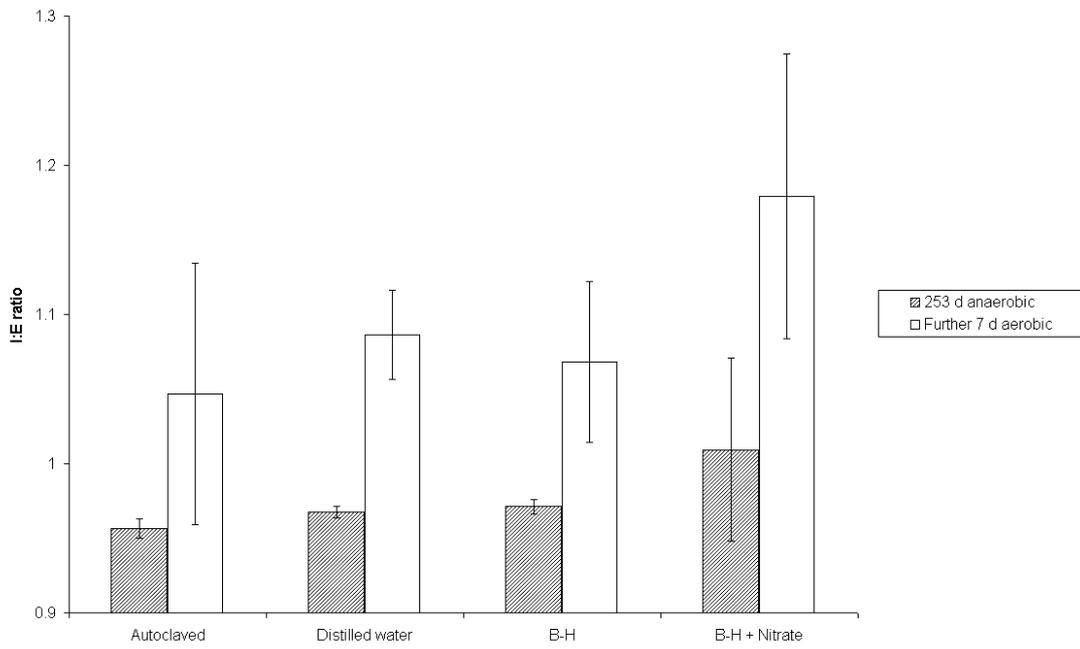


Figure 8.6 Mean nC₁₂ I:E ratio (error bars = 1 SD, n = 3)

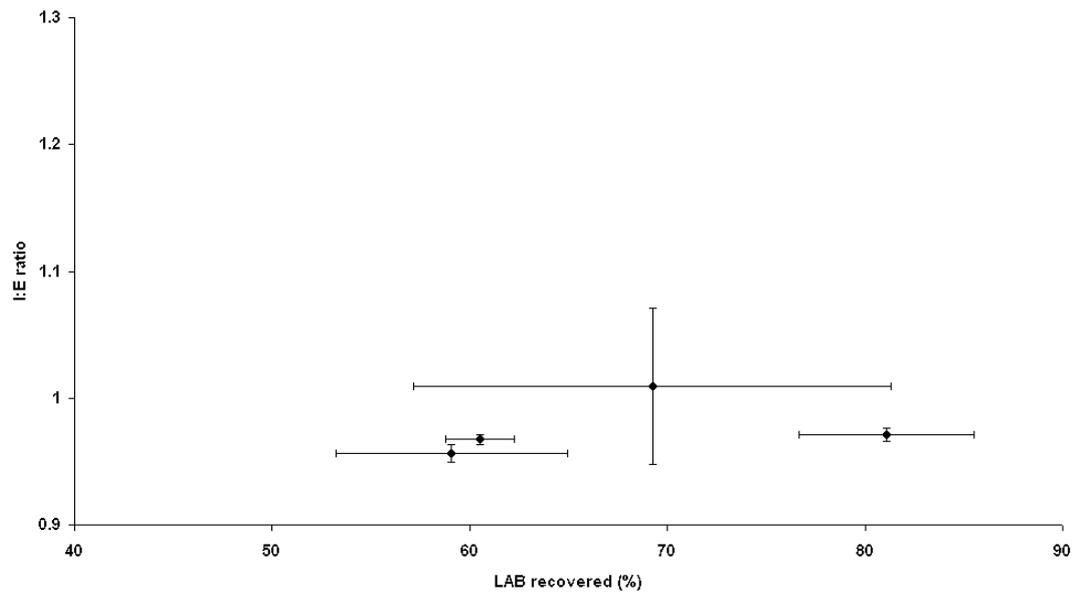


Figure 8.7 I:E vs. LAB recovery after 253 d anaerobic (error bars = 1 SD, n = 3)

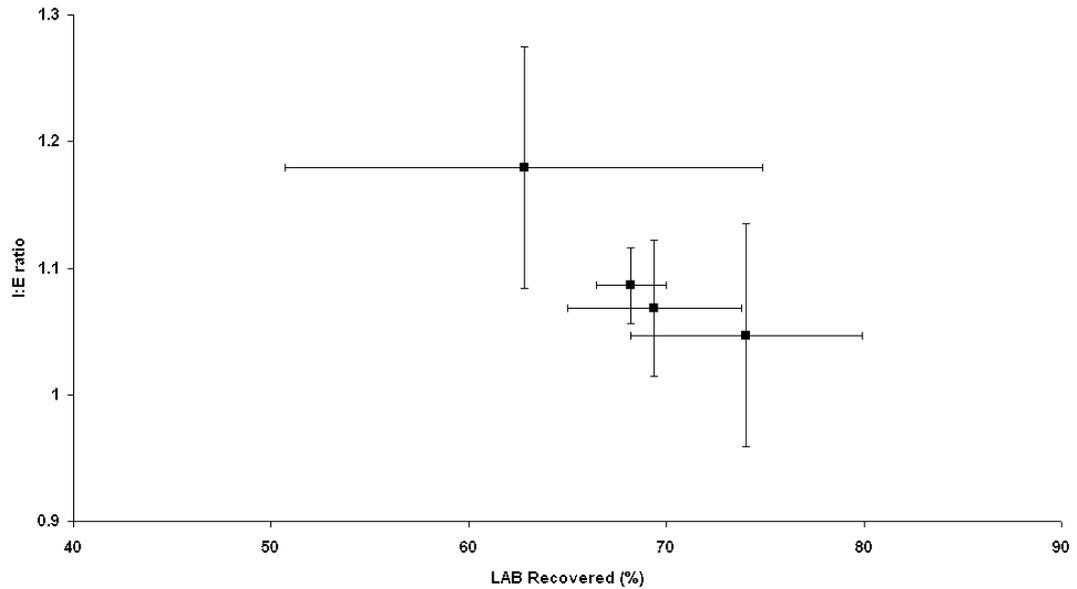


Figure 8.8 I:E vs. LAB recovery after 253 d anaerobic + 7 d aerobic (error bars = 1 SD, n = 3)

The rapid aerobic degradation of LAB following the imposition of aerobic conditions suggests that there was already a population of cable oil-degrading microorganisms (CDM) present in the soil, even though the soil had not previously been exposed to LAB or inoculated with known CDMs.

8.3 Conclusions

- A microbial community of organisms tolerant to the presence of LAB may persist in LAB-contaminated soil
- Gas production in the unsterile columns indicated that there was some microbial activity but there was no significant change in isomeric composition of recovered LAB so it is likely that the majority of the gas was derived from other carbon sources in the soil.

- LAB is degraded rapidly when previously anaerobic soil is exposed to aerobic conditions
- Either the microbial community includes facultative anaerobes, presumably nitrate-reducers that are able to metabolise LAB under aerobic condition, or spore-forming aerobes are able to survive anaerobic conditions, then metabolise LAB when aerobic conditions occur
- The LAB is available to aerobic organisms and so the poor degradation under anaerobic conditions is due either to lack of anaerobic microorganisms with the requisite metabolic pathways, preferential metabolism of other carbon sources, or low availability of LAB under reducing conditions

9 Analysis of GC data from a cable-oil contaminated site

Laboratory experiments have led to the suggestion that nC_{12} LAB internal:external isomeric ratio may provide a tool for identifying and monitoring biodegradation of LAB in soil. In order to test this hypothesis, Dr Daxaben Patel of National Grid Company plc was approached with a view to gaining access to a field site where a cable oil leak had occurred. She was unable to provide access to a suitable site but she did have some historical data from a site that had been assessed by the Environment Agency. It was decided to make use of this data to try to assess the usefulness of I:E ratio in the field.

Data provided on hydrocarbons recovered from the contaminated site were analysed for evidence of biodegradation. There was no evidence of degradation of cable oil in the immediate vicinity of the leaking cable. The composition of linear alkylbenzene recovered from a nearby stream was dissimilar to the new cable oil, and did not represent the major hydrocarbon component. The differences in the LAB may have been due to alternative sources (such as detergent residues) or because of changes in the composition of the oil between the point of release and the stream.

The most likely explanation, however, was that the cable had originally been filled with an older mixture of linear and branched alkylbenzenes and it was this mixture that was seen in the stream, the oil in the cable having been replaced with the newer, better defined mixture. There were differences between the LAB in the stream water and those in sediments from the stream that were suggestive of biodegradation occurring in the sediment.

9.1 Introduction

Gas chromatography – Flame Ionisation Detection (GC-FID) data from the Environment Agency (EA) were obtained from the National Grid Company plc (NGC). The data relate to an un-named site where a leaking underground cable has been excavated. A Microsoft Excel spreadsheet was provided; containing GC-FID peak information for unused linear alkylbenzene cable oil and extracts from water from the excavation, as well as water and sediment from a nearby stream. Details of the extraction and analytical protocols were not provided.

9.2 Methods

The retention time and peak area for each sample were plotted in Excel to visualise the chromatograms. GC-FID does not provide enough information to identify the compound represented by individual peaks so previous GC-Mass Spectroscopy (GC-MS) data from new oil were used to identify peaks in the EA data for fresh oil. The GC signature of fresh LAB is quite distinctive so it was possible to say with confidence that the sequence of peaks in the EA data for new oil was the same as that obtained using GC-MS, even though the method data for the former were unavailable.

Using the retention time information, the equivalent peaks in the chromatograms for oil extracted from water and sediments were also identified. Peaks representing other compounds could not be identified and so were discarded for the purposes of this analysis.

9.3 Results

Figure 9.1 is a graph of peak area for all the supplied data, plotted against retention time. Large peaks eluting in the first few minutes represent the solvent used to extract the oil. The peaks associated with undegraded C₁₀₋₁₃ LAB cable oil are well resolved and elute between 20.16 and 26.55 min. It is clear that these peaks are not the dominant features of the chromatograms from the excavation site or stream water. This is particularly evident in the stream water where there are several major peaks between about 27 and 30 min. The more diffuse elevation in general background seen in the excavation water trace is more likely to be an artefact of the chromatography such as column bleed at higher temperatures.

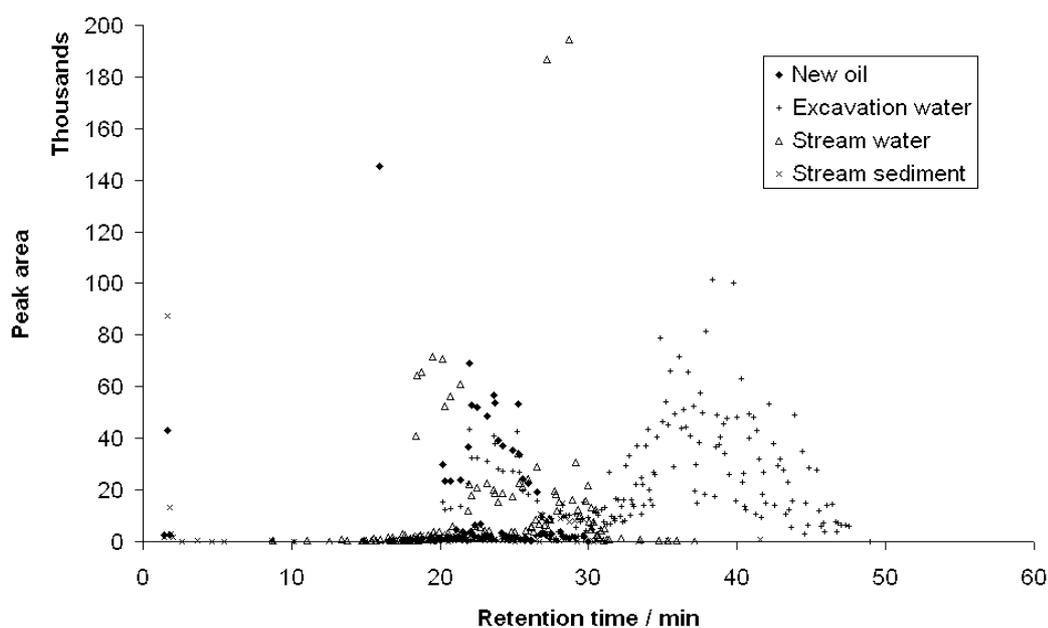


Figure 9.1 Raw data GC data from “new” cable oil and hydrocarbons extracted from a contaminated site, from data provided by National Grid

Table 9.1 shows the retention times of the peaks associated with isomers of linear alkylbenzene cable oil, along with the amount of each isomer expressed as a percentage of the total oil, and the ratio of internal to external isomers of C₁₂ LAB (I:E ratio = $(6C_{12} + 5C_{12}) / (1C_{12} + 2C_{12} + 3C_{12})$).

Table 9.1 Isomeric composition of LAB by % of recovered oil

Retention Time / min	Homologue	“New” oil	Excavation water	Stream water	Stream sediment
20.17	5C ₁₀	4.06	3.04	12.69	13.49
20.34	4C ₁₀	3.20	2.45	9.44	10.10
20.67	3C ₁₀	3.20	2.54	10.07	9.62
21.37	2C ₁₀	3.22	2.74	10.94	9.29
21.89	6C ₁₁	5.00	4.50	2.16	2.46
21.95	5C ₁₁	9.38	8.70	3.97	4.76
22.13	4C ₁₁	7.19	6.51	3.18	3.42
22.49	3C ₁₁	7.10	6.43	3.78	3.32
23.17	2C ₁₁	6.62	6.23	4.04	3.72
23.59	6C ₁₂	7.72	8.14	3.57	4.29
23.66	5C ₁₂	7.30	7.61	3.36	4.23
23.88	4C ₁₂	5.33	5.63	2.75	2.96
24.23	3C ₁₂	5.06	5.42	3.40	2.64
24.90	2C ₁₂	4.79	5.41	3.15	2.79
25.22	7+6C ₁₃	7.25	8.53	6.13	7.28
25.33	5C ₁₃	4.57	5.39	4.02	4.56
25.54	4C ₁₃	3.28	3.92	3.86	3.75
25.90	3C ₁₃	3.08	3.67	4.33	3.48
26.55	2C ₁₃	2.63	3.16	5.18	3.84
	TOTAL	100	100	100	100
	C₁₂ I:E ratio	0.99	0.96	0.75	1.01

The difference between the hydrocarbons recovered from the stream and those seen in the excavation and in unused LAB cable oil was so marked that enquiries were made into the history of the cable. It transpired that the cable had originally been filled with a mixture of linear and branched alkylbenzenes (D. Patel pers. comm.). As the cable had leaked, this had been replaced with the current mixture of C₁₀₋₁₃ LAB homologues.

An unused sample of the “old” cable oil was obtained and analysed by GC-MS as described previously (Figure 9.2). It was compared to an image of a chromatogram of the “old” oil provided by National Grid (Figure 9.3). A sample of “new” LAB cable oil (Figure 9.5) was analysed in the same way.

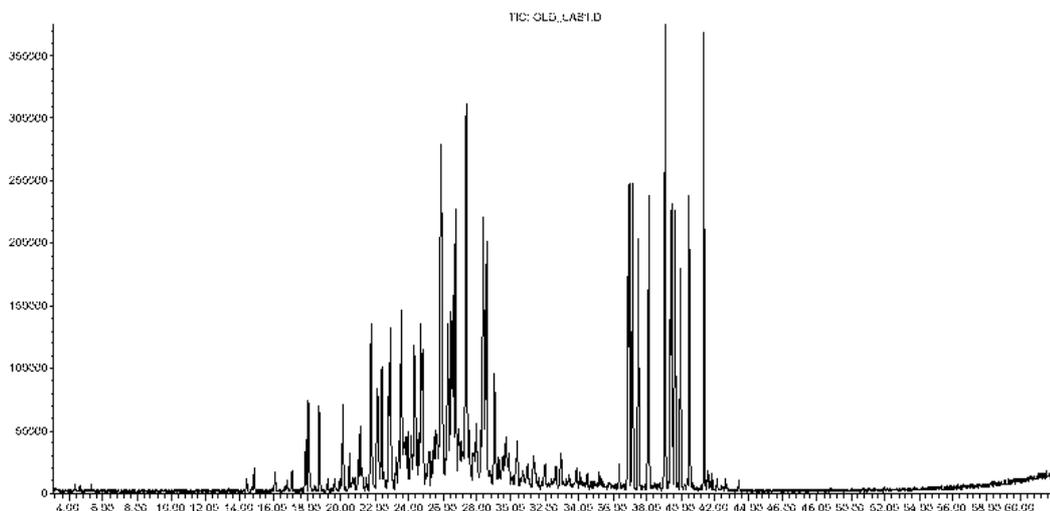


Figure 9.2 Total ion current chromatogram of a sample of unused “old” cable oil

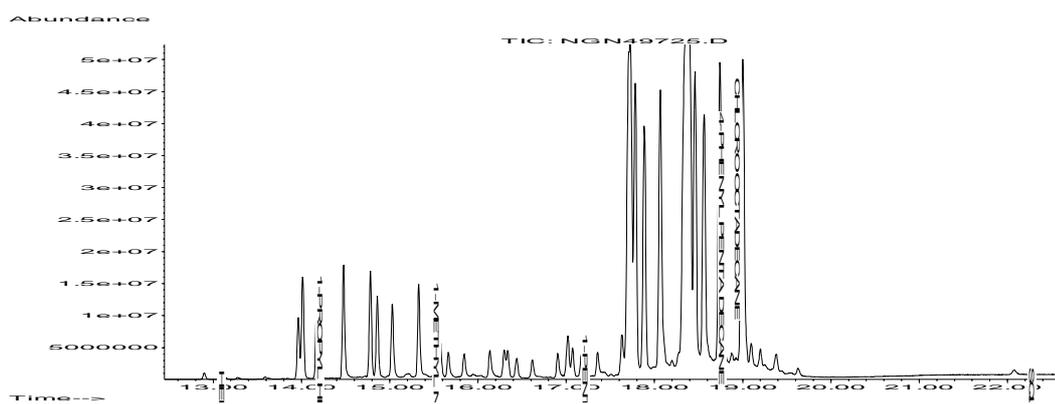


Figure 9.3 Chromatogram of "old" cable oil (courtesy National Grid Transco plc)

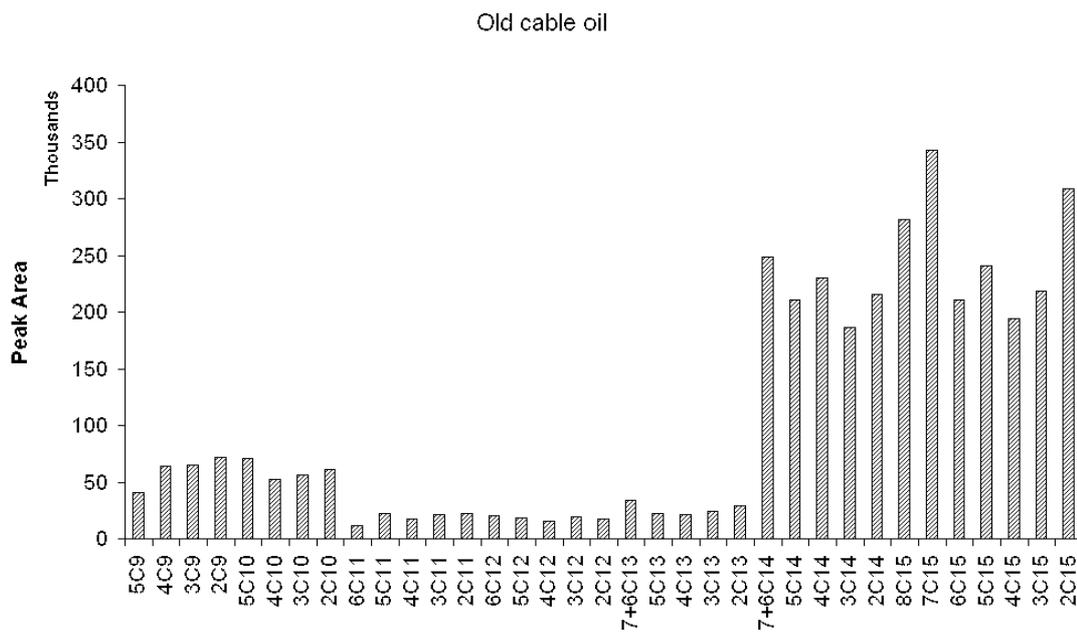


Figure 9.4 Isomeric composition of “old” oil, calculated from data provided by National Grid

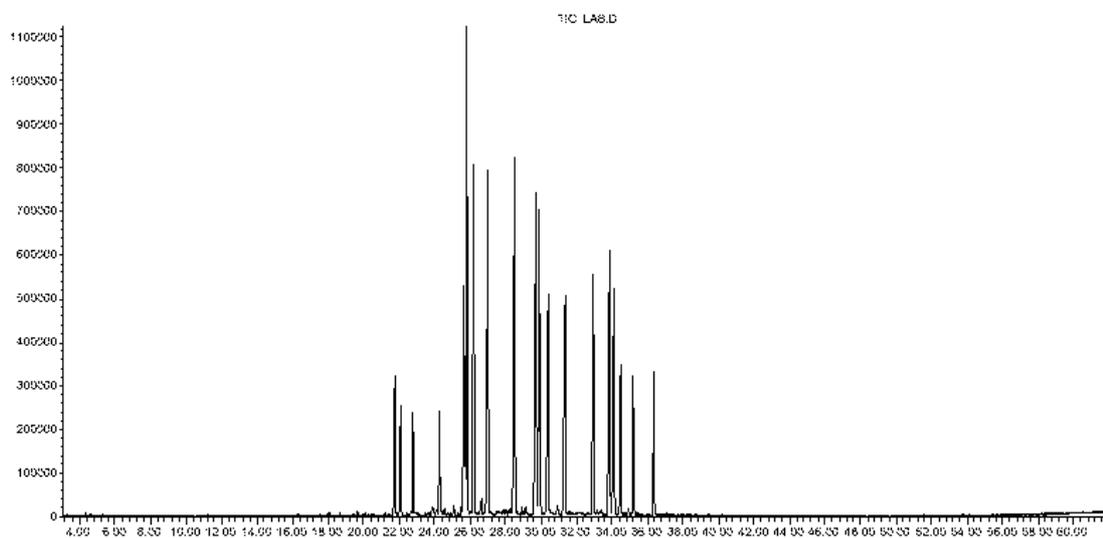


Figure 9.5 Total ion current chromatogram of unused "new" LAB cable oil

The peak areas are plotted for each LAB isomer in the supplied data in Figure 9.6-

Figure 9.9. Note the differing scales on the y-axes. The supplied data did not indicate the amount of water and sediment represented.

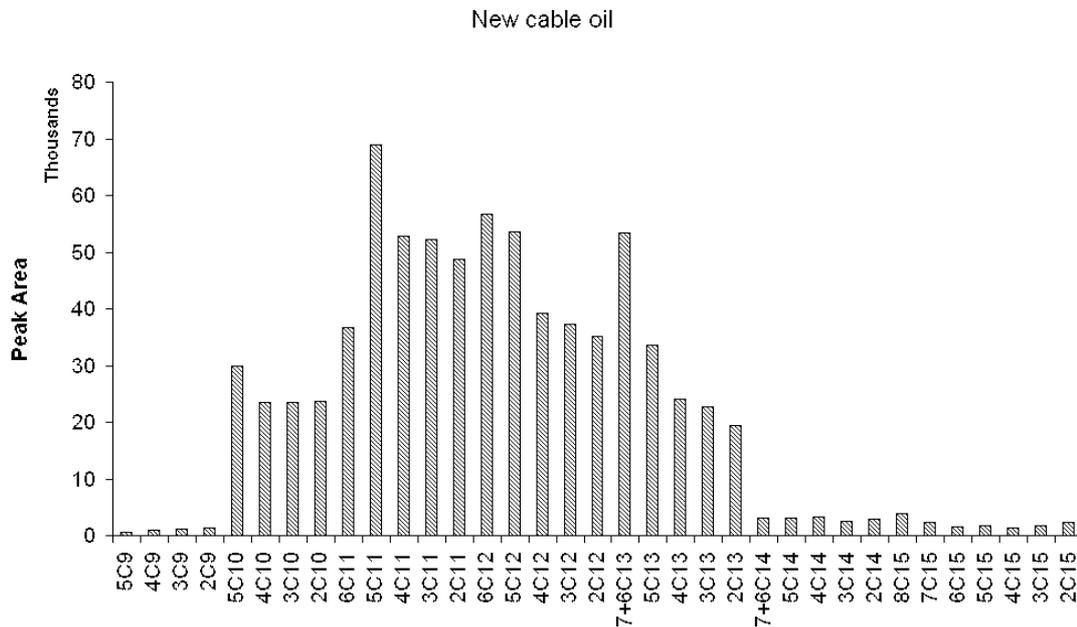


Figure 9.6 Isomeric composition of “new” unused cable oil, calculated from data provided by National Grid

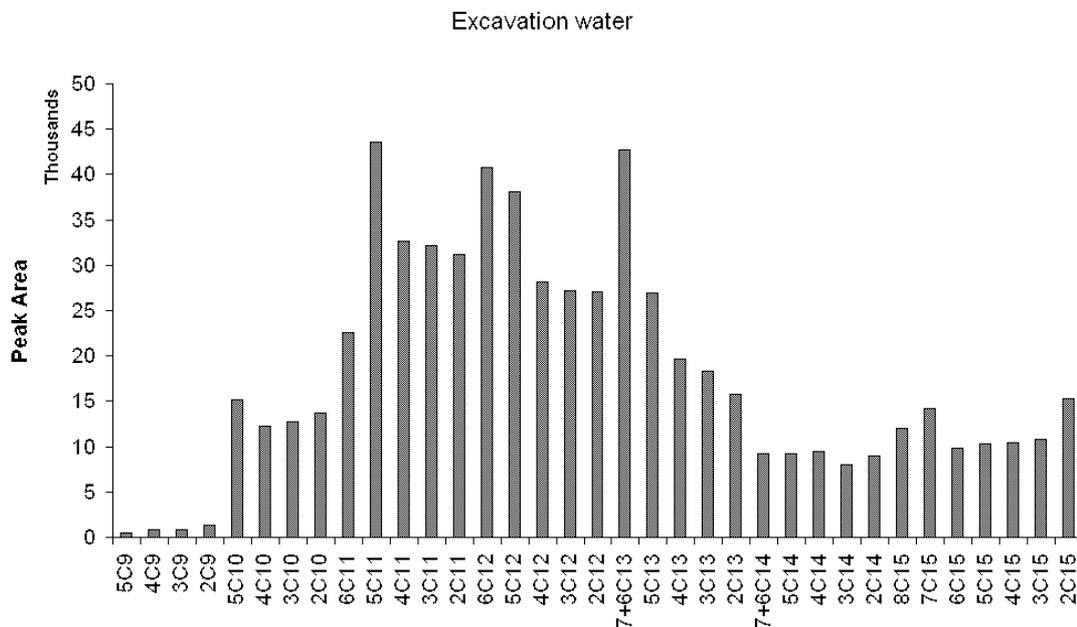


Figure 9.7 Isomeric composition of cable oil recovered from excavation water, from data provided by National Grid

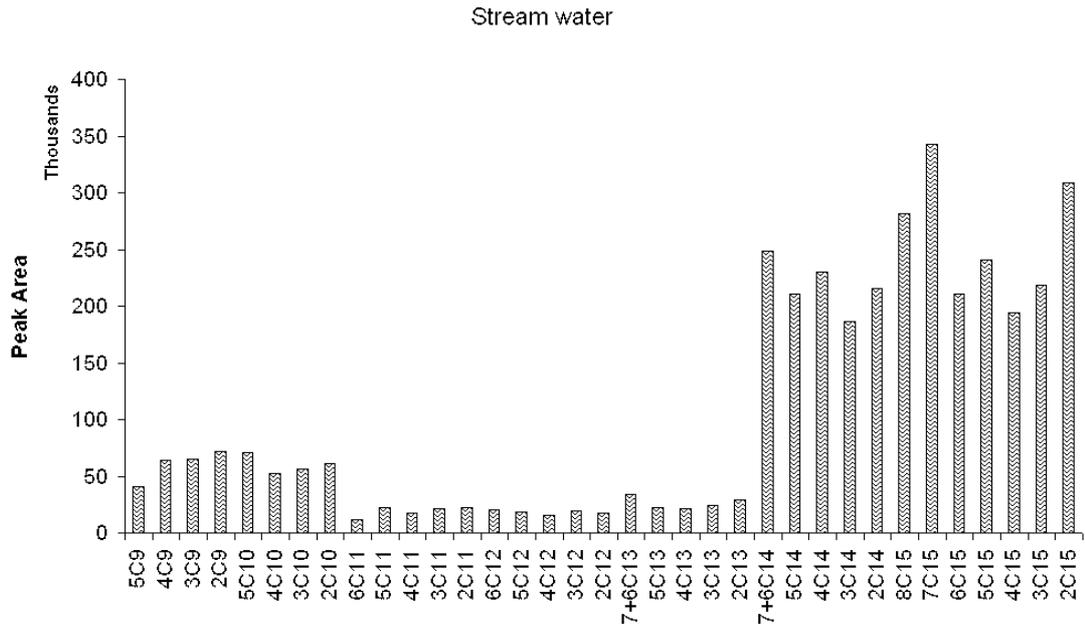


Figure 9.8 Isomeric composition of cable oil recovered from stream water, from data provided by National Grid

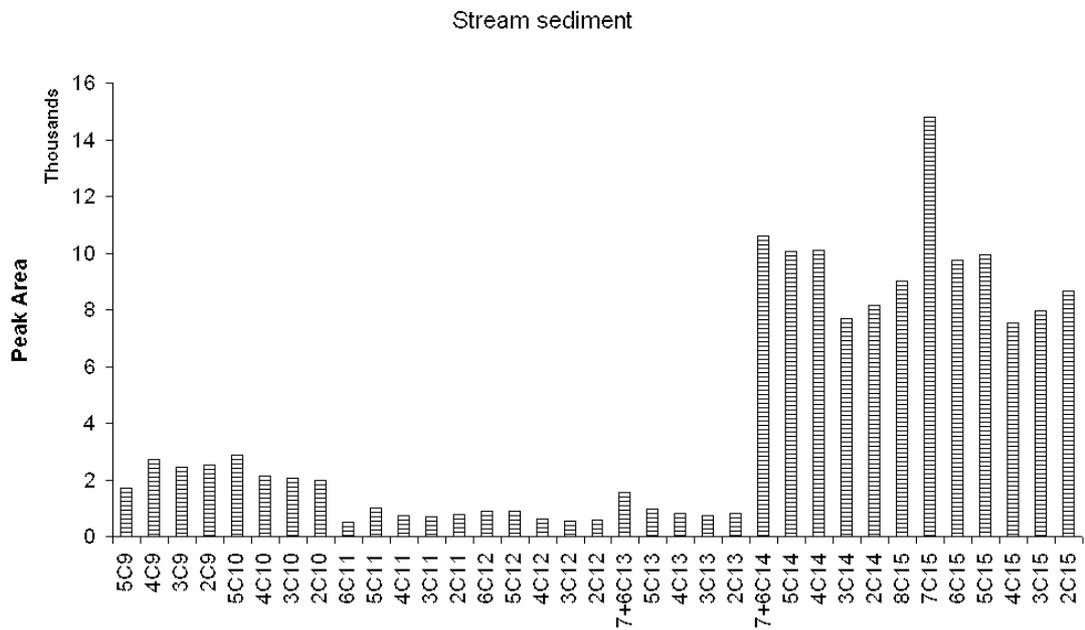


Figure 9.9 Isomeric composition of cable oil recovered from stream sediment, from data provided by National Grid

9.4 Discussion and conclusions

Any substances that elute simultaneously from the column will contribute to the resulting peak. No identifying information beyond the retention time for each peak is available, so it is not possible to say whether the peaks of interest represent only isomers of LAB or if other compounds are also represented. The data must therefore be interpreted with a degree of caution. Peaks other than those from LAB isomers probably represent humic matter (possibly including cable oil breakdown products) and other hydrocarbons such as fuel oils.

A comparison of cable oil from the excavation with that from new oil shows that there is no significant difference. There is, if anything, a small depression of C₁₂ I:E ratio in the oil recovered from the site, but this is within the margin of error for the analytical technique. There is thus no evidence for biodegradation of oil in the immediate vicinity of the leaking cable. This is to be expected since the oil nearest the leak is likely to be fresh from the cable. Not only will there have been a very short contact time between this oil and the extra-cable environment, but also the bulk oil is strongly hydrophobic and tends to form lenses within the soil column, minimizing contact between the oil and the biosphere (Peter Matthews, pers. comm.).

There is a distinct difference between the isomeric composition of LAB recovered from the excavation and that from stream water and sediment. Most notably, there is a large increase in the relative abundance of C₁₀ isomers, and peaks representing higher homologues dominate.

This may be due to a number of factors. The LAB in the stream may be from several current and historical sources, which may include the leaking cable, but might also represent residual LAB from linear alkylbenzene sulphonate detergents. If the oil in the stream is from the leaking cable, there may have been preferential sorption of longer-chain isomers to soil particles between the leak and the point where the oil reaches the stream, though this seems unlikely, based on the findings of Preston and Raymundo (1993).

Whatever processes are at work, it is impossible to say whether the LAB in the stream water represents a degraded or otherwise attenuated sample of that released from the cable based on the available data. To confirm whether the leaking cable is the sole or dominant source of LAB in the stream, it would be necessary to take samples from up and downstream of the supposed point of entry of oil from the cable, as well as soil and groundwater samples from between the leak site and the stream. Comparing the chromatogram of unused “old” cable oil with those from the stream suggests strongly that the LAB from the stream is indeed from the cable.

If the sole input of LAB to the stream sediment is the overlying water column, there is some evidence, in the form of an elevated C₁₂ I:E ratio, that biological degradation of this oil is occurring.

Bulk oil in the immediate vicinity of the leak is indistinguishable from new cable oil, indicating that conditions close to the source are unsuitable for biodegradation.

LABs are not the sole, or even the dominant hydrocarbon present in the stream water. If cable oil is the major input of LAB to the stream, it undergoes profound changes in its isomeric composition between the cable and the stream. This probably reflects a change in the oil used to replace that lost through leakage, rather than appreciable degradation in the soil.

LAB in the stream sediment may be slightly degraded compared to that in the water column. The isomeric shift in sediment LAB is suggestive, but by no means confirmatory, of microbial degradation.

The major conclusion reached from this limited data set is that while isomeric composition is a useful tool in identifying biodegradation of uncontaminated LAB, it must be used with care, taking into account the history of the site. In particular, contamination of LAB with other cable oils such as branched alkylbenzenes or mineral oil will seriously impair the usefulness of the technique.

10 Molecular techniques

10.1 Introduction

The methods used so far have provided a great deal of information on the conditions under which LAB is degraded and on the patterns of degradation seen. However, efforts to isolate and characterise the organisms responsible for the degradation were unsuccessful.

Towards the end of the project, a preliminary investigation was made into the suitability of common molecular biology techniques to assess the diversity of bacterial populations in contaminated soil. The techniques involved were extraction of total DNA from soil, followed by polymerase chain reaction (PCR) amplification of eubacterial 16S ribosomal DNA. The size of the resulting products was confirmed by agarose gel electrophoresis and the products were separated using denaturing gradient gel electrophoresis (DGGE).

Both time and resources were limited by this point and it was unlikely that high quality data would be obtained, but it was felt that it would be a useful exercise to gain experience and an insight into the potential of these techniques for future use.

10.1.1 Isolation of DNA from soil

A variety of methods for obtaining DNA from environmental soil and sediment samples has been described. They can be broadly divided into cell extraction, where whole cells are removed from the matrix and purified before being lysed to release

the DNA within; and direct lysis, where the cells are lysed while still in the matrix and the DNA isolated from the resulting lysate (Miller *et al.* 1999). Cell extraction may result in artificially low yields and the DNA extracted may not be representative of the whole community because of cells adhering to or invading soil particles (Frostegård *et al.* 1999; Courtois *et al.* 2001). Direct lysis is therefore preferable but may result in the co-extraction of PCR-inhibitory substances, though these may be removed chemically or by centrifuging through a filter or a spin column (Jackson *et al.* 1997). Direct lysis generally consists of a physical disruption of the soil or sediment, for instance by bead-mill homogenization, freeze-thaw, ultrasonication, or freezing and grinding in liquid N₂, followed by chemical and/or enzymatic lysis. The best yields are found from bead-mill homogenization but this must be optimised to minimise shearing of the DNA and remove PCR-inhibiting humic acids (Miller *et al.* 1999).

10.1.2 Polymerase chain reaction

The polymerase chain reaction (Mullis & Faloona 1987; Saiki *et al.* 1988) is a powerful means of amplifying small quantities of DNA to yield analytical quantities. It makes use of a thermostable DNA polymerase (“*Taq* polymerase” isolated from the thermophilic bacterium *Thermus aquaticus*), to extend single-stranded “primer” oligonucleotides that are annealed to heat-denatured single-stranded DNA. By repeatedly cycling the temperature, to denature, extend and anneal DNA, the number of copies increases exponentially.

Judicious design of PCR primer sequences can allow great specificity – for instance, individual species of *Rhodococcus* can be discriminated (Bell *et al.* 1999). Alternatively, higher taxa can be amplified by choosing a more conservative primer. In the latter case, discriminatory techniques such as DGGE can be combined with the general abilities of PCR to investigate complex bacterial communities (Ranjard *et al.* 2000; Colores *et al.* 2000; Ibekwe *et al.* 2002; Nicolaisen & Ramsing 2002).

General considerations in the choice of PCR primers are:

- The primer targets are unique and found in the sequence of interest
- Targets are conserved within the taxon of interest
- Approximately 20 bases and same length for both primers
- Sequence contains approximately 50 % A:T and 50 % C:G base pairs
- Two or three C's or G's at the 3' end to ensure specificity
- No complementary sequences within or between primers to avoid folding or dimerisation of primers
- Forward primer is complementary to the 3' end of the sequence
- Reverse primer is reversed and complementary to the 5' end of the sequence

For DGGE use, a high-melting point domain (“a G-C clamp”) is appended to the 5' end of the forward primer to prevent the PCR product becoming completely denatured in the denaturing gel.

10.1.3 Electrophoresis

10.1.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA fragments based on length. It involves loading the DNA into wells at one end of an agarose gel slab submerged in a conductive buffer, and applying an electric field parallel to the gel. DNA carries a net negative charge and so will migrate towards the anode at a rate dependant on its size. The gel structure provides both a stationary phase that modifies the movement of the DNA in a manner analogous to chromatography, and a substrate on which the separated DNA can be handled. The concentration of agarose in the gel can be varied to accommodate a wide range of fragment sizes.

10.1.3.2 Denaturing gradient polyacrylamide gel electrophoresis

The melting temperature of a double-stranded DNA molecule is strongly dependent on its nucleotide base sequence. Guanine - cytosine pairs with three hydrogen bonds are considerably stronger than the two bonds between adenine and thymine and it is possible to calculate the temperature range over which a given sequence will transition between the helical, double stranded and the random, single stranded forms (Lerman & Silverstein 1987). Temperature-gradient gel electrophoresis has been used to separate DNA polymers of similar length but different base sequences. Practical difficulties in maintaining a thermal gradient with the heating effect of the electrophoretic field has lead to the development of a chemical analogue – denaturing gradient gel electrophoresis (Fischer & Lerman 1983; Myers *et al.* 1987), which is capable of separating DNA fragments differing by a single base-pair substitution. Results must be treated with some caution however as since the

technique effectively works on a combination of GC:AT ratio and fragment length, it can cause fragments differing by more than one base to form a single band (Jackson *et al.* 2000). Nevertheless, it has become a common technique in the study of molecular diseases in humans (Fodde & Losekoot 1994) and in monitoring complex bacterial assemblages (Ciric *et al.* 2003).

10.2 Materials and methods

Twelve soil samples from the soil column experiment previously described and seven aqueous cultures were selected. Total DNA was extracted and amplified using PCR primers for eubacterial 16S rDNA. The PCR products were visualised by agarose gel electrophoresis and denaturing gradient gel electrophoresis (DGGE).

10.2.1 Extraction of DNA from soil

Initially it was intended to trial the extraction method described by Miller *et al.* (1999) which consists of a brief bead-mill homogenization step in buffered SDS-chloroform, followed by purification on a Sephadex G-200 column to remove PCR-inhibiting substances. However, time was short and so DNA was extracted from soil samples using a MoBio UltraClean™ Soil DNA Isolation kit (Cam-Bio catalogue #UC-12800-100) which was already available, and which uses a broadly similar protocol of glass-bead homogenization followed by a centrifugal filtration. The kit was several years old but had been stored according to the manufacturers instructions. More recent versions of the kit have included a PCR inhibitor removal solution (IRS). A free sample (2 preps) of the updated kit (#UC-12800-01) was obtained and used to compare the efficiency of extraction with and without the IRS

(Section 10.3.1). Because of the optimisation experiments, a supply of the IRS (#UC-12800-IRS) was obtained and used in all subsequent extractions.

10.2.2 Extraction of DNA from bacterial cultures

Aqueous cultures consisting either of enrichment cultures of LAB-contaminated soil (Johnson 2000), or isolates from these cultures, all grown under anaerobic conditions in B-H medium with LAB as the sole carbon source, were centrifuged to separate the cells from undegraded LAB. The supernatant was discarded and the pellet was resuspended in 1 ml sterile dH₂O. To ensure consistent treatment, DNA was extracted from a 400 ml aliquot of the cell suspension using the same protocol as for soil samples.

10.2.3 16S rDNA Polymerase Chain Reaction

Following extraction of total DNA from soil, part of the bacterial genome coding for the 16S component of ribosomal RNA was amplified using eubacterial primers to yield enough DNA to analyse using DGGE.

10.2.3.1 PCR primers

Choice of the primer sequences to use was aided by the existence of a substantial body of work using 16S rDNA to investigate bacterial diversity in environmental samples. Primers were selected from a selection of commonly used sequences listed by Muyzer, Brinkhoff, *et al.* (1998) and were constructed by MWG Biotech AG:

Forward primer 341F + GC

5'- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC
TAC GGG AGG CAG CAG-3' (57 nucleotides including 40 nucleotide G-C clamp)

Reverse primer 907R

5'-CCG TCA ATT CMT TTG AGT TT-3' where M is an A/C degeneracy (20 nucleotides)

The suitability of the primers for the amplification of eubacterial 16S rDNA was confirmed by searching for matches with the BLAST Basic Local Alignment Search Tool (Altschul *et al.* 1997) on the National Center for Biotechnology Information web site (NCBI 9 April 2003).

Extracted DNA, primers and other PCR components were mixed in a thin-walled PCR tube as per Table 10.1.

Table 10.1 Requirements for PCR of bacterial 16S rDNA

Requirement per tube	Notes	Volume / μl
Biomix	Buffer, nucleotides and Taq polymerase	25
100 pmol/ μ l forward primer	341F+GC	1
100 pmol/ μ l reverse primer	907R	1
Target DNA		1
dH ₂ O		22

10.2.3.2 Thermal cycler program

Amplification was carried out using a Perkin Elmer GeneAmp2400 thermal cycler.

The thermal cycler was programmed as detailed in Table 10.2.

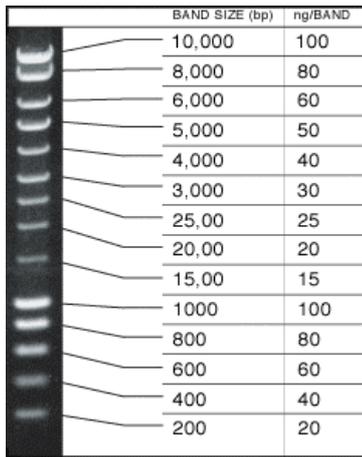
Table 10.2 Thermal cycler program used for PCR amplification of bacterial 16S rDNA

Step	Temperature/°C	Time (min:s)	Notes
1	95	2:00	Denature
2	95	1:00	Denature
3	55	0:45	Extend
4	72	3:00	Anneal
5	Repeat Steps 2-4, 30 times		
6	72	7:00	Anneal
7	4	Indefinite	

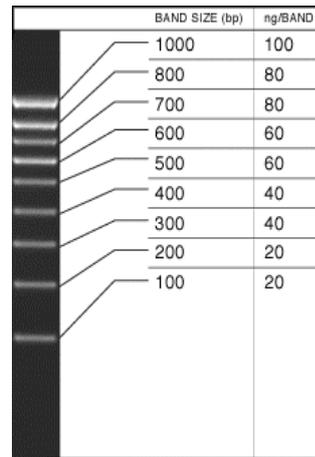
10.2.4 Electrophoresis

10.2.4.1 Mini gel

A 0.8 % agarose mini gel was used to visualise amplified DNA. The size of the band was checked against a calibrated DNA ladder (Figure 10.1) and found to be approximately 600-700 bp. Based on this, a 1.0 % agarose mini gel and a more appropriate ladder (Figure 10.2) were used in subsequent experiments to check that amplification had been successful.



5µl HyperLadder per lane, 1% molecular-biology grade agarose in 1xTAE stained with Ethidium Bromide



5µl HyperLadder per lane, 2% molecular-biology grade agarose in 1xTAE stained with Ethidium Bromide

Figure 10.1 Bioline Hyperladder I

Figure 10.2 Bioline Hyperladder IV

10.2.4.2 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gels were cast using the Bio-Rad DCode System. All gradients were parallel to the vertical axis of the gel.

10.2.4.2.1 Assembly of gel

Glass plates, 1 mm spacers and combs were washed in soapy water, rinsed with dH₂O and ethanol and allowed to dry before the gel sandwich was assembled. The spacers were smeared with a thin layer of silicone grease to ensure a watertight fit. Spacers were laid on the longer glass plate and the shorter plate was laid on top (Plate 10.1).

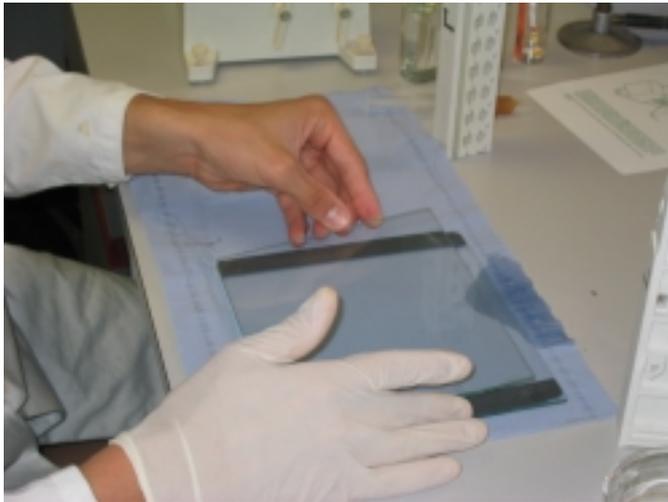


Plate 10.1 Assembling DGGE gel sandwich

The plates were placed in the clamp assembly and the clamps tightened, taking care to ensure that the spacers were vertical, and that they were flush with the bottom edge of the glass plates. Once assembled, the gel sandwich was placed in the pouring frame and cams were tightened to ensure a seal between the glass plates and the grey rubber gasket at the bottom. A thin layer of silicone grease was also used here (Plate 10.2).

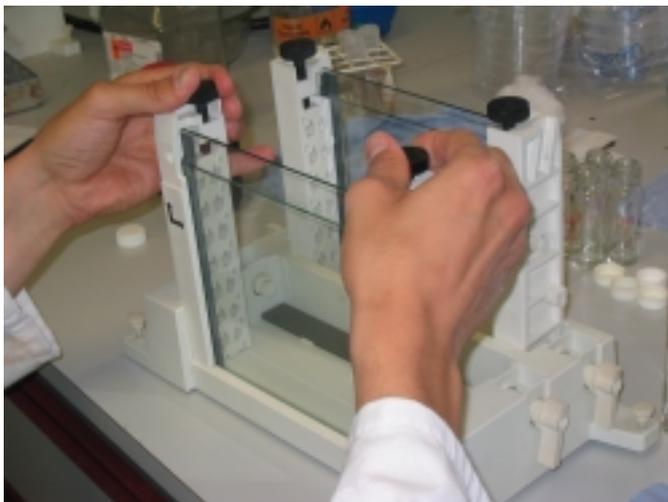


Plate 10.2 Clamping glass plates

The denaturing gradient gel was formed from two solutions (Table 10.3). The DNA fragments produced from the PCR step had been determined to be 600-700 bp so, as per the DCode™ manual, a 6% polyacrylamide gel was poured.

15 ml of each solution were required for each 16 cm gel when using 1 mm spacers and combs. To each solution, immediately prior to casting the gel, 45 µl of tetramethylethylenediamine (TEMED) and 20 µl of a 10% (w/v) aqueous solution of ammonium persulphate were added to catalyse the polymerisation of the gel. Optionally, 300 µl of 0.05 % (w/v) bromophenol blue in × 1 tris(hydroxymethyl)aminomethane/acetic acid/EDTA (TAE) buffer was added to one of the solutions to allow the gradient to be visualised.

Table 10.3 Solutions for 6% polyacrylamide denaturing gradient gel

Component	0% Denaturant (Low density solution)	30 % Denaturant (Alternative low density solution)	100% Denaturant (High density solution)
40% acrylamide/bis acrylamide	15 ml	15 ml	15 ml
× 50 TAE buffer	2 ml	2 ml	2 ml
Deionised formamide		12 ml	40 ml
Urea		12.6 g	42 g
dH ₂ O	to 100 ml	to 100 ml	to 100 ml

A Bio-Rad Model 475 gradient delivery system was used to cast a parallel gradient gel as per the manufacturers instructions (Plate 10.3). This system makes use of a pair of syringes coupled by a calibrated stand and cam arrangement. The syringes are

filled with the appropriate solutions and the cam is turned by hand to deliver the contents of each syringe in the correct proportions.

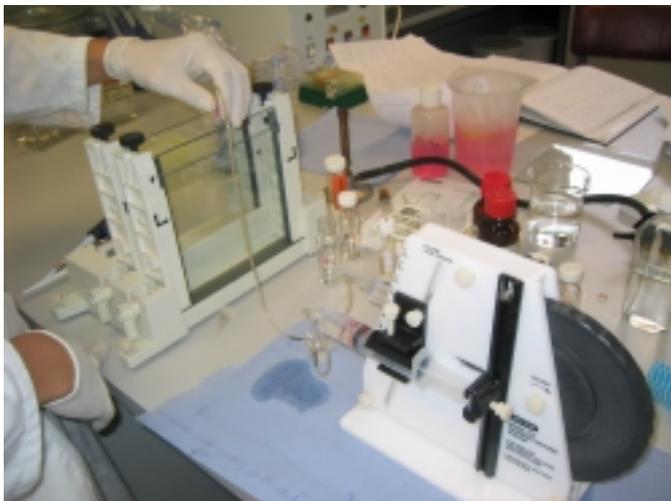


Plate 10.3 Casting denaturing gradient gel. Note gradient former in foreground

Following the pouring of the gel, a 1 mm comb was inserted to the required depth and the gel was left to polymerise overnight (Plate 10.4).

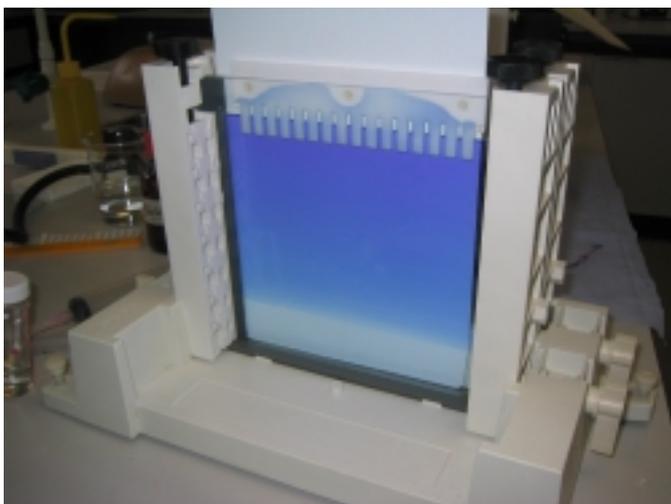


Plate 10.4 Gradient gel incorporating bromophenol blue to visualise gradient

10.2.4.2.2 Electrophoresis

The electrophoresis tank was filled with 7 l of $\times 1$ TAE buffer and preheated to 60°C. The gel sandwiches were mounted in the electrophoresis tank core and placed in the electrophoresis tank.

The buffer-circulating pump was turned on and the upper buffer chamber, formed by the glass plates sealing against the white U-shaped gasket on the inner core, was checked to make sure it was not leaking. The gel was left like this for several minutes to ensure that any unpolymerised acrylamide was washed out of the wells.

Wells were loaded with 180-300 ng DNA with 50 to 70 % loading dye and the electrophoresis was run at 100 V for 18 h (Plate 10.5).

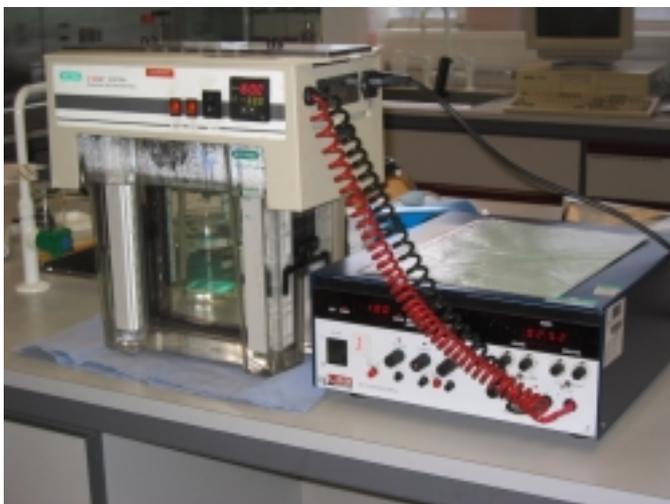


Plate 10.5 Electrophoresis tank and power supply

10.2.4.2.3 Staining

After electrophoresis, the power supply was disconnected from the tank and the core was removed and disassembled. The clamps were removed from the gel sandwich assembly and the spacers and short glass plate carefully removed.

The gel, still on the longer glass plate was washed in ethidium bromide (25 μ l of 10 mg/ml ethidium bromide in 250 ml of $\times 1$ TAE buffer) for 15 min, then destained in 250 ml of $\times 1$ TAE buffer for 15 min (Plate 10.6).

10.2.4.2.4 Gel handling

Before removing the gel from the glass plate, the top right-hand corner was cut off to ensure that the orientation of the gel was known. In order to move the gel from the glass plate to the transilluminator without damage, a sheet of Whatman Number 1 filter paper large enough to completely cover the gel was laid on top of it. The air bubbles were smoothed out and the filter paper lifted up. The gel adhered to the paper, greatly facilitating the handling (Plate 10.7). To remove the gel from the paper, it was placed gel down on a smooth clean surface (such as a transilluminator plate) and the paper moistened with dH₂O. This allowed the paper to be peeled off the gel. This operation can be repeated several times, using a fresh sheet of filter paper each time, without damage to the gel.

10.2.4.2.5 Visualisation

Gels were transilluminated and photographed using the GeneSnap System, and SynGene GeneTools software version 2.11.03 was used to detect lanes and bands. The data was exported to a Microsoft Excel spreadsheet for further analysis.



Plate 10.6 Staining with ethidium bromide



Plate 10.7 Gels are fragile but may be handled on a filter paper support

10.3 Results and discussion

10.3.1 Method optimisation

To optimise extraction and PCR methods, total DNA was extracted from soil using the MoBio UltraClean™ Soil DNA Isolation kit both with and without inhibitor removal solution (IRS). Eubacterial 16S rDNA in neat extract and two tenfold dilutions was amplified using either 25 or 100 pmol of the forward and reverse primers. A culture of *Escherichia coli* was also amplified.

The PCR products (Table 10.4) were loaded and run at 70 V for 1 h on a 0.8% agarose minigel containing ethidium bromide. The gel was documented using the GeneSnap system (Plate 10.8).

Each sample was visible as a band corresponding to a DNA fragment length of about 600 bp. The brightest bands were seen with undiluted extract, using IRS to remove PCR inhibitory materials, and using 100 pmol of each primer. Accordingly, this was the protocol used for subsequent samples. A diffuse band of shorter DNA presumably corresponded to unreacted primers.

Table 10.4 Optimisation of extraction and PCR parameters (see Plate 10.8)

Lane	Description
1	Undiluted soil extract + 100 pmol primers
2	Undiluted soil extract + IRS + 100 pmol primers
3	10 ⁻¹ diluted soil extract + 100 pmol primers
4	10 ⁻¹ diluted soil extract + IRS + 100 pmol primers
5	10 ⁻² diluted soil extract + 100 pmol primers
6	10 ⁻² diluted soil extract + IRS + 100 pmol primers
7	Undiluted soil extract + 25 pmol primers
8	Undiluted soil extract + IRS + 25 pmol primers
9	10 ⁻¹ diluted soil extract + 25 pmol primers
10	10 ⁻¹ diluted soil extract + IRS + 25 pmol primers
11	10 ⁻² diluted soil extract + 25 pmol primers
12	10 ⁻² diluted soil extract + IRS + 25 pmol primers
13	E. coli + 100 pmol primers
14	E. coli + 25 pmol primers
15	Bioline Hyperladder IV



Plate 10.8 Extraction efficiency of MoBio soil DNA extraction kit, with and without IRS (see Table 10.4 for legend)

10.4 PCR and confirmation of products

16S rDNA from a variety of sources (Table 10.5, Table 10.6) was amplified and visualised in a 1.0% agarose gel (Plate 10.9, Plate 10.10) to confirm that the PCR had been successful. The DNA was obtained from aqueous enrichment cultures and isolates described in Chapter 6; and soil columns described in Chapter 1.

Table 10.5 PCR amplification of 16S rDNA from a variety of sources (see Plate 10.9)

Lane	Description
1	Bioline Hyperladder IV
2	Soil column 1, Sample 1 (Autoclaved soil saturated with dH ₂ O, 0-50 mm)
3	Soil column 1, Sample 3 (Autoclaved soil saturated with dH ₂ O, 100-150 mm)
4	Soil column 1, Sample 5 (Autoclaved soil saturated with dH ₂ O, 200-250 mm)
5	Soil column 4, Sample 1 (Soil, saturated with dH ₂ O, 0-50 mm)
6	Soil column 4, Sample 3 (Soil, saturated with dH ₂ O, 100-150 mm)
7	Soil column 4, Sample 5 (Soil, saturated with dH ₂ O, 200-250 mm)
8	Soil column 7, Sample 1 (Soil, saturated with B-H, 0-50 mm)
9	Soil column 7, Sample 3 (Soil, saturated with B-H, 100-150 mm)
10	Soil column 7, Sample 5 (Soil, saturated with B-H, 200-250 mm)
11	Soil column 10, Sample 1 (Soil, saturated with B-H + additional nitrate, 0-50 mm)
12	Bioline Hyperladder IV



Plate 10.9 Agarose gel electrophoresis of 16S rDNA from a variety of sources (see Table 10.5 for legend)

Table 10.6 PCR amplification of 16S rDNA from different sources (see Plate 10.10)

Lane	Description
1	Bioline Hyperladder IV
2	Soil column 10, Sample 3 (Soil, saturated with B-H + additional nitrate, 100-150 mm)
3	Soil column 10, Sample 5 (Soil, saturated with B-H + additional nitrate, 200-250 mm)
4	NIT enrichment
5	ANA enrichment
6	MET enrichment
7	B-H/NIT/ANA C1 isolate
8	B-H/NIT/ANA W1 isolate
9	B-H/NIT/MET C1 isolate
10	B-H/NIT/MET W1 isolate
11	Bioline Hyperladder IV



Plate 10.10 Agarose gel electrophoresis of 16S rDNA from a variety of sources (see Table 10.6 for legend).

Each lane has a band at the position consistent with the size of product expected. There are no additional bands. Autoclaved soil gives a faint band due either to undegraded DNA or a reduced bacterial population. Unsterile soil saturated with dH₂O yields a clear band, as do samples from unsterile soil saturated with Bushnell-Haas medium, both with and without additional nitrate.

Enrichment cultures and isolates generally give a rather faint band, although the NIT enrichment and BH/NIT/MET W1 isolate are quite clear. This is presumably due to the amount of DNA in the original PCR mixture.

10.4.1 DGGE of PCR products

In order to investigate the diversity of the 16S rDNA in the samples, they were resolved by DGGE as described in Section 10.2.4.2 (Table 10.7 to Table 10.9 and Plate 10.11 to Plate 10.13).

Since no bands appeared in the top half of the gel, it was decided to expand the gradient in subsequent gels to provide better resolution. The 0% denaturant was replaced with 30%.

Table 10.7 Samples applied to first DGGE gel (see Plate 10.11)

Lane	Description
1	Column 1, Sample 3 (Autoclaved soil saturated with dH ₂ O, 100-150 mm)
2	None
3	Column 4, Sample 3 (Soil saturated with dH ₂ O, 100-150 mm)
4	None
5	Column 7, Sample 3 (Soil saturated with B-H, 100-150 mm)
6	None
7	Column 10, Sample 3 (Soil saturated with B-H + additional nitrate, 100-150 mm)
8	None
9	ANA enrichment
10	None
11	BH/NIT/ANA W1 isolate
12	None
13	<i>E. coli</i>

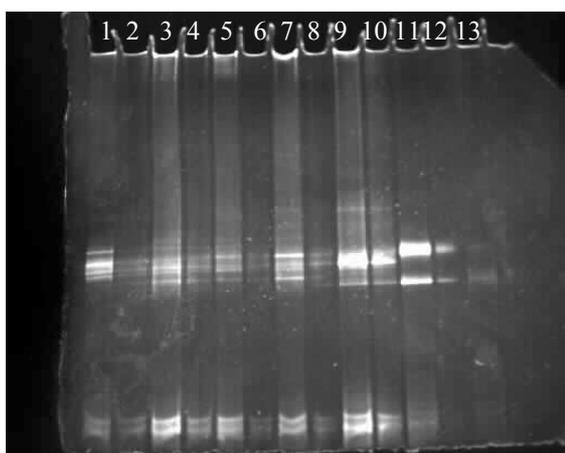


Plate 10.11 DGGE of selected PCR products (see Table 10.7 for legend). Gradient is 0% denaturant at top to 100% at bottom of gel.

Table 10.8 DGGE samples (see Plate 10.12)

Lane	Description
1	Column 1, Sample 1 (Autoclaved soil saturated with dH ₂ O, 0-50 mm)
2	Column 1, Sample 3 (Autoclaved soil saturated with dH ₂ O, 100-150 mm)
3	Column 1, Sample 5 (Autoclaved soil saturated with dH ₂ O, 200-250 mm)
4	Column 4, Sample 1 (Soil saturated with dH ₂ O, 0-50 mm)
5	Column 4, Sample 3 (Soil saturated with dH ₂ O, 100-150 mm)
6	Column 4, Sample 5 (Soil saturated with dH ₂ O, 200-250 mm)
7	Column 7, Sample 1 (Soil saturated with B-H, 0-50 mm)
8	Column 7, Sample 3 (Soil saturated with B-H, 100-150 mm)
9	Column 7, Sample 5 (Soil saturated with B-H, 200-250 mm)
10	Column 10, Sample 1 (Soil saturated with B-H + additional nitrate, 0-50 mm)
11	Column 10, Sample 5 (Soil saturated with B-H + additional nitrate, 200-250 mm)
12	NIT
13	ANA
14	MET
15	Column 1, Sample 1 (Autoclaved soil saturated with dH ₂ O, 100-150 mm)

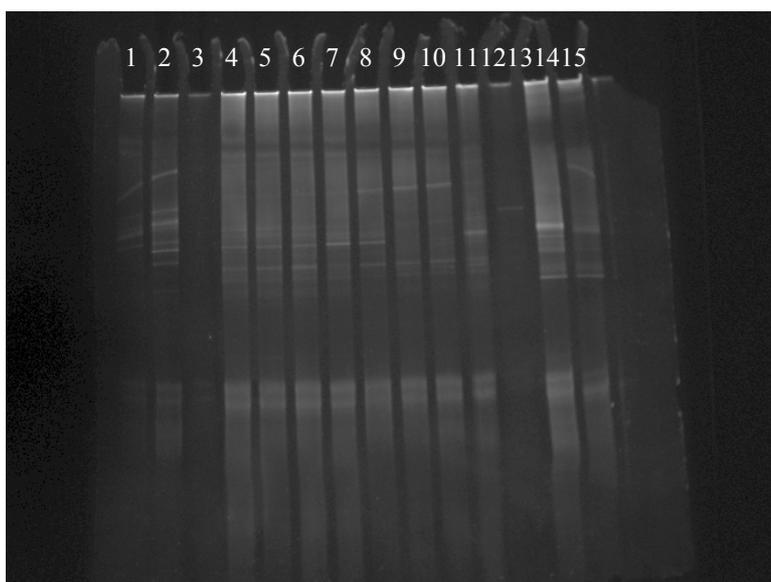


Plate 10.12 DGGE gel (see Table 10.8 for legend). Gradient is 30% denaturant at top of gel to 100% at bottom.

Table 10.9 DGGE samples (see Plate 10.13)

Lane	Description
1	BH/NIT/ANA C1 isolate
2	BH/NIT/ANA W1 isolate
3	BH/NIT/MET C1 isolate
4	BH/NIT/MET W1 isolate
5	Column 1, Sample 1 (Autoclaved soil saturated with dH ₂ O, 0-50 mm)
6	Column 4, Sample 1 (Soil saturated with dH ₂ O, 0-50 mm)
7	Column 4, Sample 3 (Soil saturated with dH ₂ O, 100-150 mm)
8	Column 7, Sample 1 (Soil saturated with B-H, 0-50 mm)
9	Column 7, Sample 3 (Soil saturated with B-H, 100-150 mm)
10	Column 10, Sample 1 (Soil saturated with B-H + additional nitrate, 0-50 mm)
11	Column 10, Sample 3 (Soil saturated with B-H + additional nitrate, 100-150 mm)
12	NIT enrichment
13	ANA enrichment
14	MET enrichment
15	BH/NIT/ANA C1 isolate
16	BH/NIT/ANA W1 isolate
17	BH/NIT/MET C1 isolate
18	BH/NIT/MET W1 isolate

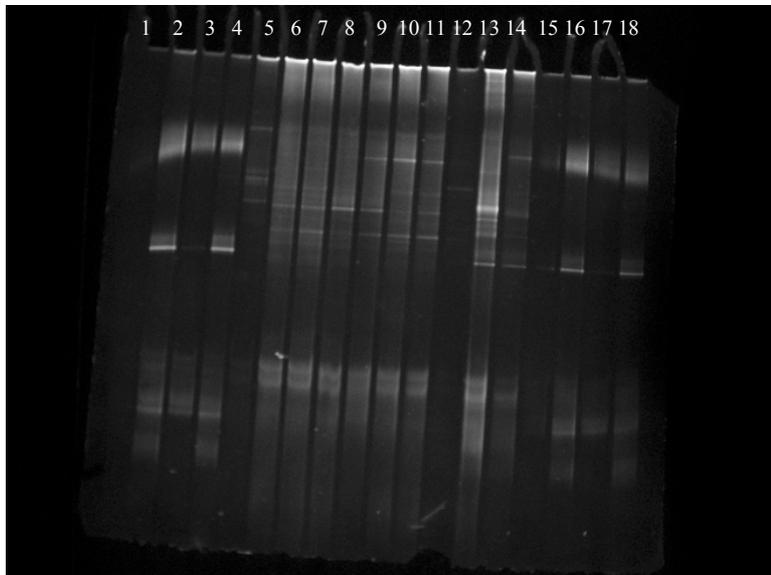


Plate 10.13 DGGE gel (see Table 10.9 for legend). Gradient is 30% denaturant at top of gel to 100% at bottom.

The microbial flora in both the soil columns and enrichment cultures are unknown and no pure strains were included in the gels so the bands in the DGGE gel were arbitrarily assigned alphabetical codes, starting from the topmost band. The results for soil columns were treated separately since there seemed to be few if any bands in common. This was unsurprising since the soil used to inoculate the enrichment cultures was not the same as that used in the soil columns. It is of course possible that some of the same strains were present, however the quality of the results is not good enough to confirm whether this is the case.

The results for soil columns are shown in Figure 10.3 to Figure 10.6. Enrichment culture and isolates were similarly treated and results are shown in Figure 10.7 to Figure 10.12

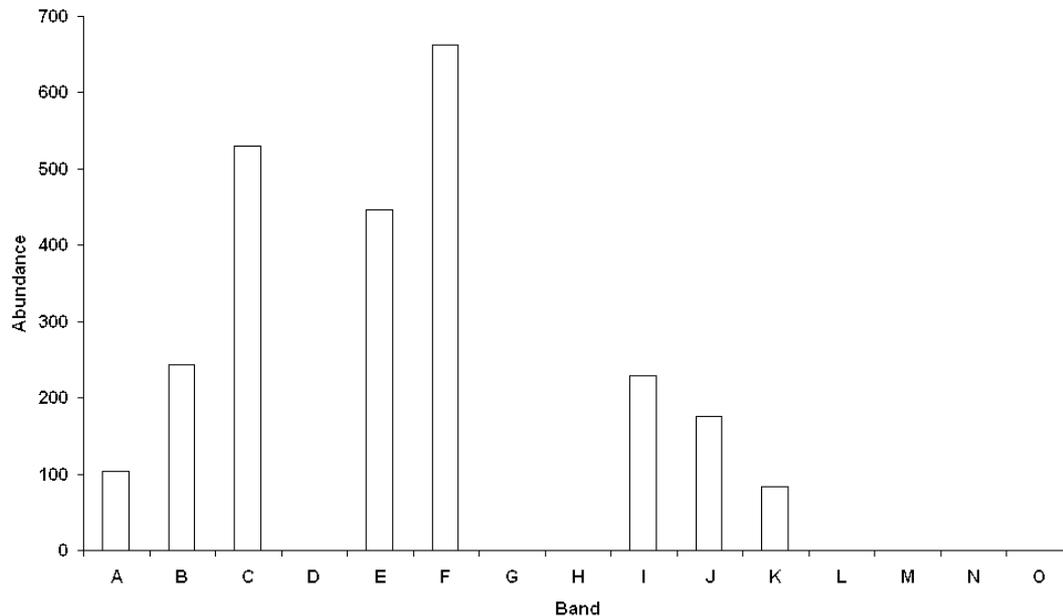


Figure 10.3 Bands seen in DGGE of 16S rDNA PCR products from autoclaved soil columns

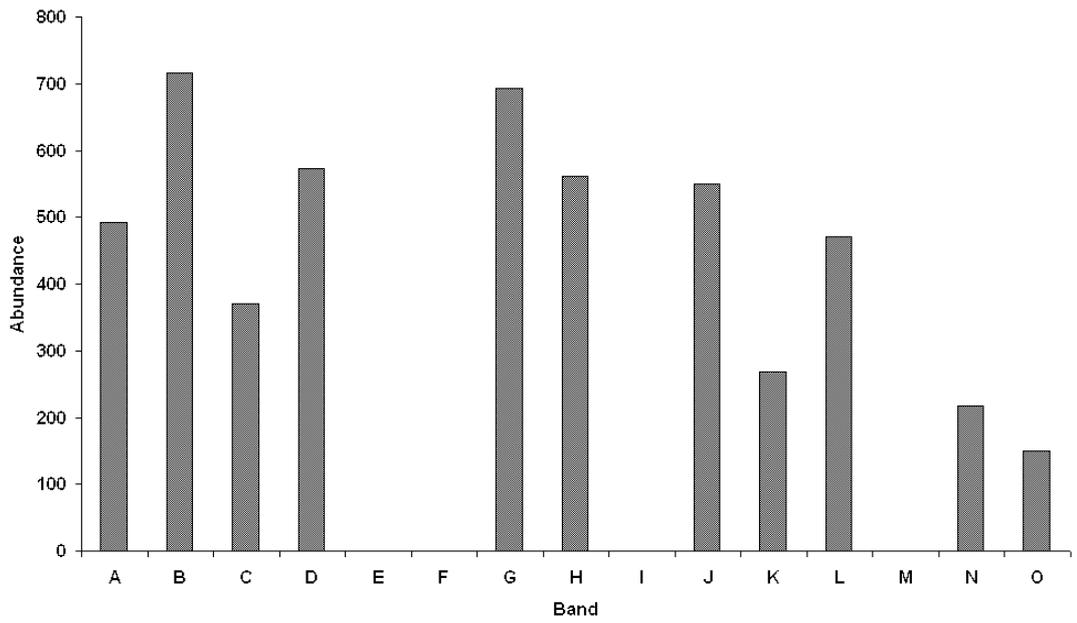


Figure 10.4 Bands seen in DGGE of 16S rDNA PCR products from unsterile soil columns

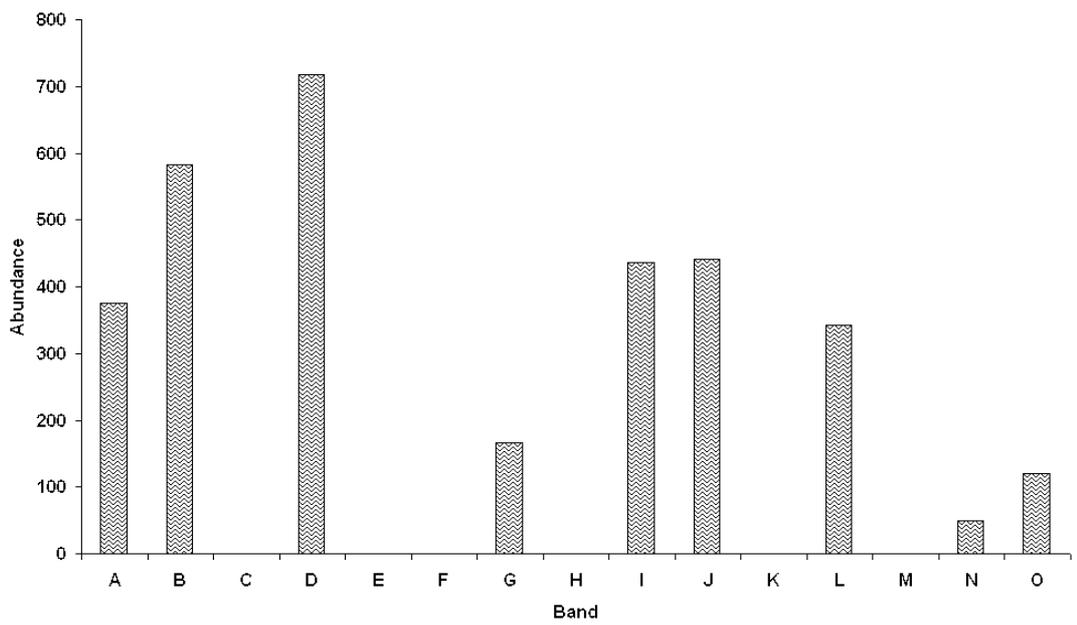


Figure 10.5 Bands seen in DGGE of 16S rDNA PCR products from columns containing unsterile soil plus Bushnell-Haas medium

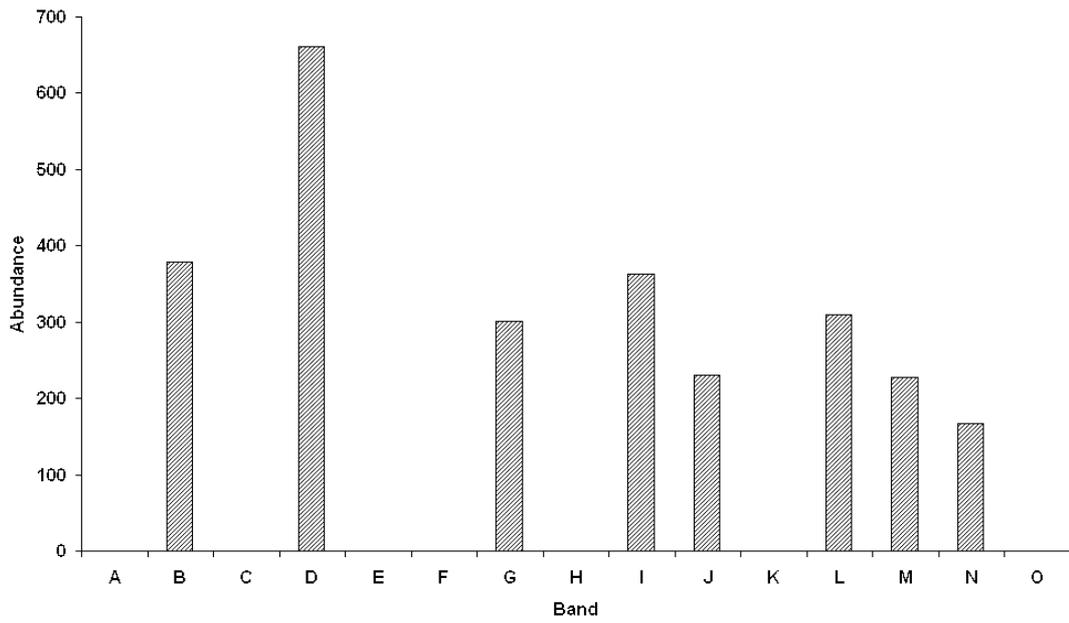


Figure 10.6 Bands seen in DGGE of 16S rDNA PCR products from soil columns containing unsterile soil plus B-H plus additional nitrate

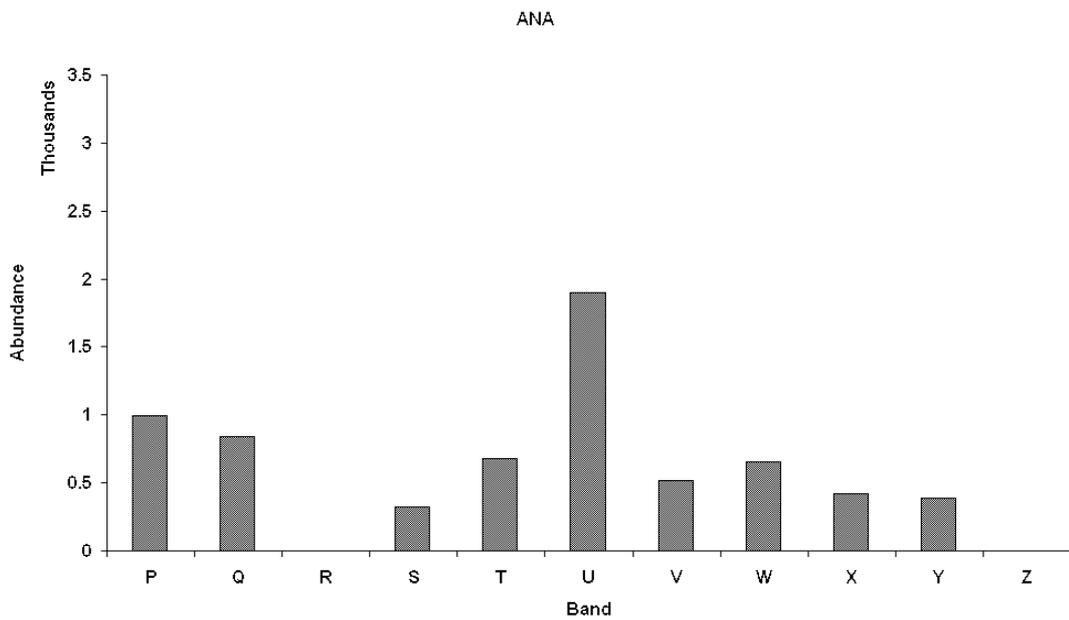


Figure 10.7 Bands seen following DGGE of 16S rDNA PCR products from ANA enrichment culture LAB-contaminated soil

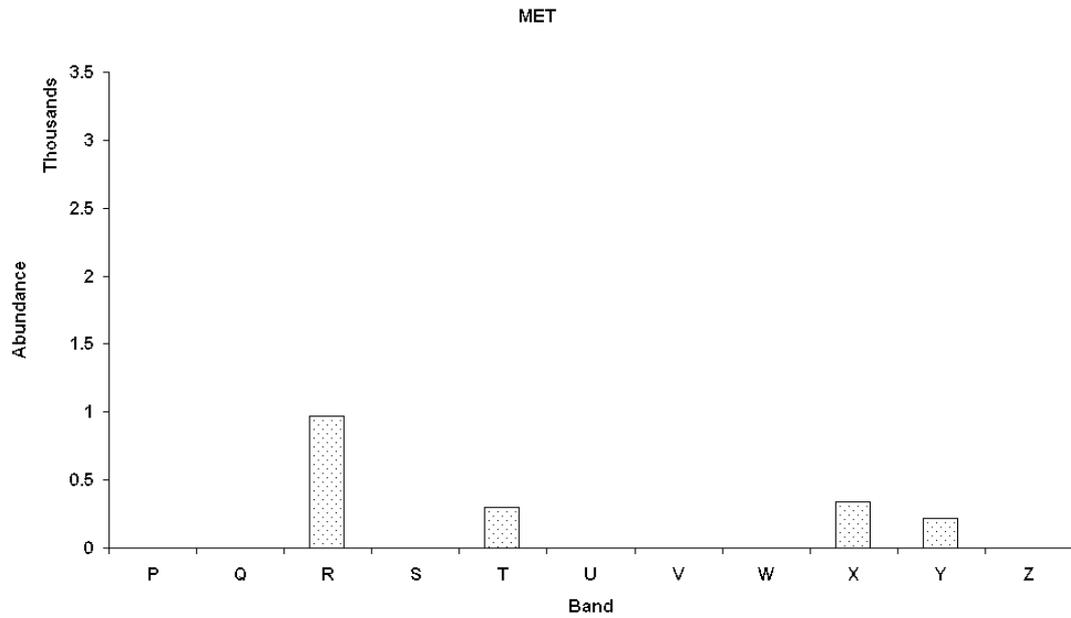


Figure 10.8 Bands seen following DGGE of 16S rDNA PCR products from MET enrichment culture from LAB-contaminated soil

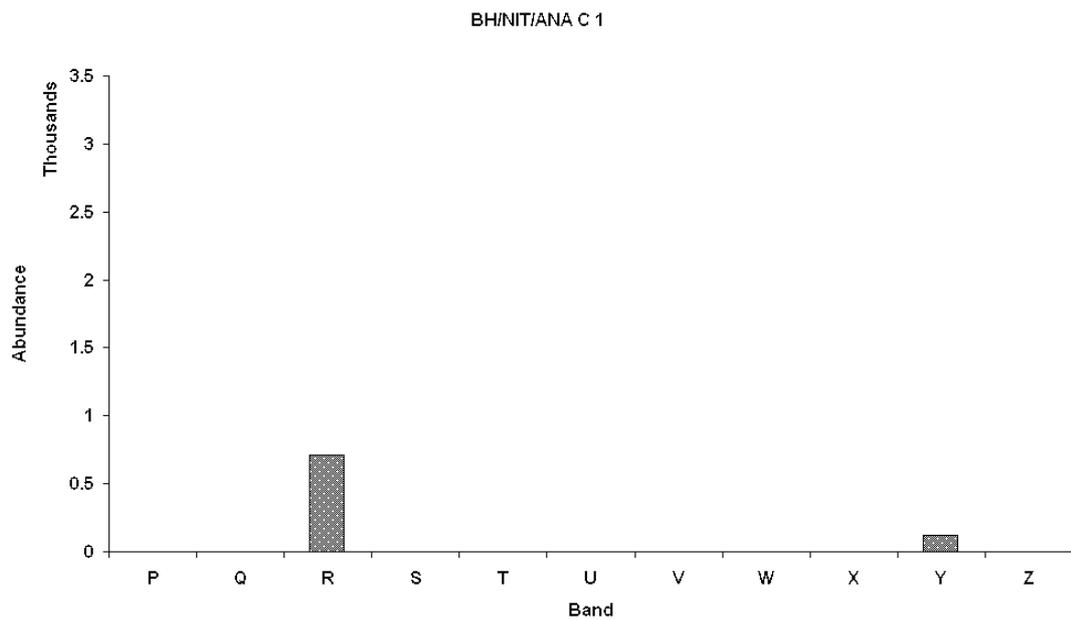


Figure 10.9 Bands seen following DGGE of 16S rDNA PCR products from isolate BH/NIT/ANA C 1 from LAB-contaminated soil

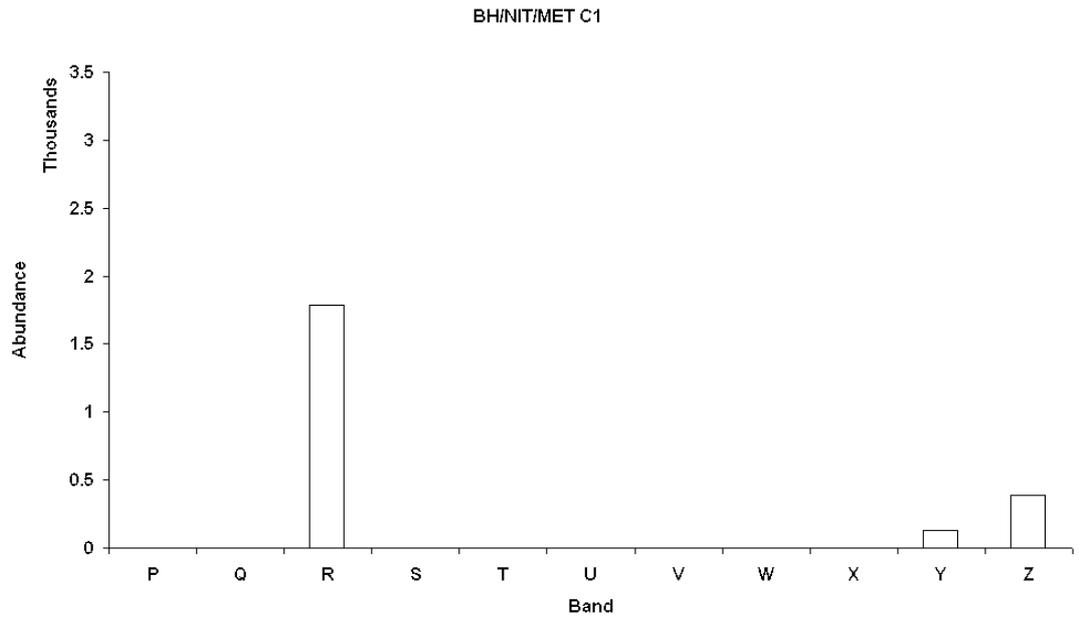


Figure 10.10 Bands seen following DGGE of 16S rDNA PCR products from isolate BH/NIT/MET C1 from LAB-contaminated soil

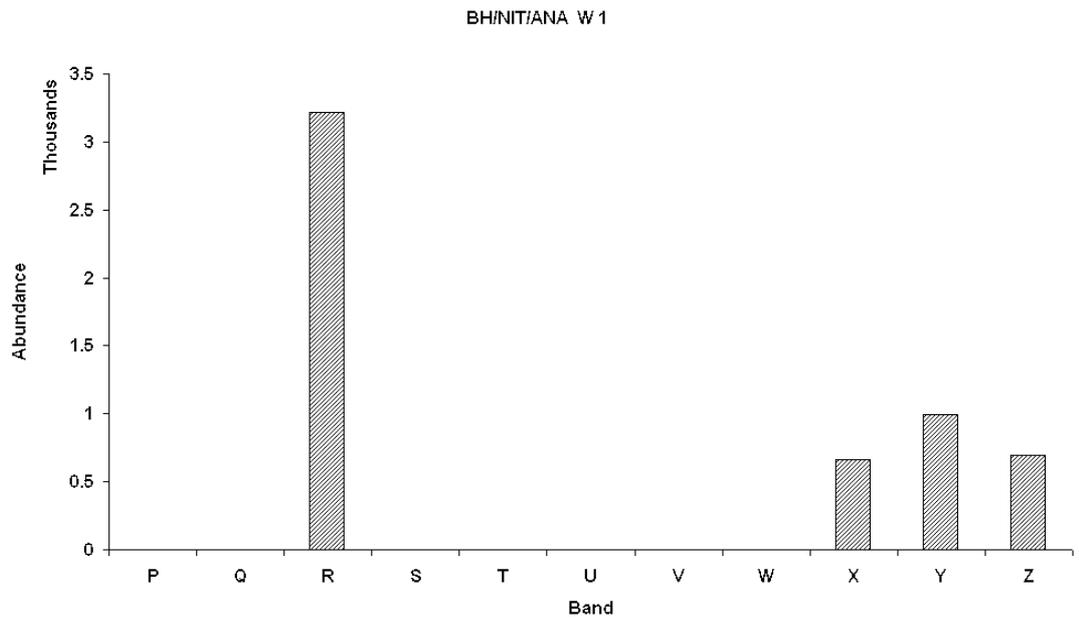


Figure 10.11 Bands seen following DGGE of 16S rDNA PCR products from isolate BH/NIT/MET W1 from LAB-contaminated soil

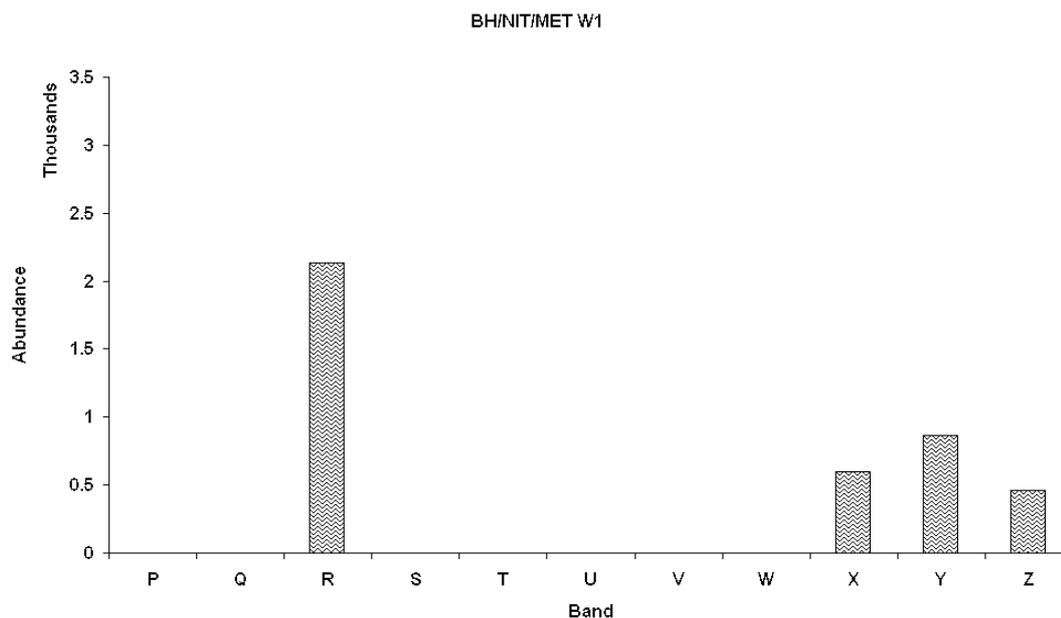


Figure 10.12 Bands seen following DGGE of 16S rDNA PCR products from isolate BH/NIT/MET W1 from LAB-contaminated soil

10.5 Conclusions

This work was carried out late in the project when it became clear that traditional microbiological techniques were not capable of isolating anaerobic cable oil-degrading organisms. It was not expected that DNA extraction, PCR amplification and DGGE analysis would yield publishable results in the time available but it was considered to be a valuable opportunity to learn these standard molecular biology techniques, and to assess their likely utility in monitoring the changes that occur in the microbial community of a contaminated soil.

In addition, the samples used for DGGE analysis, especially those from the soil columns, had been generated to investigate degradation, rather than analysis of changes in bacterial community, so there were no uncontaminated columns to

provide a true picture of the community in the absence of cable oil. In hindsight, this would have been a useful control in any case. The closest alternative was soil that had been autoclaved prior to contamination.

Subject to these provisos, tentative conclusions may be drawn from the results of the investigation:

10.5.1 Soil columns

- Autoclaved soil has a less diversity in eubacterial 16S rDNA than unsterilised soil. This may indicate that the DNA in the soil was degraded by autoclaving, or it may represent a genuine shift in microbial community following contamination
- Soil saturated with additional nutrients and NO_3^- shows a slightly lower diversity than those containing water alone. This is probably due to the added nitrate poisoning the redox potential to favour nitrate-reducers.

10.5.2 Enrichments and isolates

- Nitrate- and sulphate-containing enrichments are more diverse than those with no additional electron acceptors, presumably a reflection of the wide variety of nitrate-reducing organisms found in soils.
- Colonies that appeared identical on plates from each of the enrichments produced similar patterns of banding when separated by DGGE
- There were also striking similarities between visually distinct colony types, suggesting that, despite their gross differences, the organisms were closely related.

- Isolated microorganisms presented multiple bands on the DGGE gels.
 - The colonies may have represented a consortium of organisms
 - If the colonies represented a single organism, their genome may have contained multiple, slightly different copies of the section of DNA coding for 16S rDNA
 - The eubacterial genome may contain unrelated sequences with sufficient similarity that they were amplified by the same primers
 - Additional bands that appear at a high concentration of denaturant may represent a small, high melting point sequence such as might be produced by dimerisation of excess forward primer with its G-C clamp.
- DGGE analysis of PCR products isolated from soil is also very susceptible to poor operator technique. Performance would therefore be expected to improve markedly with practice.

11 Discussion

This project has made use of a wide range of techniques to gain a broad understanding of the effects and fate of cable oil in the subsurface. The main findings of each phase are summarised below.

11.1 Extraction and analysis of cable oil

The simple hexane extraction protocol used throughout this study has the advantage of speed, allowing a large number of samples to be processed in a short period. It is very effective in aqueous samples but is rather dependant upon moisture content in soil samples. In particular, soils that have become very dry, such as small volumes used in aerobic investigations have very low oil recovery rates. This can be partially mitigated by rehydrating the soil before extraction.

It is likely that this is due to a combination of two factors. The most obvious is that in soil slurries, the soil structure is largely disrupted, resulting in intimate contact between the solvent, water and soil particles. In addition, it is likely that as the water in the soil evaporates, capillary forces draw oil into pores in and between soil particles.

This has not been investigated in this study, but is the subject of as-yet unpublished work at the University of Plymouth (P. Matthews, pers. comm.). Once the oil is in place, it will not be fully displaced by rehydration and so a more aggressive

extraction procedure will be required. This was not a major issue in this work since most of the samples were either aqueous or saturated soils.

GC-MS is a proven tool for analysis of linear alkylbenzenes extracted from environmental samples. It has a slightly higher detection limit than GC-FID but the ability of the mass selective detector to positively identify individual peaks makes it preferable for extracts from complex samples.

The inclusion of an internal standard transforms the technique from a comparative to a truly quantitative tool. The quantitative data, along with analysis of the relative amounts of individual homologues, allowed the positive identification both of biodegradation under aerobic and nitrate-reducing conditions, and the common isomeric shift associated with biological degradation in both cases. That this relationship exists suggests that the efficiency of the extraction technique is not critical in the assessment of whether LAB extracted from field samples has undergone biodegradation.

Breakdown products were not seen using GC-MS. This is presumably because the primary degradation of LAB homologues will result in products that are more polar and so have a far greater aqueous solubility. These will not be picked up in a hexane extraction, which is strongly selective for hydrophobic species. In addition, the column used to separate the extract is optimised for non-polar materials.

11.2 Toxicology

Previous work on the environmental toxicology of linear alkylbenzenes has rightly concentrated on its impact on aquatic organisms. By far the largest input of LAB into the environment is from linear alkylbenzenesulphonate detergents and this will remain the case. In addition, there are abundant data on the effects of LAB in mammals.

The ecotoxic effects of LAB in soil has been neglected so far and so the calculation of an LC50 for cable oil to *Caenorhabditis elegans*, a common soil invertebrate, provides an important indicator that LAB can have an adverse effect on soil communities.

There do not seem to be any published data for toxicity of LAB to terrestrial plants. However, anecdotal evidence indicates that grass over a leaking cable dies back (D. Patel, pers. comm.). It is not clear whether this is a toxic effect of the LAB, or if it is a result of disruption to the transpiration stream caused by a lens of oil interrupting the flow of groundwater to the roots in an area with a shallow water table.

11.3 Culture methods

Enrichment cultures and isolates were used to investigate the degradation of cable oil in both aqueous and soil cultures with a variety of electron acceptors. It was not possible to isolate a single organism capable of anaerobic degradation of LAB, however aqueous enrichment cultures from a previously contaminated soil did degrade the oil under both aerobic and nitrate-reducing conditions.

A relationship between isomeric composition and degree of biodegradation was found and shown to hold under both aerobic and anaerobic conditions. This was compared to published data on the aerobic degradation of LAB (Takada & Ishiwatari 1991) and shown to be statistically identical. It was suggested that this relationship could be used to identify biodegradation in the field where neither the initial amount of LAB nor the redox history of the site was known.

11.4 Anaerobic respirometry

Anaerobic soil slurries contaminated with radiolabelled LAB did not show appreciable degradation over a six-month period, confirming that LAB is likely to persist in contaminated sites where anaerobic conditions prevail. Similarly, parallel experiments using non-labelled oil under similar conditions did not show a significant isomeric shift.

There was some evidence for primary degradation in anaerobic conditions but, paradoxically, soil slurry amended with ORC[®] not only had reduced degradation, but also a pronounced immobilization of LAB – possibly due to sorption onto the magnesium phosphate or toxic effects at the concentration used here.

Given that the soil slurries probably represented optimal conditions in anaerobic soil, with added nutrients an electron acceptors and thorough mixing, it is likely that degradation rates in the field may be even slower.

Conversely, aerobic organisms that form hyphae, such as the actinomycetes already associated with aerobic degradation in soil, may not react well to the mechanical disruption introduced by shaking.

11.5 Soil columns

Anaerobic soil columns showed that while a microbial community of organisms tolerant to the presence of LAB persists in a contaminated site, anaerobic biodegradation is so slow as to be negligible. This may be due to the relative abundance of other, more readily metabolised carbon sources in the soil. It is likely that the community includes facultative anaerobes, presumably nitrate-reducers that are able to metabolise LAB under aerobic conditions. Rapid degradation of the oil following exposure to aerobic conditions suggests that this may be the case.

11.6 Analysis of data from a cable oil-contaminated site

The isomeric composition of linear alkylbenzenes extracted from a leaking cable joint bay was compared to that found in a nearby stream to assess the usefulness of isomeric composition as a measure of biodegradation in the field. While it was possible to identify the individual components of the extracts, it was not possible to demonstrate evidence for biodegradation of the oil.

There were several features of the contaminated site that prevented the use of isomeric ratio as the sole measure of biodegradation. First, the cable had originally been filled with oil of a different composition. As this had leaked over an

undisclosed period, it had been replenished by the addition of the more defined mixture that has been the subject of this project. This had lead to a situation where the backfill immediately surrounding the leak was dominated by freshly released “new” oil whereas that in the nearby stream was almost exclusively “old” oil.

Analysis of an archived sample of the “old” oil and comparison to the field data did not show any significant differences. There was a slight enrichment of internal nC12 LAB in the stream sediment but since this was a minor component of the “old” oil, it is not possible to assign significance to this.

As well as the different oils used over the history of the cable, the samples were taken while the cable was being excavated for repair. Therefore, there was a recent input of fresh contamination, which might be expected to mask any isomeric shift in oil that had been released earlier.

If the technique of measuring the isomeric composition of oil in soil is to be of use, it must be restricted to ongoing monitoring of sites that have been historically contaminated with oil of a single, known composition and where the source of contamination has been removed.

11.7 Molecular techniques

Time and resources were only available for a preliminary investigation of the biodiversity of enrichment cultures, isolates and soil columns using denaturing gradient gel electrophoresis (DGGE) of eubacterial 16S rDNA polymerase chain

reaction (PCR) products. Accordingly, the data are limited but they do reveal some basic information about the eubacterial diversity under different conditions. Specifically, the diversity seen in contaminated soil is markedly less than in uncontaminated soil.

11.7.1 Soil columns

The soil columns had been set up to look at LAB degradation, rather than analysis of changes in bacterial community, so there were no uncontaminated columns to provide a true picture of the community in the absence of cable oil. In hindsight, this would have been a useful control in any case. The closest alternative was soil that had been autoclaved prior to contamination. This showed a less diverse community than the unsterilised soil, which may simply indicate that the DNA in the soil was degraded by autoclaving. The columns that were saturated with additional nutrients and NO_3^- seemed to show a slightly lower diversity than those containing water alone. This may be due to the added nitrate poisoning the redox potential to favour nitrate-reducers. Gas production in the unsterile columns indicated that there was some microbial activity but there was no significant change in isomeric composition so it is likely that the majority of the gas was derived from other carbon sources in the soil.

11.7.2 Enrichments and isolates

The nitrate- and sulphate-containing enrichments were more diverse than those with no additional electron acceptors, presumably a reflection of the wide variety of nitrate-reducing organisms founds in soils. The enrichments were spread onto

nutrient agar plates and incubating anaerobically, then the two dominant colony types were picked off and further incubated anaerobically in CPSN medium. The colonies that appeared identical on plates from each of the enrichments produced similar patterns of banding when separated by DGGE. There were also striking similarities between the two colony types, suggesting that, despite their gross differences, the organisms were closely related.

One point of interest is that even the isolated microorganisms presented multiple bands on the DGGE gels. There are a number of possible explanations for this. The colonies may have represented a consortium of organisms. This is unlikely given that they were grown on nutrient agar – a relatively complete medium, and thus unlikely to select for symbiotic organisms. The organisms may have contained multiple, slightly different copies of the section of DNA coding for 16S rDNA, or may have contained unrelated sequences with sufficient similarity that they were amplified by the same primers. Finally, it is possible that the additional bands, which appear at a high concentration of denaturant, represent a small, high melting point sequence such as might be produced by dimerisation of excess forward primer with its G-C clamp. This might have been eliminated if more time had been available to develop and practice the techniques and would be a goal of further work that might be carried out.

12 Conclusions

One aim of this project was to use the knowledge gained to make a recommendation to National Grid Transco plc. on whether monitored natural attenuation was likely to be a sustainable and effective means of remediating soil contaminated following the release of LAB cable insulating oil into the subsurface.

The major conclusions that may be drawn are:

- LAB may have an appreciable effect on both the microbial community of a contaminated soil and on soil invertebrates. It is therefore of significance not only in the aquatic environment, but also as a soil pollutant.
- LAB is confirmed to be readily degraded in aerobic conditions.
- Additionally, LAB can be degraded under nitrate-reducing conditions.
- The degradation seen in different conditions varied widely and this is indicative that degradation in the field will be highly site specific.
- Isomeric composition is a good indicator of extensive biological degradation of LAB but is not useful where the LAB is contaminated with other hydrocarbons such as BAB or mineral oil.
- The relationship between nC_{12} internal:external isomer ratio and degree of biodegradation under aerobic and nitrate-reducing conditions is identical for practical purposes and so may be used to quantify biodegradation where redox history is unknown.
- Anaerobic degradation is likely to be mediated by a consortium of organisms.

- Anaerobic degradation in the field is unlikely to proceed at a significant rate due to the availability of more readily metabolised carbon and energy sources.
- Degradation in the field is expected to be mostly aerobic, either in aerobic soil where the water table fluctuates, or in aquatic environments following migration of the oil.
- The fate of LAB in the field will be heavily site-dependent.
- Natural attenuation – a combination of sorption, dilution, volatilization and biodegradation is likely to be acceptable as a remedial approach for small quantities of LAB in soil.
- Where there is a significant release of oil into soil, it may be necessary to consider an active remediation effort. The fastest would be aerobic bioremediation but since it may be impossible to excavate the soil it may be necessary to impose aerobic conditions *in situ*.
- Oxygen release compound (ORC[®]) may provide the basis of an engineered approach, either as a slurry injection, powder added during the excavation of the source, or as a reactive barrier down gradient of the source to remediate the plume before it reaches a controlled water body. However, its effectiveness should be investigated in detail since the evidence from this investigation suggests that it may actually inhibit degradation by reducing bioavailability of the contaminant.

13 Suggestions for further work

A number of areas addressed in this project would be appropriate avenues for further investigation.

13.1 Field monitoring

While it is clear that nC₁₂ LAB I:E isomeric ratio of LAB is a useful indicator of biodegradation in the laboratory, and it has been used for some time as a marker in aquatic environments, it has not been validated as a monitoring method in contaminated soils. A comprehensive survey of the soil surrounding an historically contaminated site in which the original source has been removed and into which it is known that only the mixture of C₁₀₋₁₃ LAB which has been studied in this project has been released, would be the most appropriate test of whether the oil is biodegraded in the field.

13.2 Other cable oils

Historically, other oils such as mineral oil and other mixtures of linear/branched alkylbenzenes have been used to insulate cables. In some cases, one type has been replaced with another, as the original oil has been lost from a damaged cable or failing joint bay. In such situations, the isomeric ratio is unlikely to provide a reliable indicator and it may be that a more fundamental measure such as stable isotope ratio may be useful and so this avenue should be explored.

13.3 DGGE

This method does appear to have some merit as an indicator of biodiversity, and certainly is more useful than traditional microbiological techniques where it proves difficult to culture a representative community. If it is to be of use it will be necessary to spend an appreciable amount of time on optimizing the extraction method, PCR conditions, gel composition and staining technique.

13.4 Oxygen Release Compound (ORC®)

While it is clear from this work that LAB cable oil can be broken down under nitrate-reducing conditions in aqueous enrichments from a contaminated soil, there is little evidence that LAB is used as a carbon or energy source in soils where other, more readily metabolised organic compounds may be available. It might therefore be the case in future as environmental legislation becomes more stringent, that the enforcing agencies may require active remediation to be undertaken.

As has been discussed, excavation of all the affected soil may be impossible, and the application of existing methods of providing oxygen for aerobic *in situ* bioremediation may have undesirable effects on the stability of the soil. Oxygen release compound, applied to the excavation as a powder, injected as slurry or used in boreholes down gradient of the source to form an aerobic zone cutting across the contaminant plume, could provide a solution. However, its use should be considered with caution given the inhibitory effects seen in this investigation.

14 References

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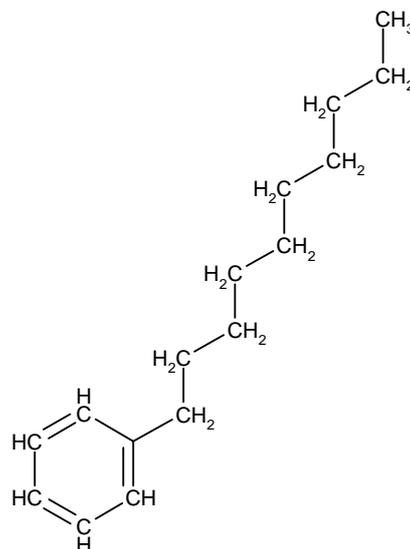
15 Appendices

15.1 Names, synonyms and molecular structures of C₁₀ – C₁₃ linear alkylbenzenes

Homologues in *italics* are not present in cable oil.

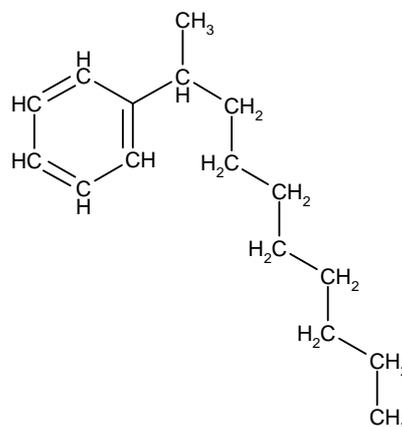
DECYLBENZENES

1C₁₀ *Decyl-1-benzene*
 1-Phenyldecane
 (decyl)-Benzene



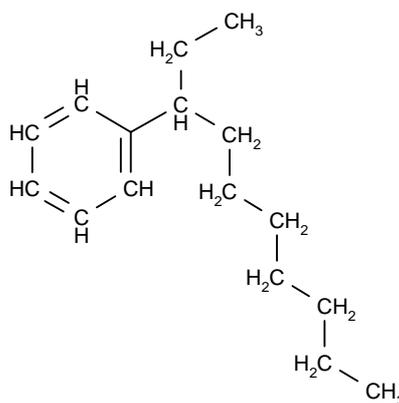
Molecular Weight =218.39
 Exact Mass =218
 Molecular Formula =C₁₆H₂₆
 Molecular Composition =C 88.00% H 12.00%

2C₁₀ *Decyl-2-benzene*
 2-Phenyldecane
 (1-methylnonyl)-Benzene



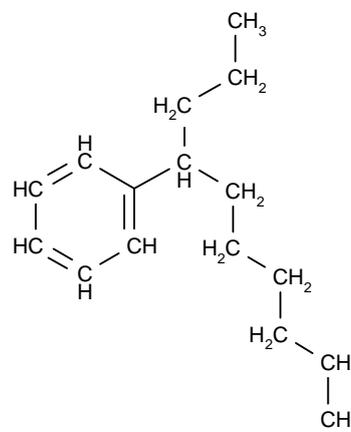
Molecular Weight =218.39
 Exact Mass =218
 Molecular Formula =C₁₆H₂₆
 Molecular Composition =C 88.00% H 12.00%

3C₁₀ Decyl-3-benzene
3-Phenyldecane
(1-ethyloctyl)-Benzene



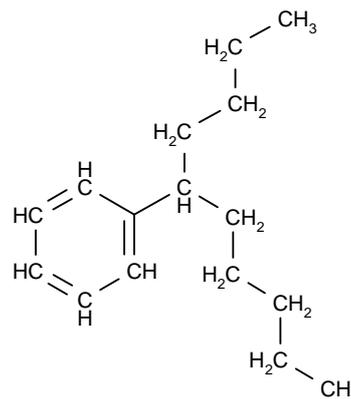
Molecular Weight =218.39
Exact Mass =218
Molecular Formula =C₁₆H₂₆
Molecular Composition =C 88.00% H 12.00%

4C₁₀ Decyl-4-benzene
4-Phenyldecane
(1-propylheptyl)-Benzene



Molecular Weight =218.39
Exact Mass =218
Molecular Formula =C₁₆H₂₆
Molecular Composition =C 88.00% H 12.00%

5C₁₀ Decyl-5-benzene
5-Phenyldecane
(1-butylhexyl)-Benzene



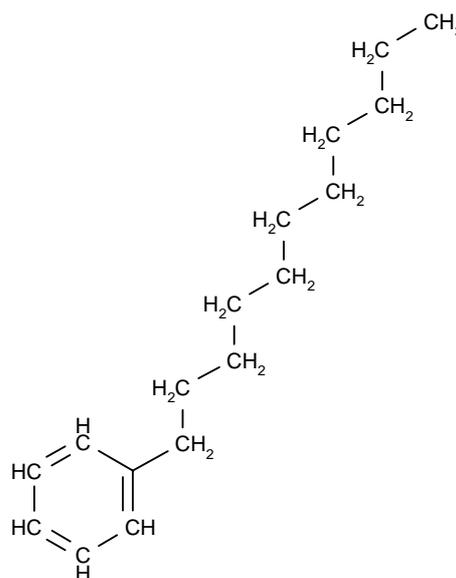
Molecular Weight =218.39
Exact Mass =218
Molecular Formula =C₁₆H₂₆
Molecular Composition =C 88.00% H 12.00%

UNDECYLBENZENES

1C₁₁ *Undecyl-1-benzene*

1-Phenylundecane

(undecyl)-Benzene



Molecular Weight =232.41

Exact Mass =232

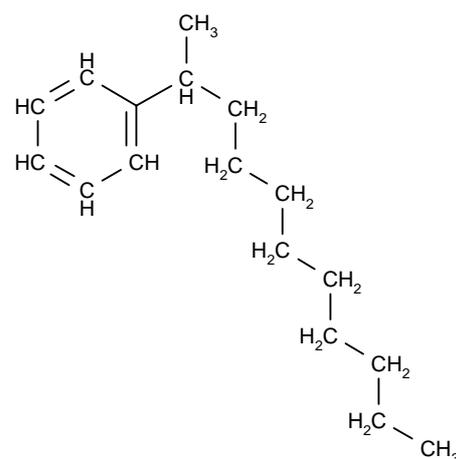
Molecular Formula =C₁₇H₂₈

Molecular Composition =C 87.86% H 12.14%

2C₁₁ *Undecyl-2-benzene*

2-Phenylundecane

(1-methyldecyl)-Benzene



Molecular Weight =232.41

Exact Mass =232

Molecular Formula =C₁₇H₂₈

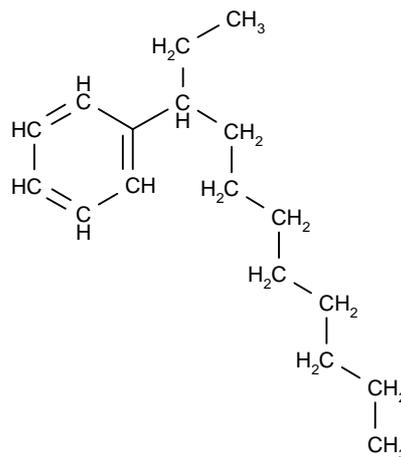
Molecular Composition =C 87.86% H 12.14%

UNDECYLBENZENES

3C₁₁ Undecyl-3-benzene

3-Phenylundecane

(1-ethylnonyl)-Benzene

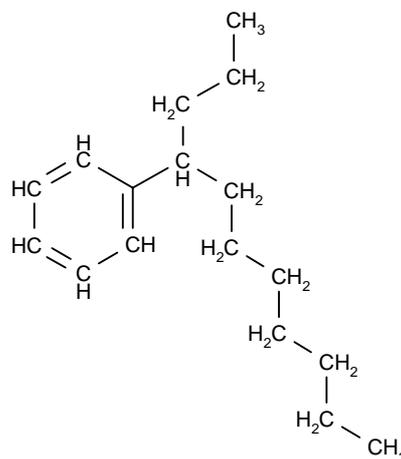


Molecular Weight =232.41
Exact Mass =232
Molecular Formula =C₁₇H₂₈
Molecular Composition =C 87.86% H 12.14%

4C₁₁ Undecyl-4-benzene

4-Phenylundecane

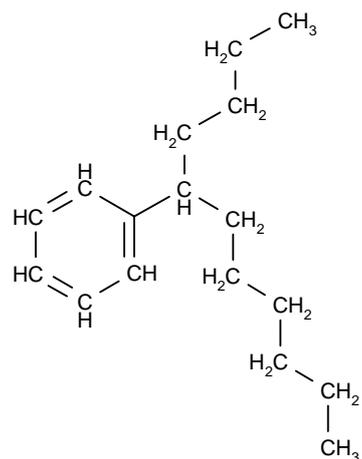
(1-propyloctyl)-Benzene



Molecular Weight =232.41
Exact Mass =232
Molecular Formula =C₁₇H₂₈
Molecular Composition =C 87.86% H 12.14%

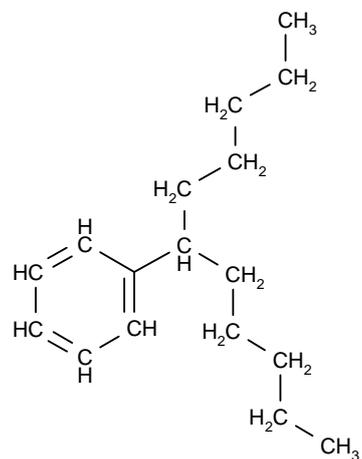
UNDECYLBENZENES

5C₁₁ Undecyl-5-benzene
5-Phenylundecane
(1-butylheptyl)-Benzene



Molecular Weight =232.41
Exact Mass =232
Molecular Formula =C₁₇H₂₈
Molecular Composition =C 87.86% H 12.14%

6C₁₁ Undecyl-6-benzene
6-Phenylundecane
(1-pentylhexyl)-Benzene



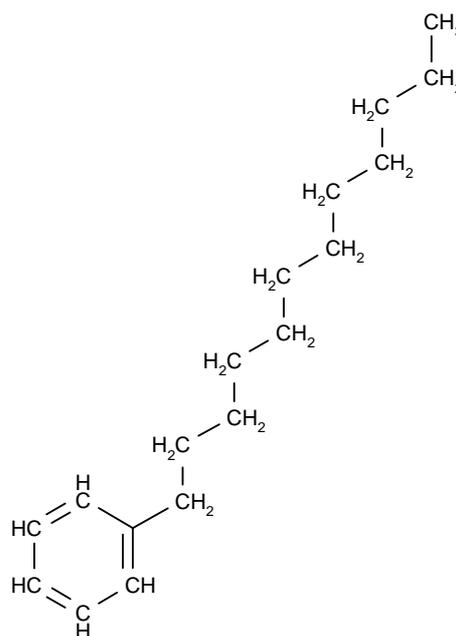
Molecular Weight =232.41
Exact Mass =232
Molecular Formula =C₁₇H₂₈
Molecular Composition =C 87.86% H 12.14%

DODECYLBENZENES

1C₁₂ Dodecyl-1-benzene

1-Phenyldodecane

(dodecyl)-Benzene



Molecular Weight =246.44

Exact Mass =246

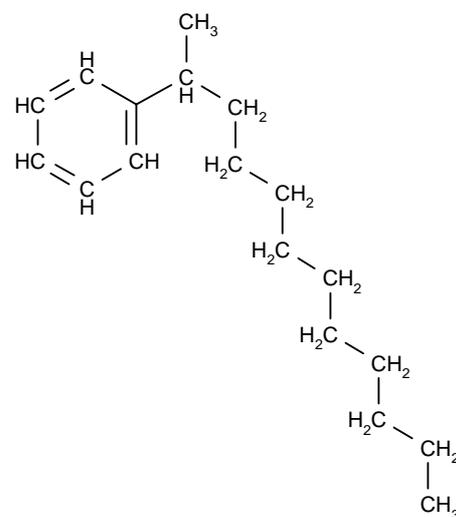
Molecular Formula =C₁₈H₃₀

Molecular Composition =C 87.73% H 12.27%

2C₁₂ Dodecyl-2-benzene

2-Phenyldodecane

(1-methylundecyl)-Benzene



Molecular Weight =246.44

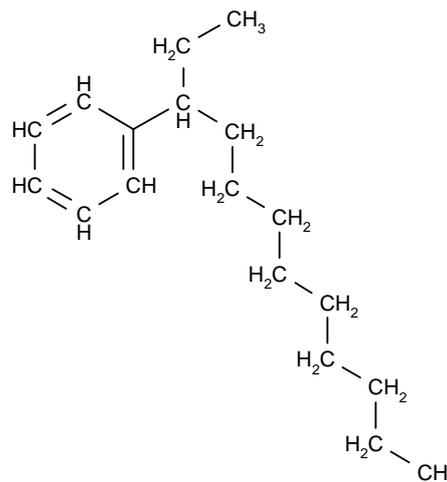
Exact Mass =246

Molecular Formula =C₁₈H₃₀

Molecular Composition =C 87.73% H 12.27%

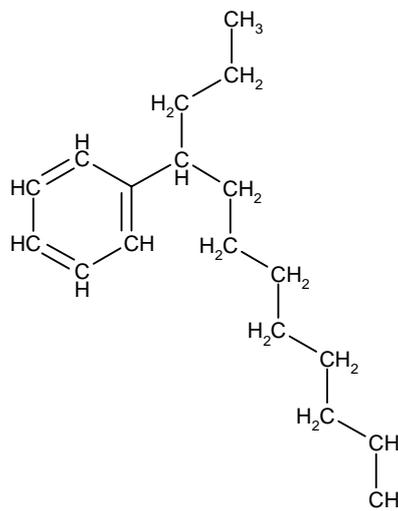
DODECYLBENZENES

3C₁₂ Dodecyl-3-benzene
3-Phenyldodecane
(1-ethyldecyl)-Benzene



Molecular Weight =246.44
Exact Mass =246
Molecular Formula =C₁₈H₃₀
Molecular Composition =C 87.73% H 12.27%

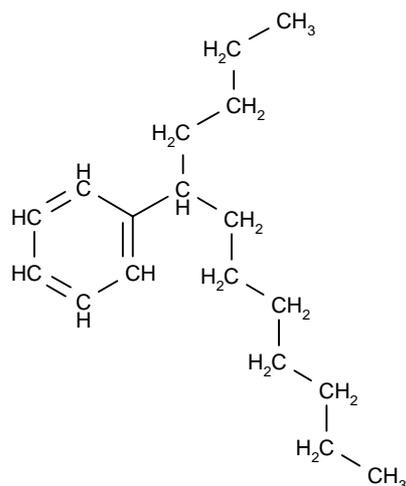
4C₁₂ Dodecyl-4-benzene
4-Phenyldodecane
(1-propylnonyl)-Benzene



Molecular Weight =246.44
Exact Mass =246
Molecular Formula =C₁₈H₃₀
Molecular Composition =C 87.73% H 12.27%

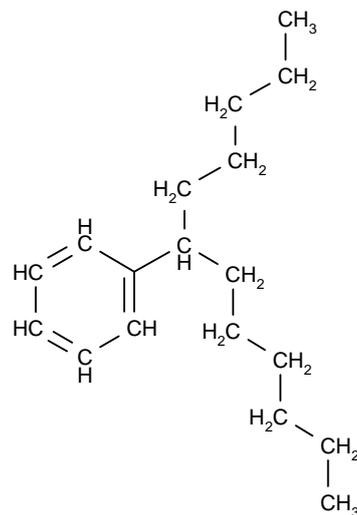
DODECYLBENZENES

5C₁₂ Dodecyl-5-benzene
5-Phenyldodecane
(1-butylloctyl)-Benzene



Molecular Weight =246.44
Exact Mass =246
Molecular Formula =C₁₈H₃₀
Molecular Composition =C 87.73% H 12.27%

6C₁₂ Dodecyl-6-benzene
6-Phenyldodecane
(1-pentylheptyl)-Benzene



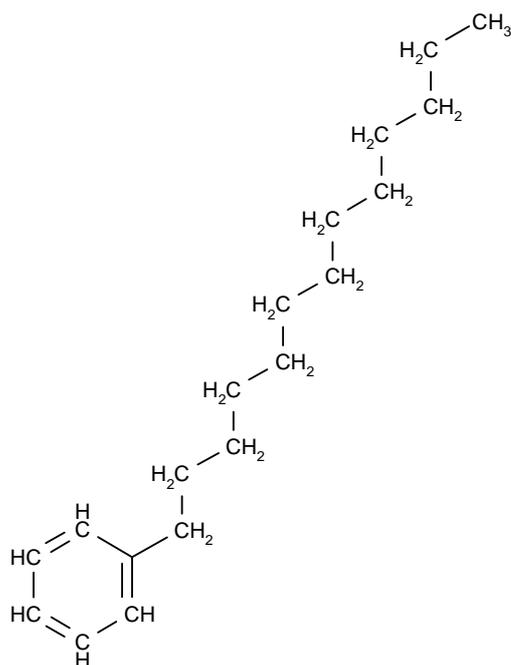
Molecular Weight =246.44
Exact Mass =246
Molecular Formula =C₁₈H₃₀
Molecular Composition =C 87.73% H 12.27%

TRIDECYLBENZENES

1C₁₃ *Tridecyl-1-benzene*

1-Phenyltridecane

(tridecyl)-Benzene

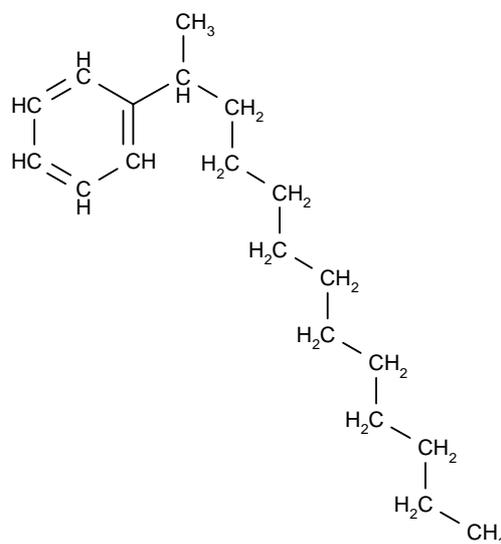


Molecular Weight =260.47
 Exact Mass =260
 Molecular Formula =C₁₉H₃₂
 Molecular Composition =C 87.62% H 12.38%

2C₁₃ *Tridecyl-2-benzene*

2-Phenyltridecane

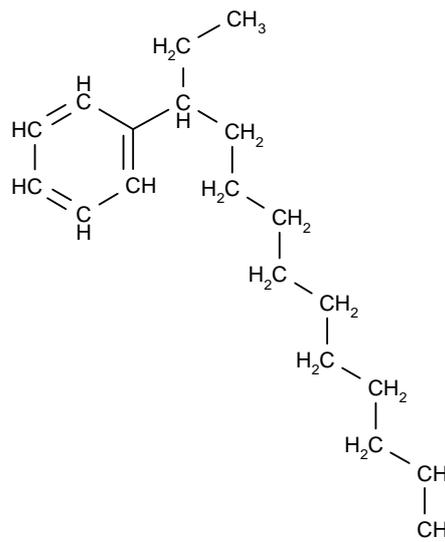
(1-methyldodecyl)-Benzene



Molecular Weight =260.47
 Exact Mass =260
 Molecular Formula =C₁₉H₃₂
 Molecular Composition =C 87.62% H 12.38%

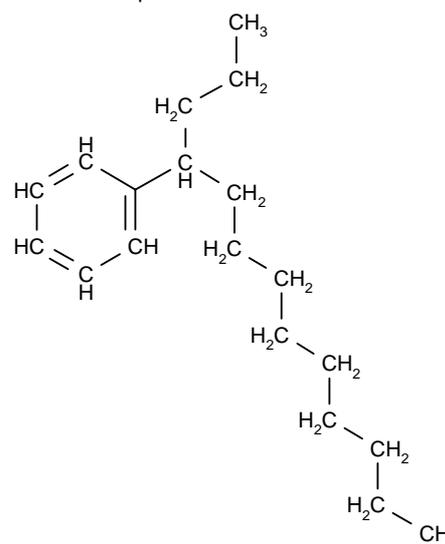
TRIDECYLBENZENES

3C₁₃ Tridecyl-3-benzene
3-Phenyltridecane
(1-ethylundecyl)-Benzene



Molecular Weight =260.47
Exact Mass =260
Molecular Formula =C₁₉H₃₂
Molecular Composition =C 87.62% H 12.38%

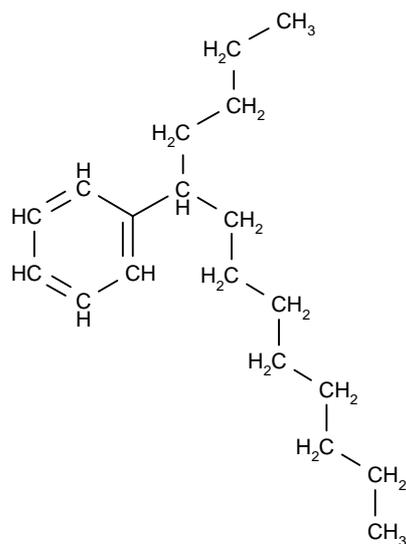
4C₁₃ Tridecyl-4-benzene
4-Phenyltridecane
(1-propyldecyl)-Benzene



Molecular Weight =260.47
Exact Mass =260
Molecular Formula =C₁₉H₃₂
Molecular Composition =C 87.62% H 12.38%

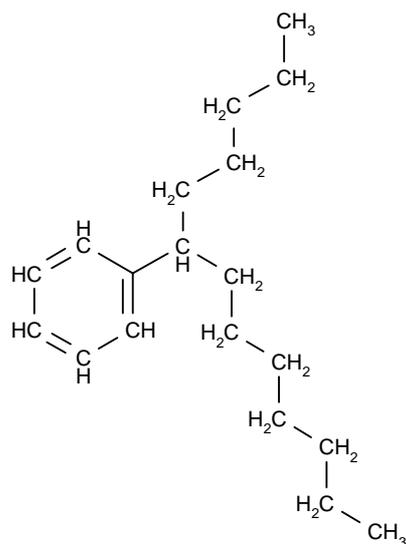
TRIDECYLBENZENES

5C₁₃ Tridecyl-5-benzene
5-Phenyltridecane
(1-butylnonyl)-Benzene



Molecular Weight =260.47
Exact Mass =260
Molecular Formula =C₁₉H₃₂
Molecular Composition =C 87.62% H 12.38%

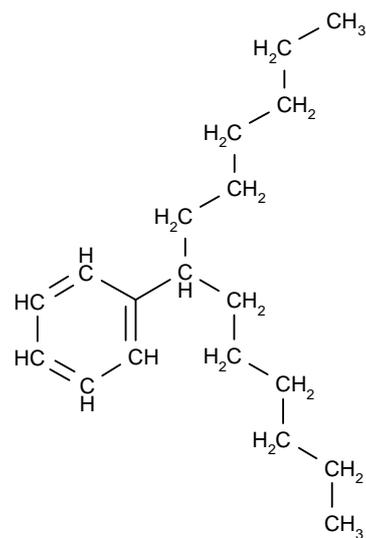
6C₁₃ Tridecyl-6-benzene
6-Phenyltridecane
(1-pentylloctyl)-Benzene



Molecular Weight =260.47
Exact Mass =260
Molecular Formula =C₁₉H₃₂
Molecular Composition =C 87.62% H 12.38%

TRIDECYLBENZENES

7C₁₃ Tridecyl-7-benzene
7-Phenyltridecane
(1-hexylheptyl)-Benzene

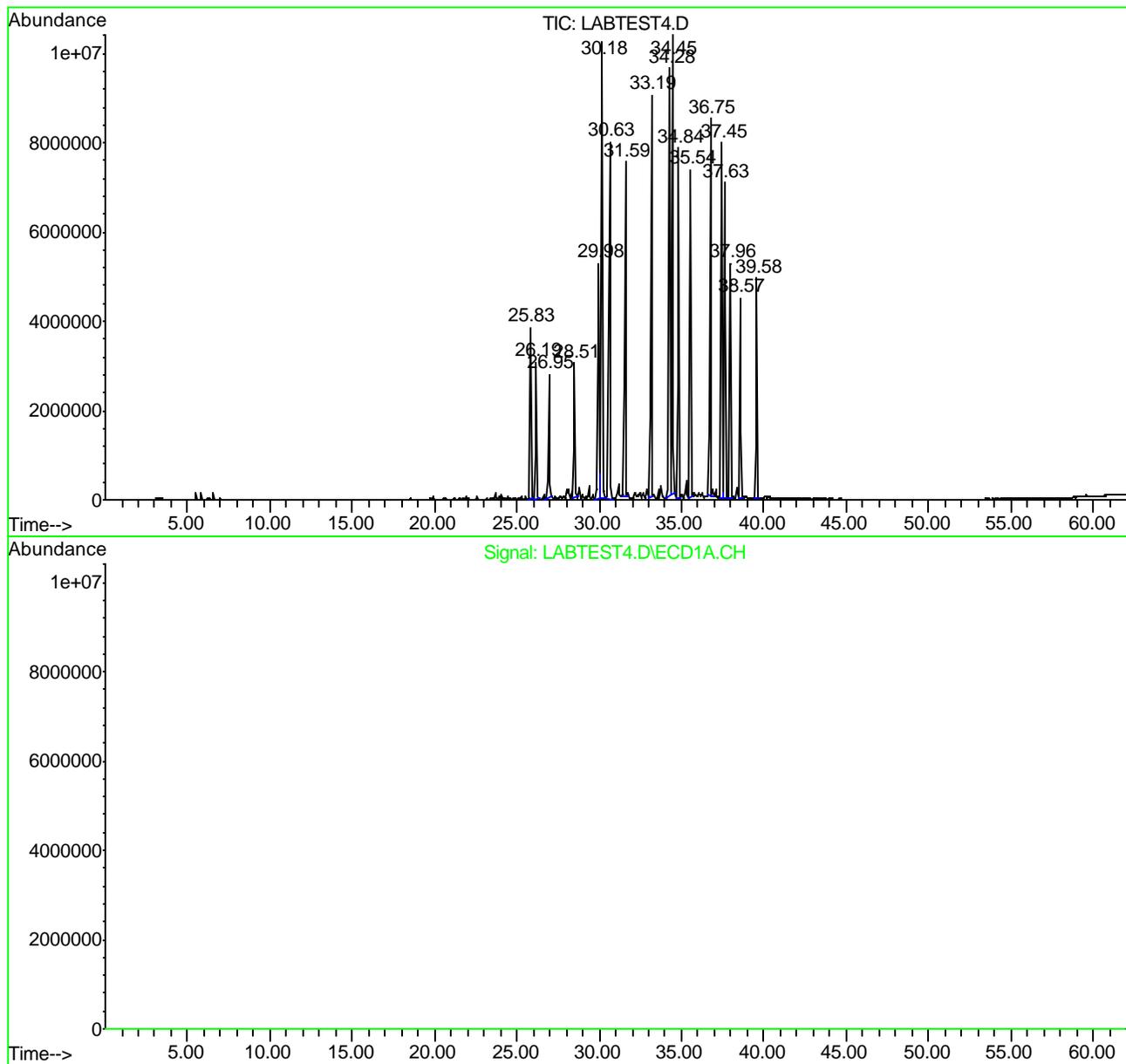


Molecular Weight =260.47
Exact Mass =260
Molecular Formula =C₁₉H₃₂
Molecular Composition =C 87.62% H 12.38%

15.2 GC-MS report

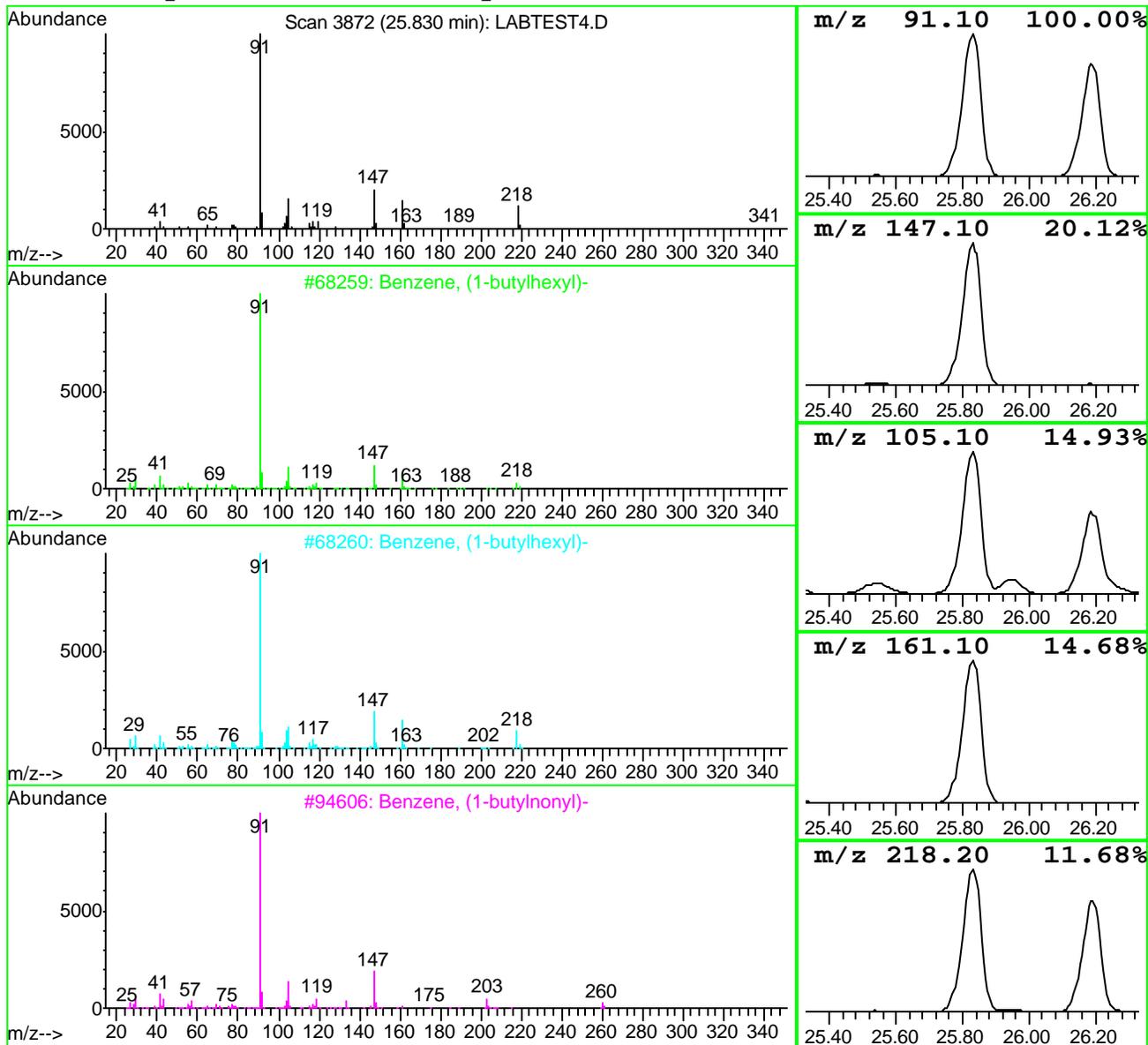
Library Search Report

Data File : C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D Vial: 1
Acq On : 19 Feb 2003 16:30 Operator: Stephen
Sample : LAB in hexane - testing new GC-MS Inst : Instrum
Misc : Multiplr: 1.00
Sample Amount: 0.00
MS Integration Params: AUTOINT1.E GC Integration Params: autoint
Method : C:\MSDCHEM\1\METHODS\SJJ_NEW.M (Chemstation Integrator)
Title :



Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 1 at 25.83 min Area: 150359105 Area % 2.86

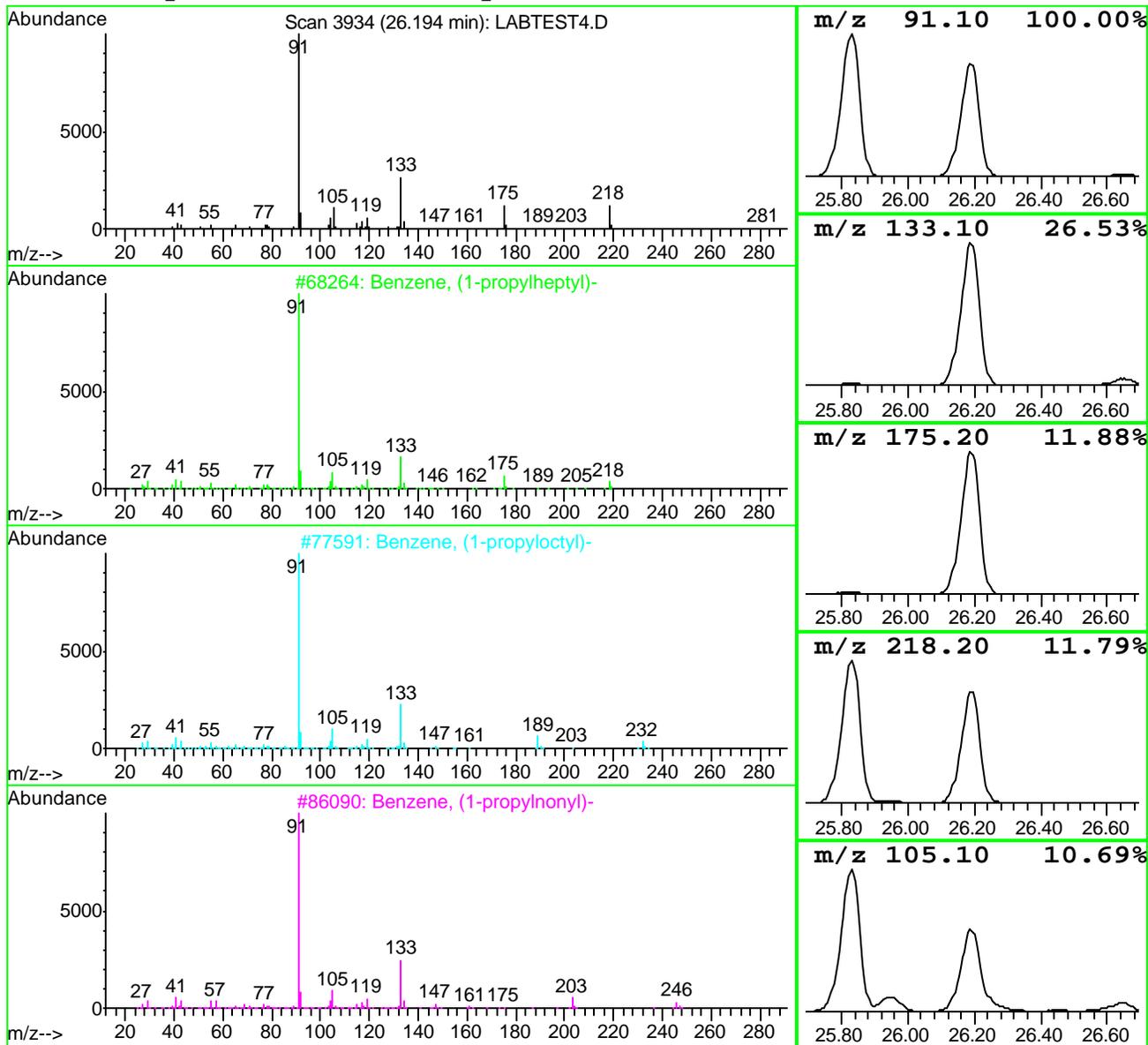
The 3 best hits from each library.

	Ref#	CAS#	Qual

nist02.L			
1 Benzene, (1-butylhexyl)-	68259	004537-11-5	94
2 Benzene, (1-butylhexyl)-	68260	004537-11-5	87
3 Benzene, (1-butylnonyl)-	94606	004534-50-3	53

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 2 at 26.19 min Area: 119482200 Area % 2.28

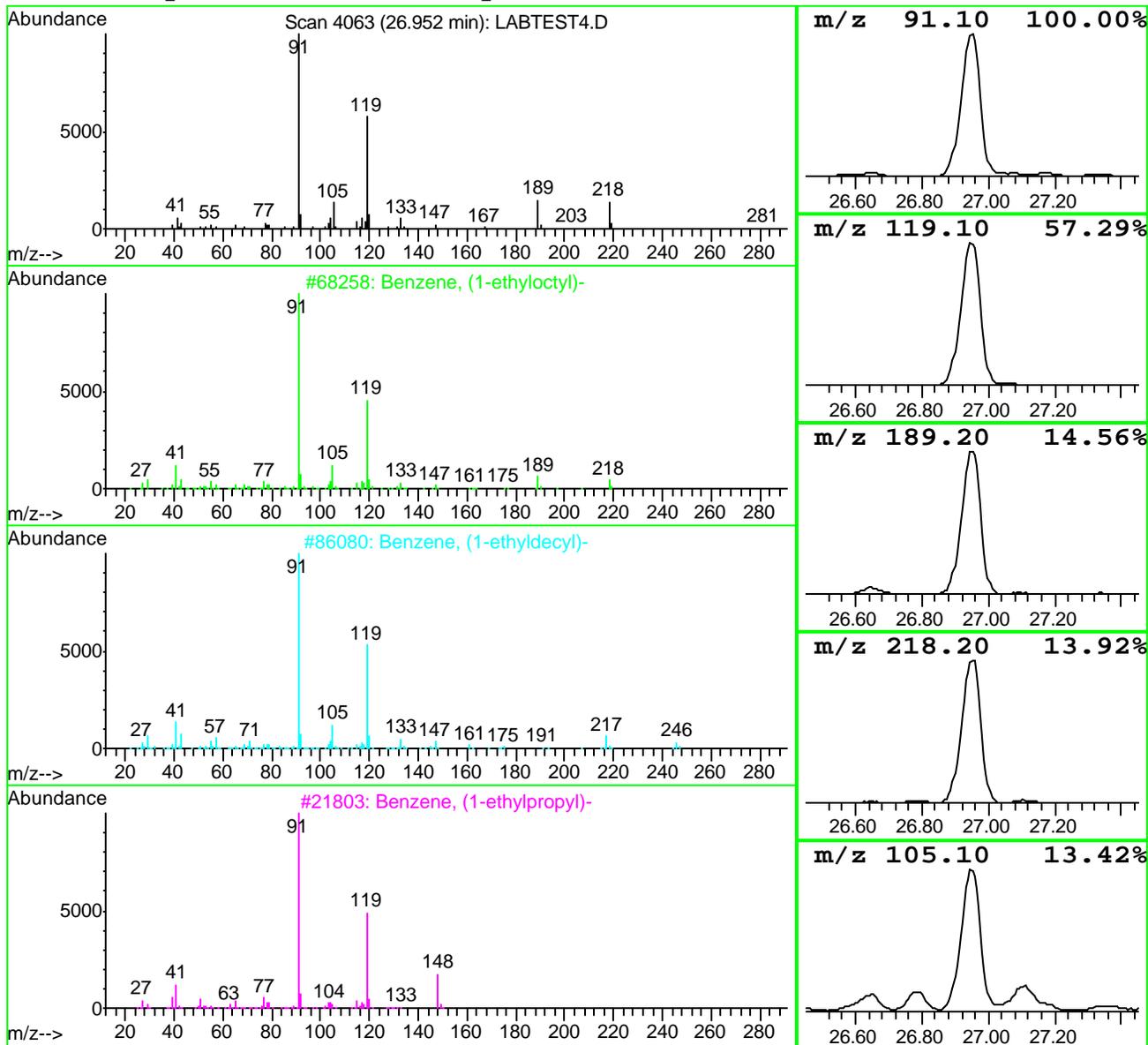
The 3 best hits from each library.

	Ref#	CAS#	Qual

nist02.L			
1 Benzene, (1-propylheptyl)-	68264	004537-12-6	95
2 Benzene, (1-propyloctyl)-	77591	004536-86-1	59
3 Benzene, (1-propylnonyl)-	86090	002719-64-4	59

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 3 at 26.95 min Area: 113199224 Area % 2.16

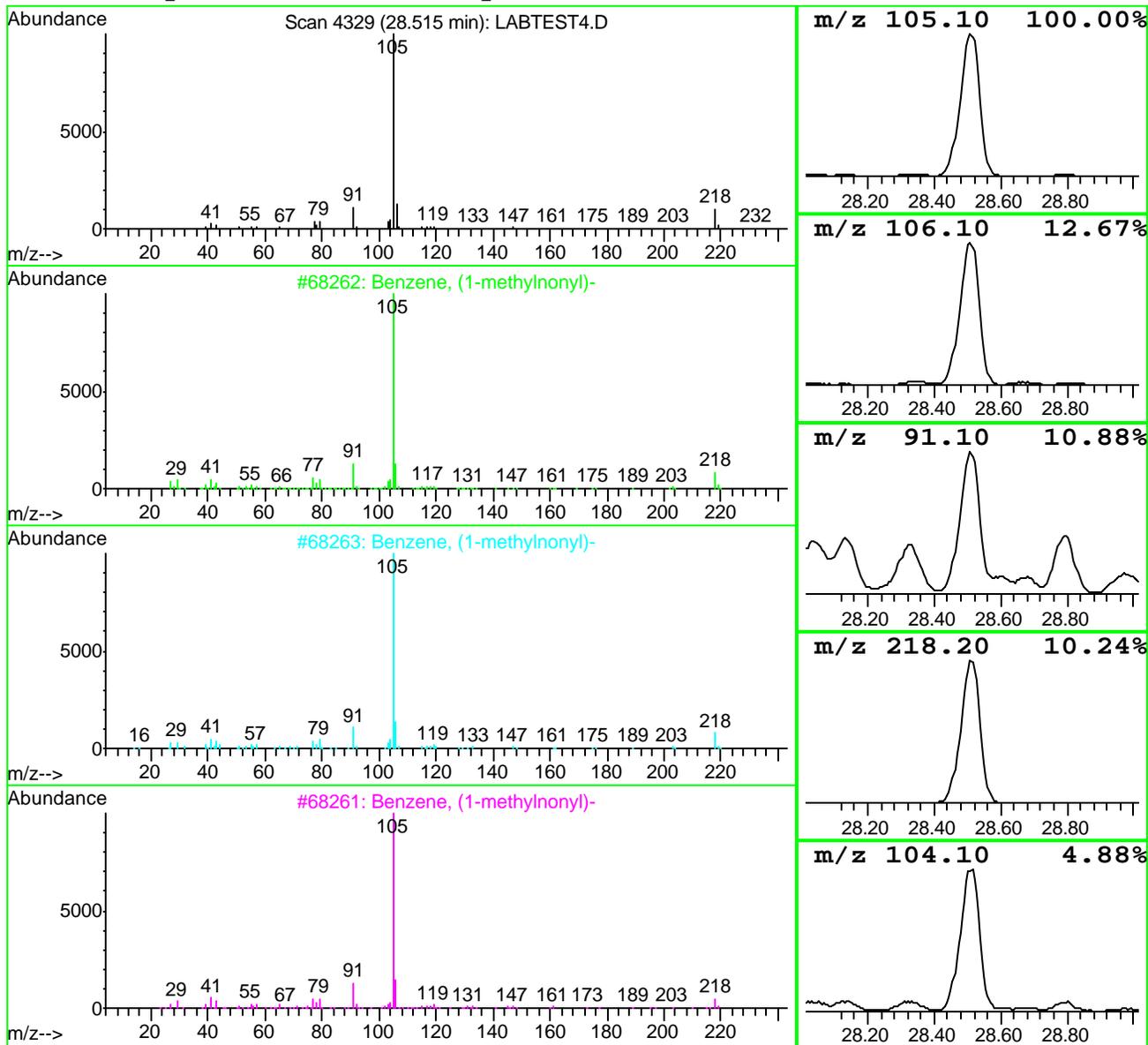
The 3 best hits from each library.

	Ref#	CAS#	Qual

nist02.L			
1 Benzene, (1-ethyloctyl)-	68258	004621-36-7	97
2 Benzene, (1-ethyldecyl)-	86080	002400-00-2	50
3 Benzene, (1-ethylpropyl)-	21803	001196-58-3	50

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

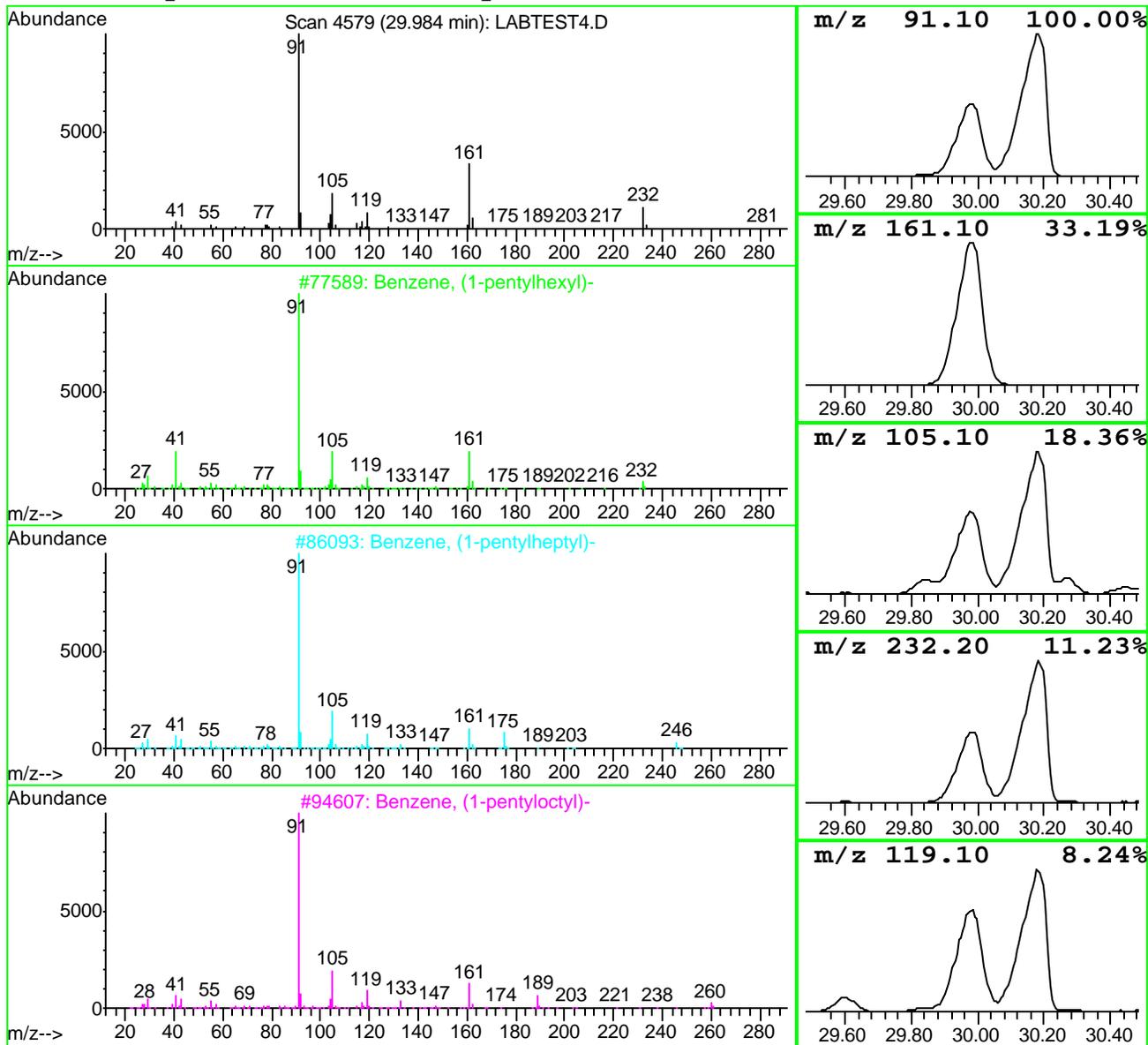
Peak Number: 4 at 28.51 min Area: 122785512 Area % 2.34

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-methylnonyl)-	68262	004537-13-7	91
2 Benzene, (1-methylnonyl)-	68263	004537-13-7	81
3 Benzene, (1-methylnonyl)-	68261	004537-13-7	74

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 5 at 29.98 min Area: 293553119 Area % 5.59

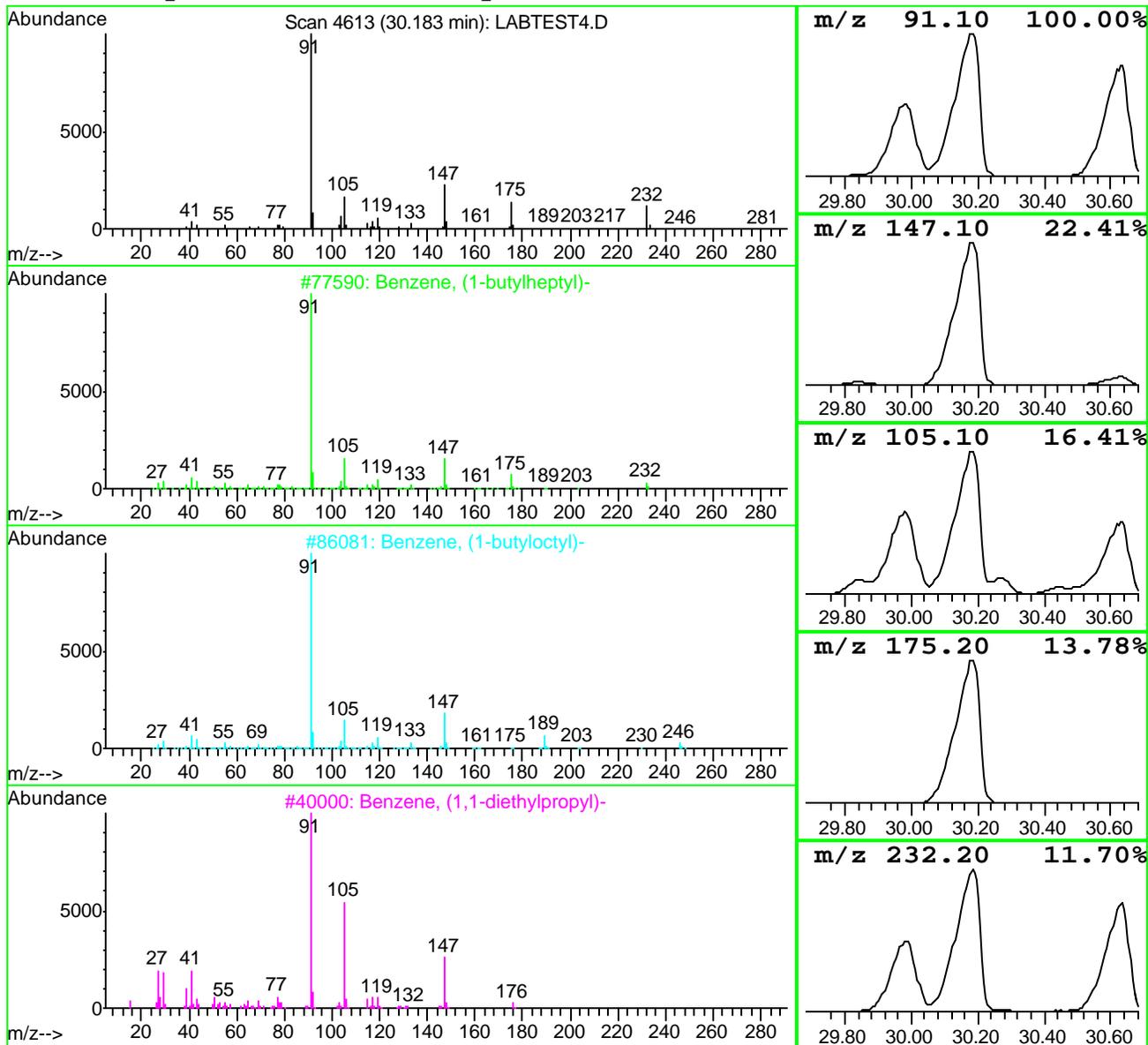
The 3 best hits from each library.

	Ref#	CAS#	Qual

nist02.L			
1 Benzene, (1-pentylhexyl)-	77589	004537-14-8	64
2 Benzene, (1-pentylheptyl)-	86093	002719-62-2	53
3 Benzene, (1-pentyl-octyl)-	94607	004534-49-0	42

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

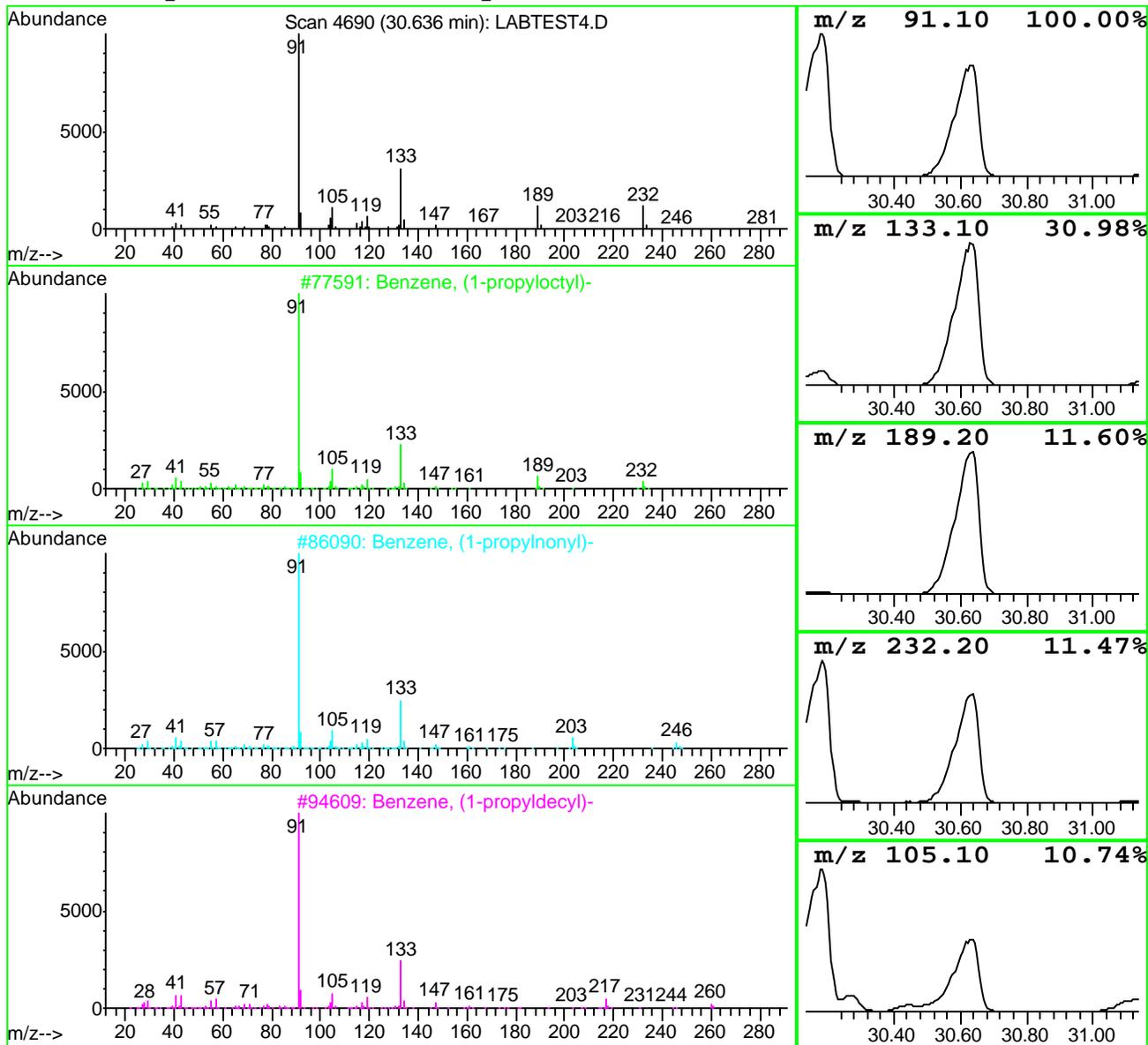
Peak Number: 6 at 30.18 min Area: 539579829 Area % 10.28

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-butylheptyl)-	77590	004537-15-9	97
2 Benzene, (1-butyloctyl)-	86081	002719-63-3	59
3 Benzene, (1,1-diethylpropyl)-	40000	004170-84-7	47

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

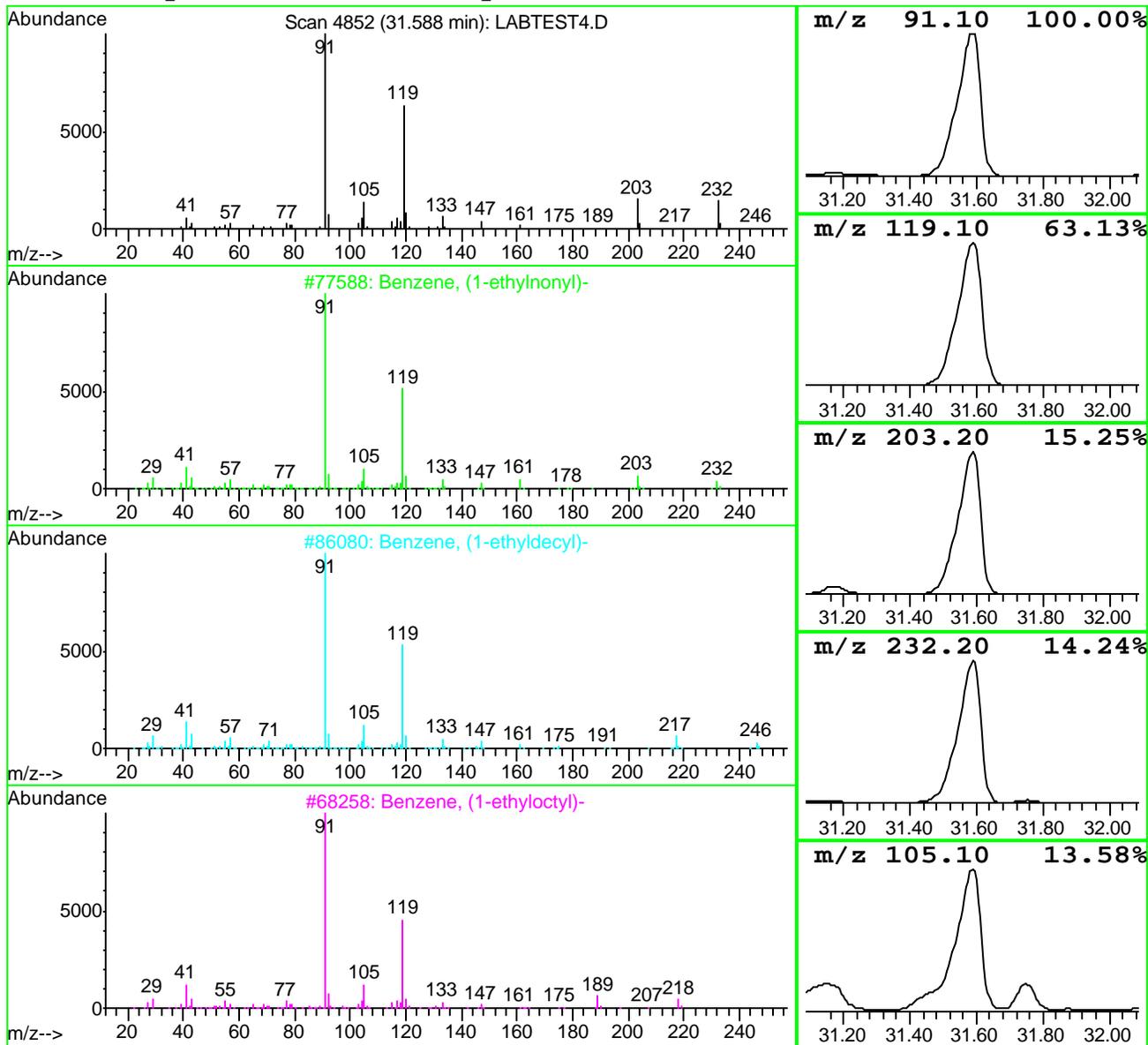
Peak Number: 7 at 30.64 min Area: 416254705 Area % 7.93

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-propyloctyl)-	77591	004536-86-1	95
2 Benzene, (1-propylnonyl)-	86090	002719-64-4	45
3 Benzene, (1-propyldecyl)-	94609	004534-51-4	45

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 8 at 31.59 min Area: 373705684 Area % 7.12

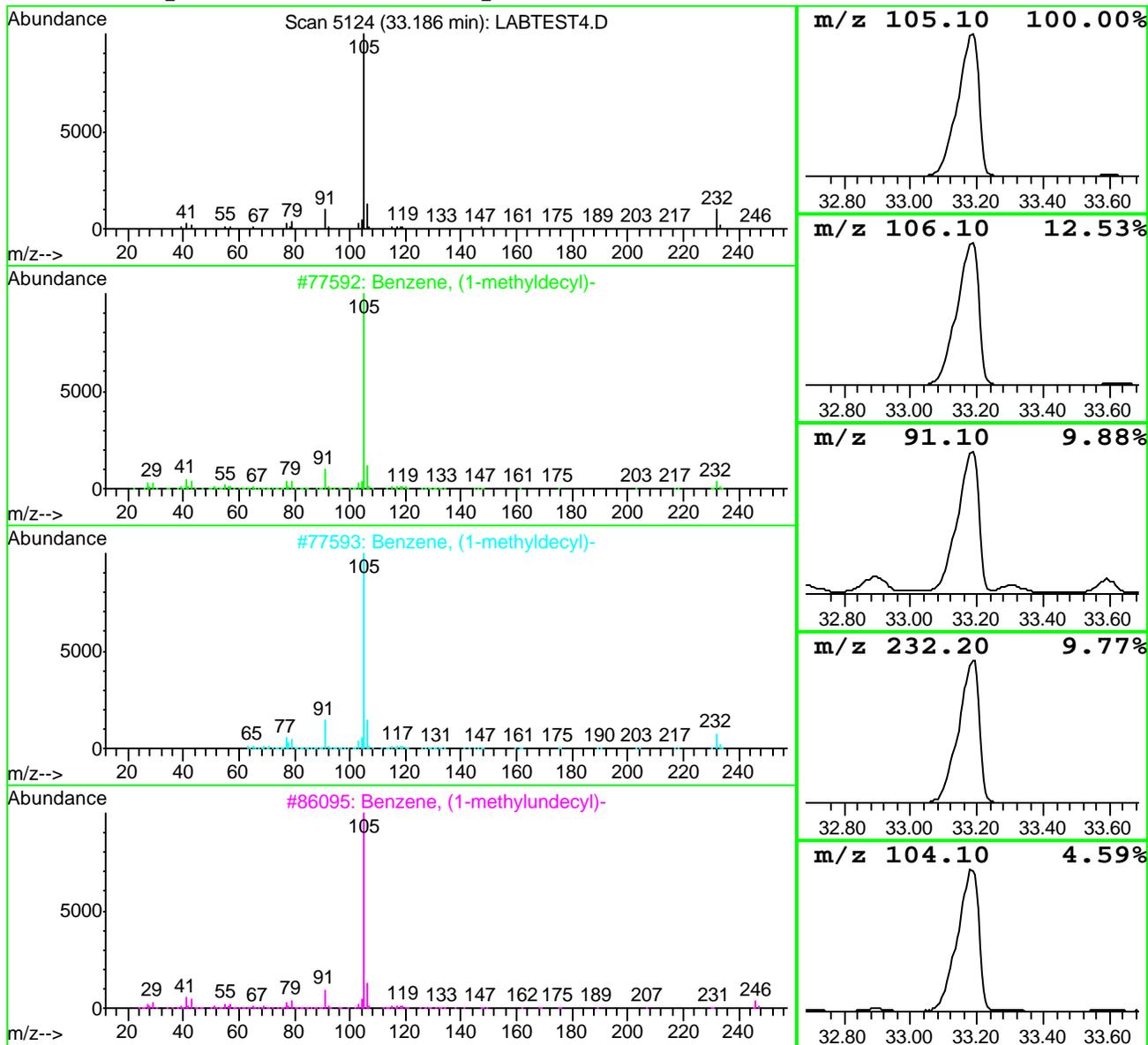
The 3 best hits from each library.

	Ref#	CAS#	Qual

nist02.L			
1 Benzene, (1-ethylnonyl)-	77588	004536-87-2	94
2 Benzene, (1-ethyldecyl)-	86080	002400-00-2	90
3 Benzene, (1-ethyloctyl)-	68258	004621-36-7	76

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 9 at 33.19 min Area: 400461535 Area % 7.63

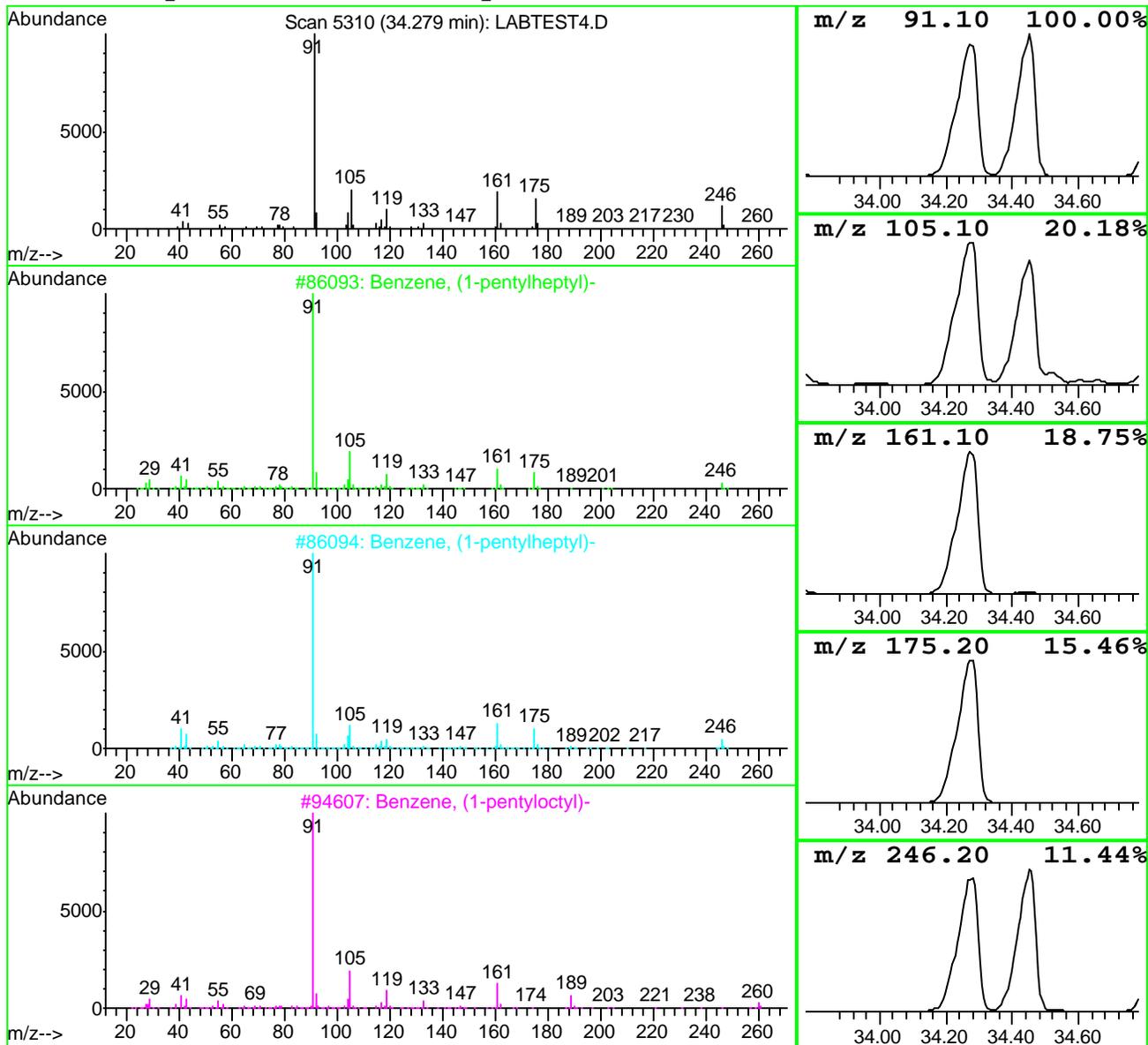
The 3 best hits from each library.

Ref#	CAS#	Qual
77592	004536-88-3	91
77593	004536-88-3	87
86095	002719-61-1	59

nist02.L

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

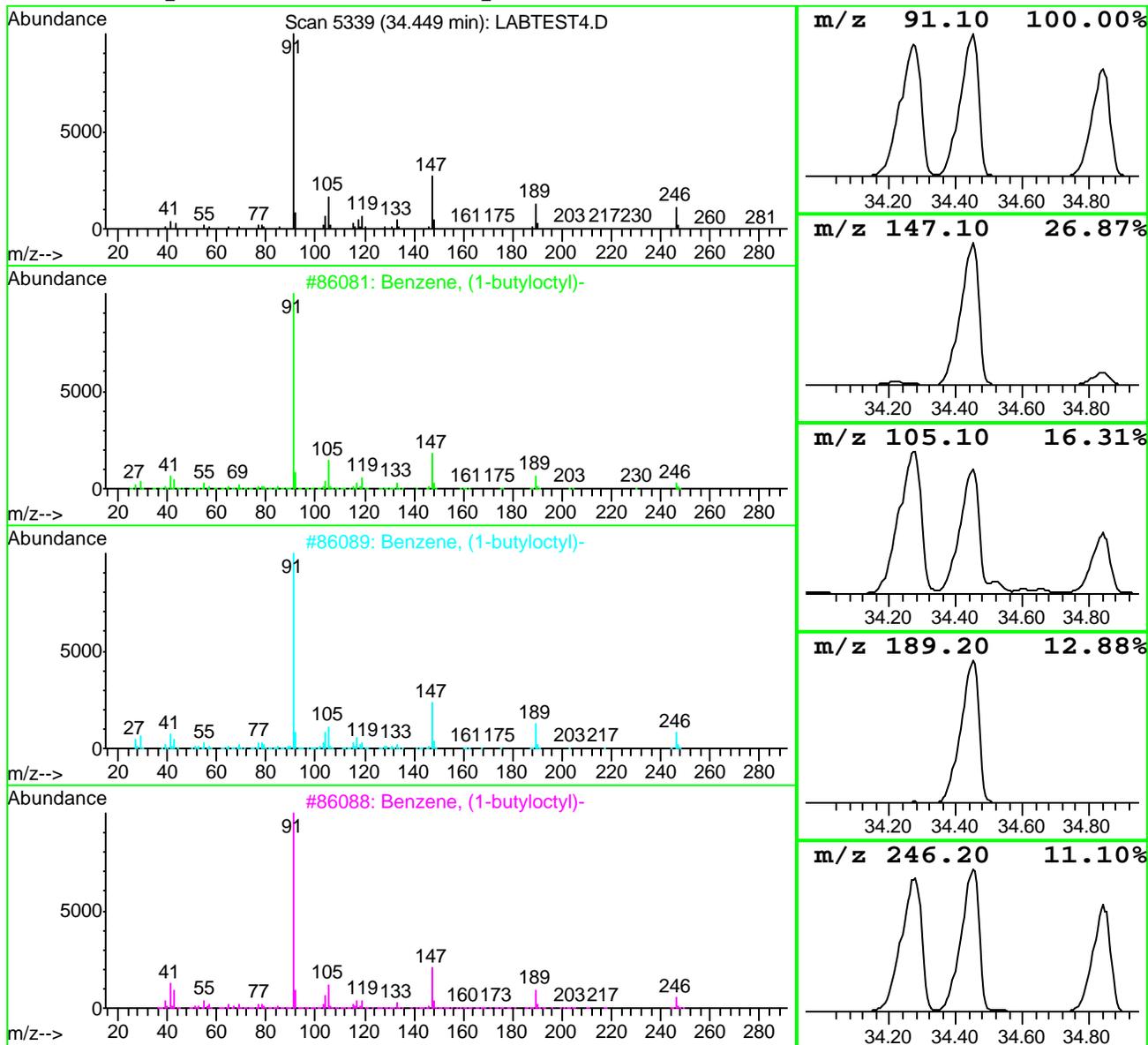
Peak Number: 10 at 34.28 min Area: 423832624 Area % 8.07

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-pentylheptyl)-	86093	002719-62-2	97
2 Benzene, (1-pentylheptyl)-	86094	002719-62-2	93
3 Benzene, (1-pentylloctyl)-	94607	004534-49-0	76

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

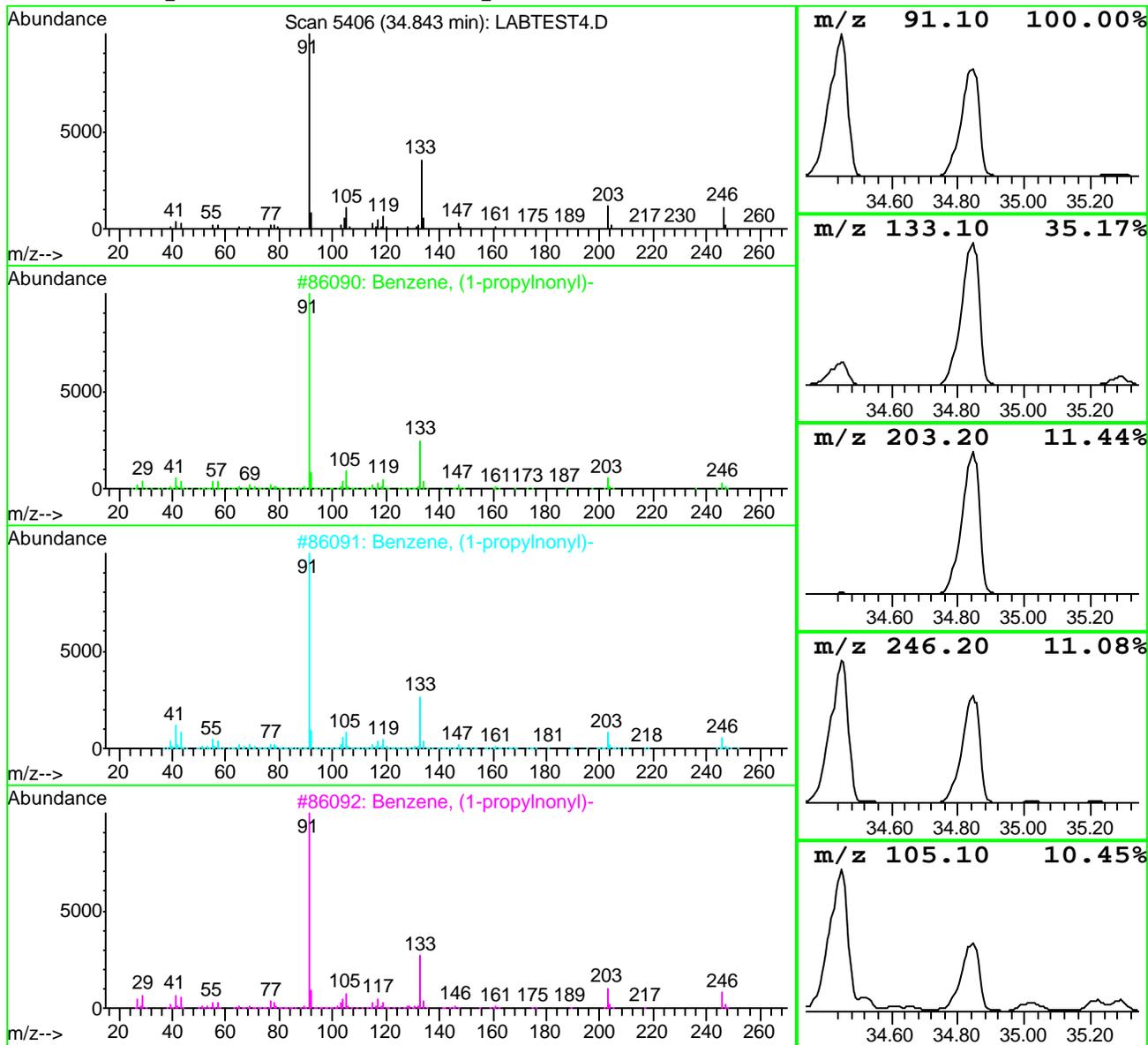
Peak Number: 11 at 34.45 min Area: 396334539 Area % 7.55

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-butyloctyl)-	86081	002719-63-3	97
2 Benzene, (1-butyloctyl)-	86089	002719-63-3	94
3 Benzene, (1-butyloctyl)-	86088	002719-63-3	93

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

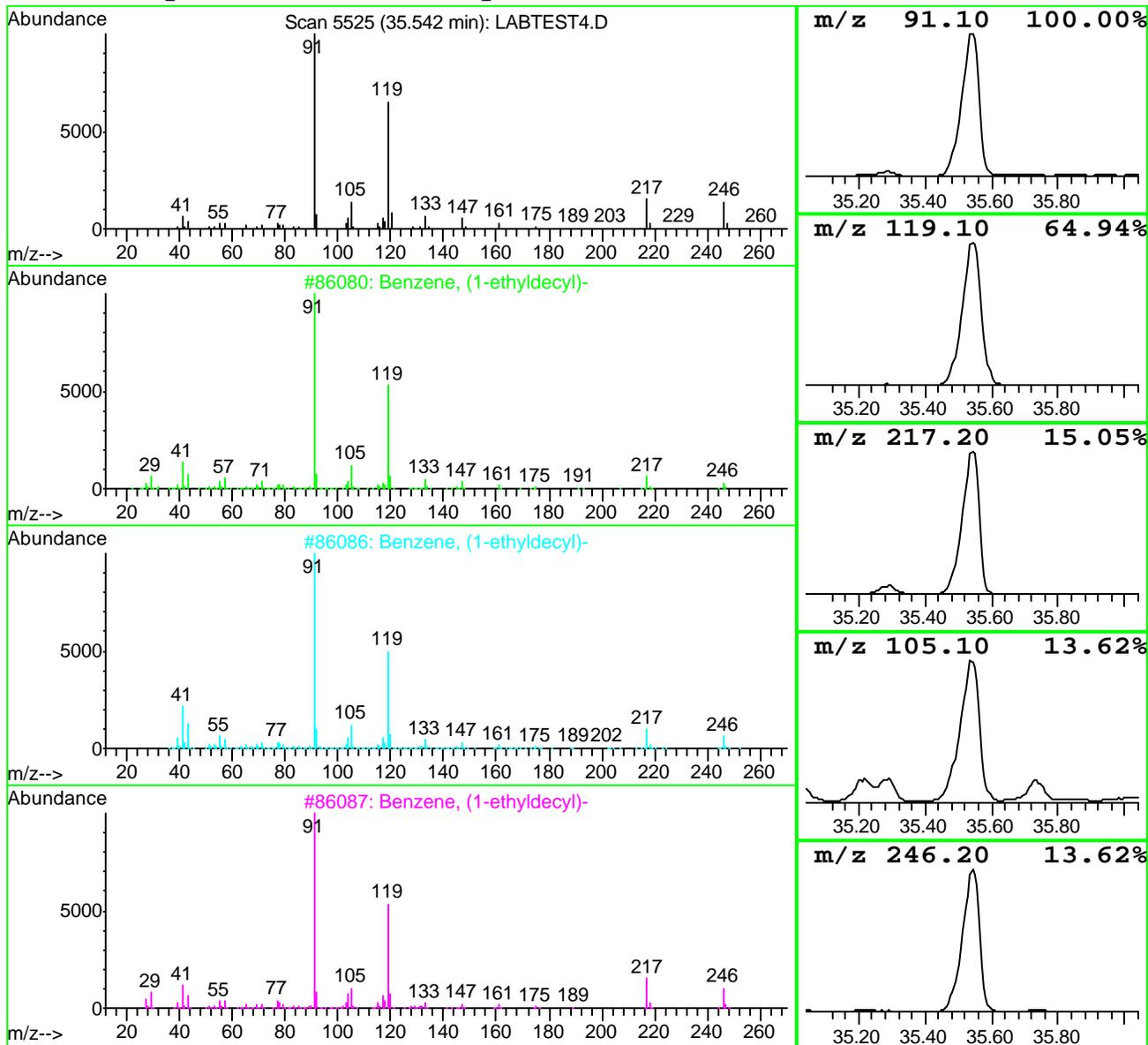
Peak Number: 12 at 34.84 min Area: 297739727 Area % 5.67

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-propylnonyl)-	86090	002719-64-4	97
2 Benzene, (1-propylnonyl)-	86091	002719-64-4	96
3 Benzene, (1-propylnonyl)-	86092	002719-64-4	94

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 13 at 35.54 min Area: 276569566 Area % 5.27

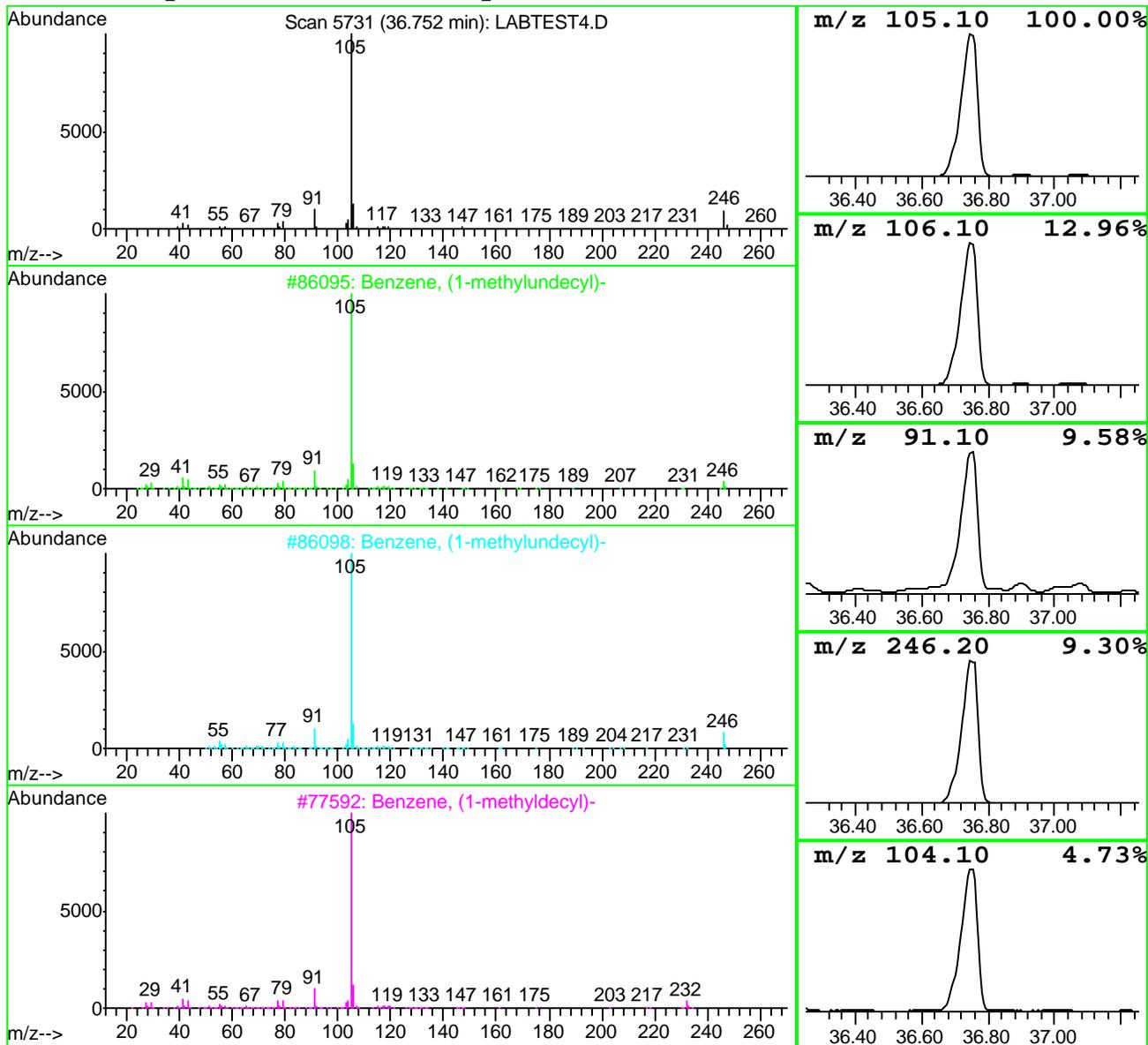
The 3 best hits from each library.

	Ref#	CAS#	Qual

nist02.L			
1 Benzene, (1-ethyldecyl)-	86080	002400-00-2	97
2 Benzene, (1-ethyldecyl)-	86086	002400-00-2	97
3 Benzene, (1-ethyldecyl)-	86087	002400-00-2	87

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

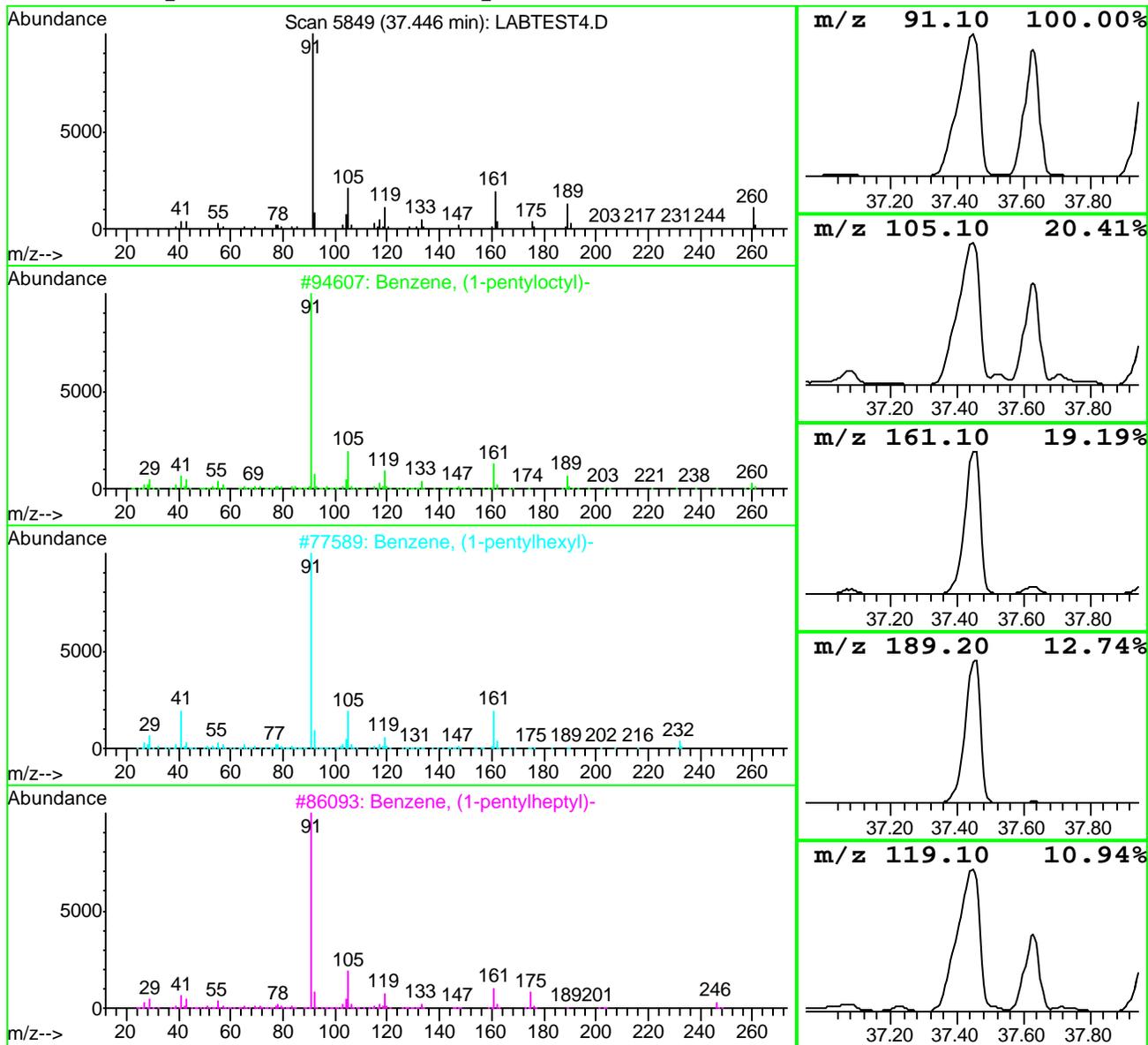
Peak Number: 14 at 36.75 min Area: 287969624 Area % 5.48

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-methylundecyl)-	86095	002719-61-1	93
2 Benzene, (1-methylundecyl)-	86098	002719-61-1	91
3 Benzene, (1-methyldecyl)-	77592	004536-88-3	70

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

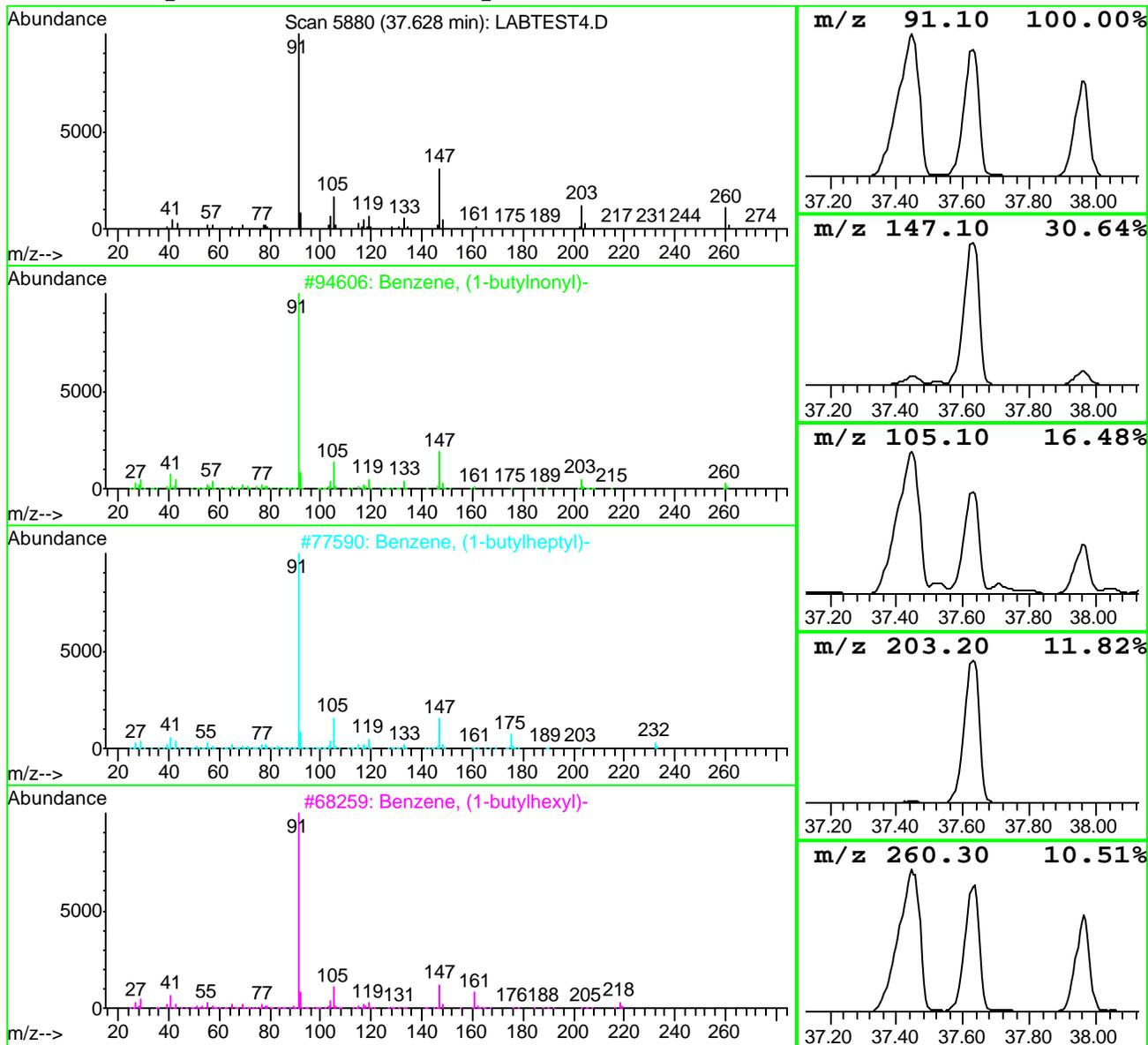
Peak Number: 15 at 37.45 min Area: 361288408 Area % 6.88

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-pentyloctyl)-	94607	004534-49-0	96
2 Benzene, (1-pentylhexyl)-	77589	004537-14-8	76
3 Benzene, (1-pentylheptyl)-	86093	002719-62-2	50

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 16 at 37.63 min Area: 229368234 Area % 4.37

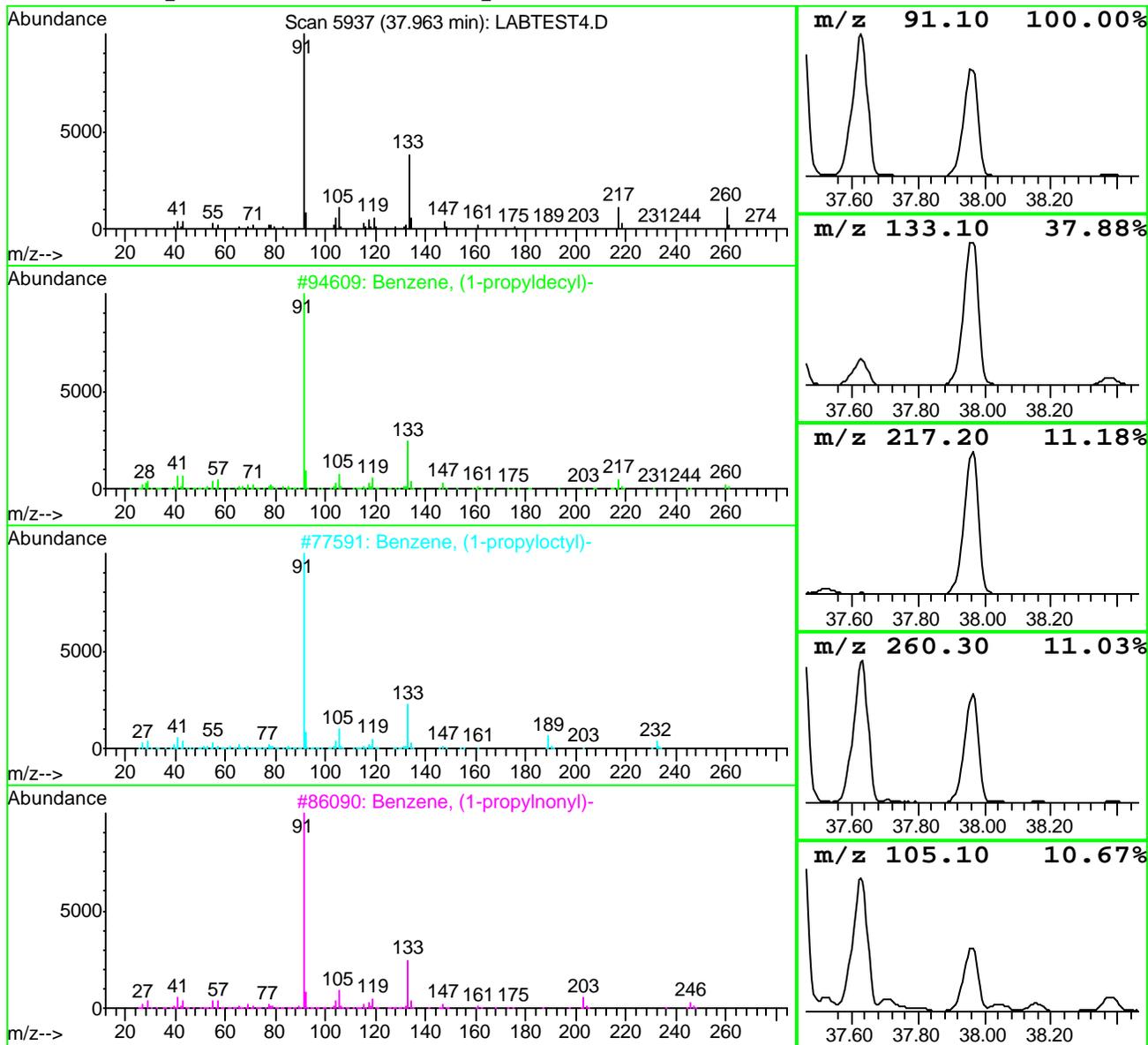
The 3 best hits from each library.

Ref#	CAS#	Qual
94606	004534-50-3	97
77590	004537-15-9	53
68259	004537-11-5	53

nist02.L

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

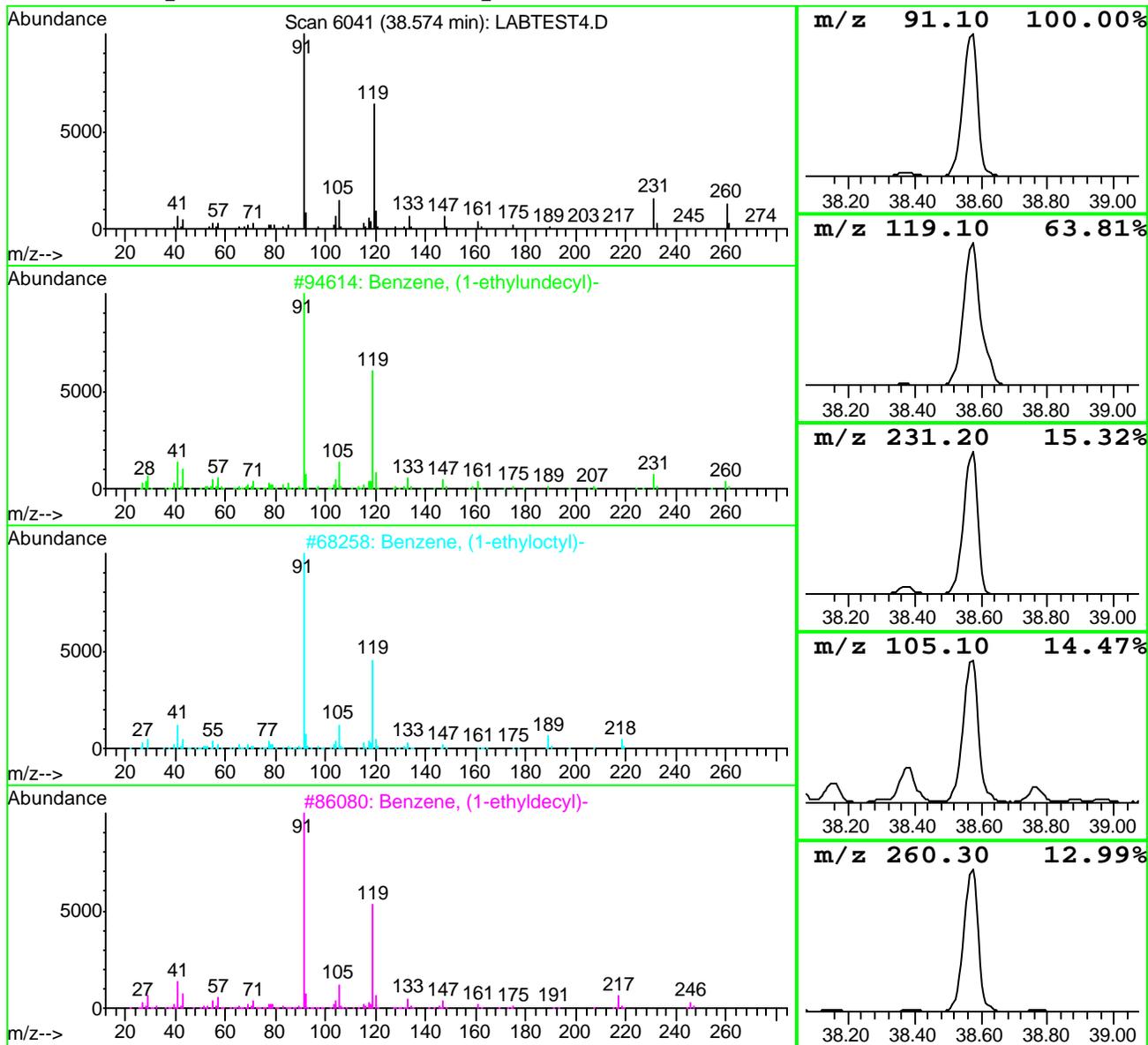
Peak Number: 17 at 37.96 min Area: 163945519 Area % 3.12

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-propyldecyl)-	94609	004534-51-4	95
2 Benzene, (1-propyloctyl)-	77591	004536-86-1	62
3 Benzene, (1-propylnonyl)-	86090	002719-64-4	59

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 18 at 38.57 min Area: 144009292 Area % 2.74

The 3 best hits from each library.

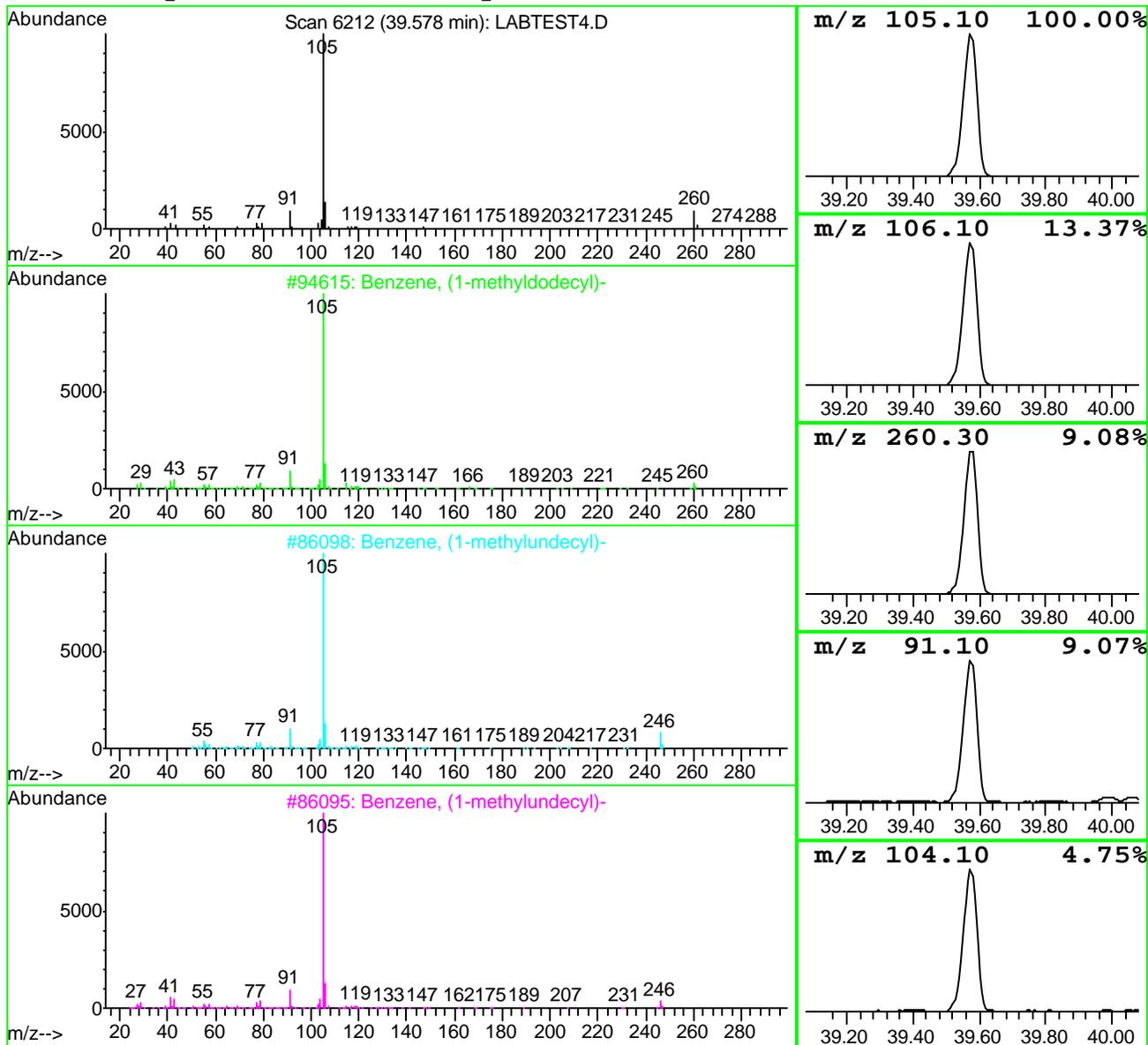
Ref#	CAS#	Qual
94614	004534-52-5	94
68258	004621-36-7	70
86080	002400-00-2	53

nist02.L

- 1 Benzene, (1-ethylundecyl)-
- 2 Benzene, (1-ethyloctyl)-
- 3 Benzene, (1-ethyldecyl)-

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex



Data File: C:\MSDCHEM\1\DATA\SJJ\20030219\LABTEST4.D

Sample : LAB in hexane - testing new GC-MS

Peak Number: 19 at 39.58 min Area: 140579381 Area % 2.68

The 3 best hits from each library.

	Ref#	CAS#	Qual
nist02.L			
1 Benzene, (1-methyldodecyl)-	94615	004534-53-6	87
2 Benzene, (1-methylundecyl)-	86098	002719-61-1	76
3 Benzene, (1-methylundecyl)-	86095	002719-61-1	76

15.3 Analysis of *C. elegans* toxicity data

15.3.1 Trimmed Spearman-Kärber analysis

15.3.1.1 Experiment 3: Raw output from TSK.EXE

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

```
ENTER DATE OF TEST:

ENTER TEST NUMBER:
3
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
1
ENTER TEST SPECIES NAME:
C. elegans
ENTER TOXICANT NAME:
LAB
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
30
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
8
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL; MAX = 10):
5
ENTER THE 5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
.01
.05
.1
.5
1
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
30
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
h
ENTER DURATION OF TEST:
24
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
8
11
16
16
20
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y
```

```
DATE:                                TEST NUMBER: 3                DURATION: 24 h
TOXICANT : LAB
SPECIES: C. elegans
```

RAW DATA:	Concentration	Number	Mortalities
---	----	Exposed	
	.00	30	8
	.01	30	8
	.05	30	11
	.10	30	16
	.50	30	16
	1.00	30	20

```
SPEARMAN-KARBER TRIM:                45.45%
```

```

SPEARMAN-KARBER ESTIMATES:      LC50:          .84
                               95% LOWER CONFIDENCE: .49
                               95% UPPER CONFIDENCE: 1.45

```

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

```

-----
WOULD YOU LIKE TO HAVE A COPY SENT TO THE PRINTER(Y/N)?
n
WOULD YOU LIKE TO CONTINUE (Y/N)?
y

```

15.3.1.2 Experiment 6: Raw output from TSK.EXE

```

ENTER DATE OF TEST:

ENTER TEST NUMBER:
6
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
l
ENTER TEST SPECIES NAME:
C. elegans
ENTER TOXICANT NAME:
LAB
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
30
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
9
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL; MAX = 10):
5
ENTER THE 5 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
.05
.1
.5
1
2
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
30
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
h
ENTER DURATION OF TEST:
24
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
11
11
15
23
28
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
y

```

```

DATE:                                TEST NUMBER: 6                DURATION: 24 h
TOXICANT : LAB
SPECIES: C.elegans

```

RAW DATA:	Concentration (%)	Number Exposed	Mortalities
---	.00	30	9
	.05	30	11
	.10	30	11
	.50	30	15
	1.00	30	23
	2.00	30	28

SPEARMAN-KARBER TRIM: 9.52%

SPEARMAN-KARBER ESTIMATES: LC50: .66
95% LOWER CONFIDENCE: .49
95% UPPER CONFIDENCE: .89

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

WOULD YOU LIKE TO HAVE A COPY SENT TO THE PRINTER(Y/N)?
n
WOULD YOU LIKE TO CONTINUE (Y/N)?
y

15.3.1.3 Experiment 3 + 6: Raw output from TSK.EXE

ENTER DATE OF TEST:

ENTER TEST NUMBER:
3+6
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
l
ENTER TEST SPECIES NAME:
C. elegans
ENTER TOXICANT NAME:
LAB
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
%
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
60
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
17
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL; MAX = 10):
6
ENTER THE 6 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):
.01
.05
.1
.5
1
2
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
n
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
30
60
60
60
60
30
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
h
ENTER DURATION OF TEST:
24
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:
8
22
27
31
43
28
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
y

DATE: TEST NUMBER: 3+6 DURATION: 24 h
TOXICANT : LAB

SPECIES: C. elegans

RAW DATA:	Concentration	Number	Mortalities
---	(%)	Exposed	
	.00	60	17
	.01	30	8
	.05	60	22
	.10	60	27
	.50	60	31
	1.00	60	43
	2.00	30	28

SPEARMAN-KARBER TRIM: 9.23%

SPEARMAN-KARBER ESTIMATES:	LC50:	.52
	95% LOWER CONFIDENCE:	.40
	95% UPPER CONFIDENCE:	.68

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

WOULD YOU LIKE TO HAVE A COPY SENT TO THE PRINTER(Y/N)?

n

WOULD YOU LIKE TO CONTINUE (Y/N)?

n

RUN COMPLETED

15.3.2 Probit analysis

15.3.2.1 Experiment 3: Raw output from PROBIT.EXE

EPA PROBIT ANALYSIS PROGRAM
 USED FOR CALCULATING LC/EC VALUES
 Version 1.5

Experiment 3

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Adjusted for Controls	Predicted Proportion Responding
Control	30	8	0.2667	0.0000	0.2512
0.0100	30	8	0.2667	0.0207	0.0778
0.0500	30	11	0.3667	0.1542	0.1866
0.1000	30	16	0.5333	0.3768	0.2538
0.5000	30	16	0.5333	0.3768	0.4470
1.0000	30	20	0.6667	0.5549	0.5377

Chi - Square for Heterogeneity (calculated) = 1.727
 Chi - Square for Heterogeneity (tabular value at 0.05 level) = 7.815

Mu = -0.125095
 Sigma = 1.320594

Parameter	Estimate	Std. Err.	95% Confidence Limits	
Intercept	5.094726	0.322641	(4.462349,	5.727103)
Slope	0.757235	0.345574	(0.079909,	1.434560)
Spontaneous Response Rate	0.251170	0.076073	(0.102068,	0.400273)

Experiment 3

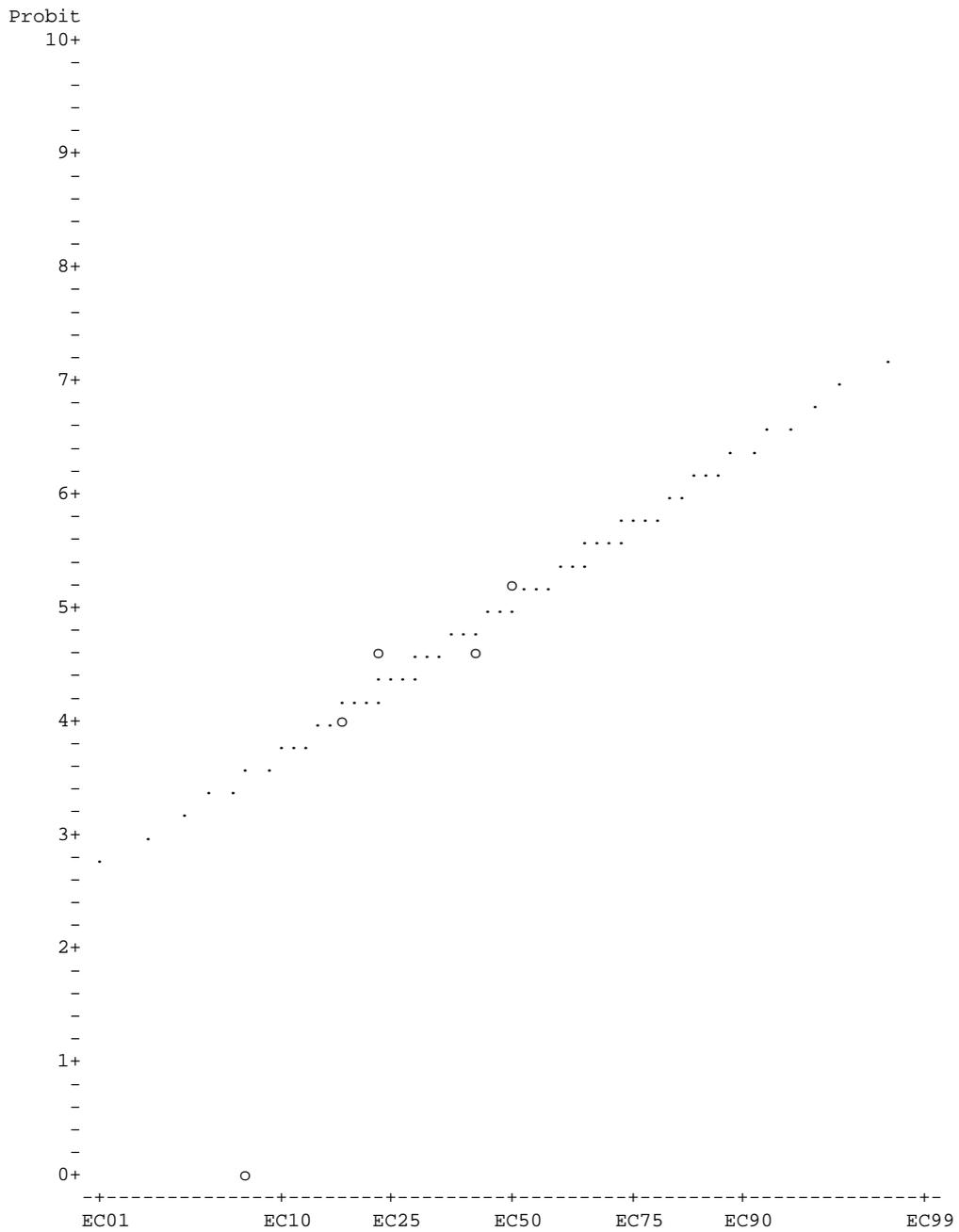
Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	95% Confidence Limits	
		Lower	Upper
LC/EC 1.00	0.001	0.000	0.019
LC/EC 5.00	0.005	0.000	0.059
LC/EC 10.00	0.015	0.000	0.114
LC/EC 15.00	0.032	0.000	0.182
LC/EC 50.00	0.750	0.076	61.313
LC/EC 85.00	17.521	2.544	%90378308943872.000
LC/EC 90.00	36.930	3.987	% 100059303.339E+09
LC/EC 95.00	111.468	7.541	% 100000002.004E+12
LC/EC 99.00	885.124	23.860	% 100000002.004E+12

NOTE - Upper limits greater than or equal to 1.E20 are really infinite

Experiment 3

PLOT OF ADJUSTED PROBITS AND PREDICTED REGRESSION LINE



15.3.2.2 Experiment 6: Raw output from PROBIT.EXE

EPA PROBIT ANALYSIS PROGRAM
 USED FOR CALCULATING LC/EC VALUES
 Version 1.5

Experiment 6

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls	Predicted Proportion Responding
Control	30	9	0.3000	0.0000	0.3431
0.0500	30	11	0.3667	0.0359	0.0000
0.1000	30	11	0.3667	0.0359	0.0016
0.5000	30	15	0.5000	0.2389	0.2545
1.0000	30	23	0.7667	0.6448	0.6269
2.0000	30	28	0.9333	0.8985	0.9045

Chi - Square for Heterogeneity (calculated) = 0.185
 Chi - Square for Heterogeneity (tabular value at 0.05 level) = 7.815

Mu = -0.099046
 Sigma = 0.305922

Parameter	Estimate	Std. Err.	95% Confidence Limits	
Intercept	5.323762	0.234062	(4.865001,	5.782524)
Slope	3.268803	1.003155	(1.302619,	5.234986)
Spontaneous Response Rate	0.343053	0.050300	(0.244465,	0.441640)

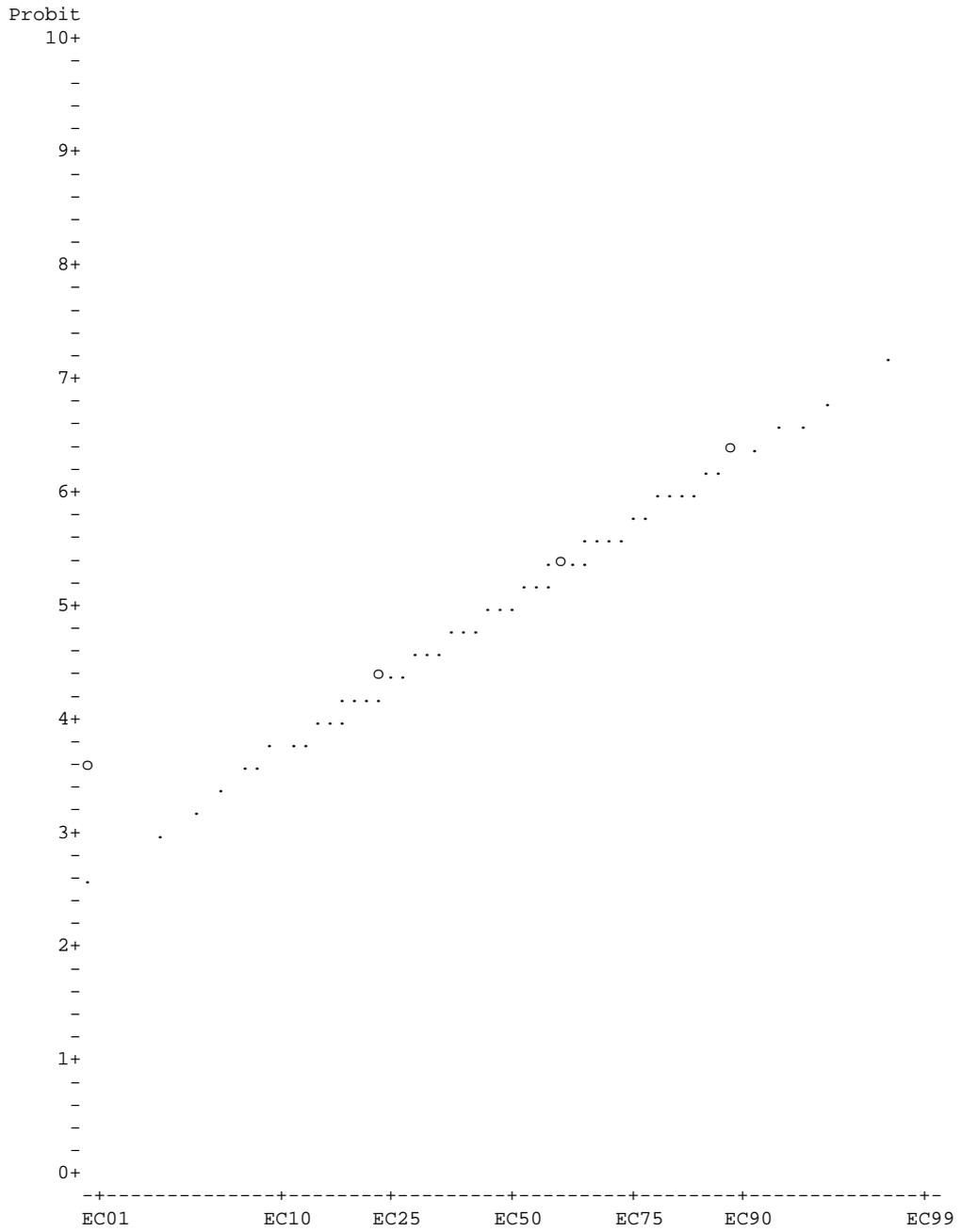
Experiment 6

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	95% Confidence Limits	
		Lower	Upper
LC/EC 1.00	0.155	0.008	0.336
LC/EC 5.00	0.250	0.026	0.460
LC/EC 10.00	0.323	0.048	0.545
LC/EC 15.00	0.384	0.074	0.614
LC/EC 50.00	0.796	0.406	1.098
LC/EC 85.00	1.652	1.192	3.680
LC/EC 90.00	1.963	1.381	5.461
LC/EC 95.00	2.536	1.677	10.029
LC/EC 99.00	4.098	2.337	32.379

Experiment 6

PLOT OF ADJUSTED PROBITS AND PREDICTED REGRESSION LINE



15.3.2.3 Experiment 3 + 6: Raw output from PROBIT.EXE

EPA PROBIT ANALYSIS PROGRAM
 USED FOR CALCULATING LC/EC VALUES
 Version 1.5

Experiment 3 + 6

Conc.	Number Exposed	Number Resp.	Observed Proportion Responding	Proportion Responding Adjusted for Controls	Predicted Proportion Responding
Control	60	17	0.2833	0.0000	0.3520
0.0100	30	8	0.2667	-.1317	0.0000
0.0500	60	22	0.3667	0.0227	0.0001
0.1000	60	27	0.4500	0.1513	0.0022
0.5000	60	31	0.5167	0.2541	0.2414
1.0000	60	43	0.7167	0.5628	0.5885
2.0000	30	28	0.9333	0.8971	0.8748

Chi - Square for Heterogeneity (calculated) = 3.650
 Chi - Square for Heterogeneity (tabular value at 0.05 level) = 9.488

Mu = -0.072779
 Sigma = 0.325252

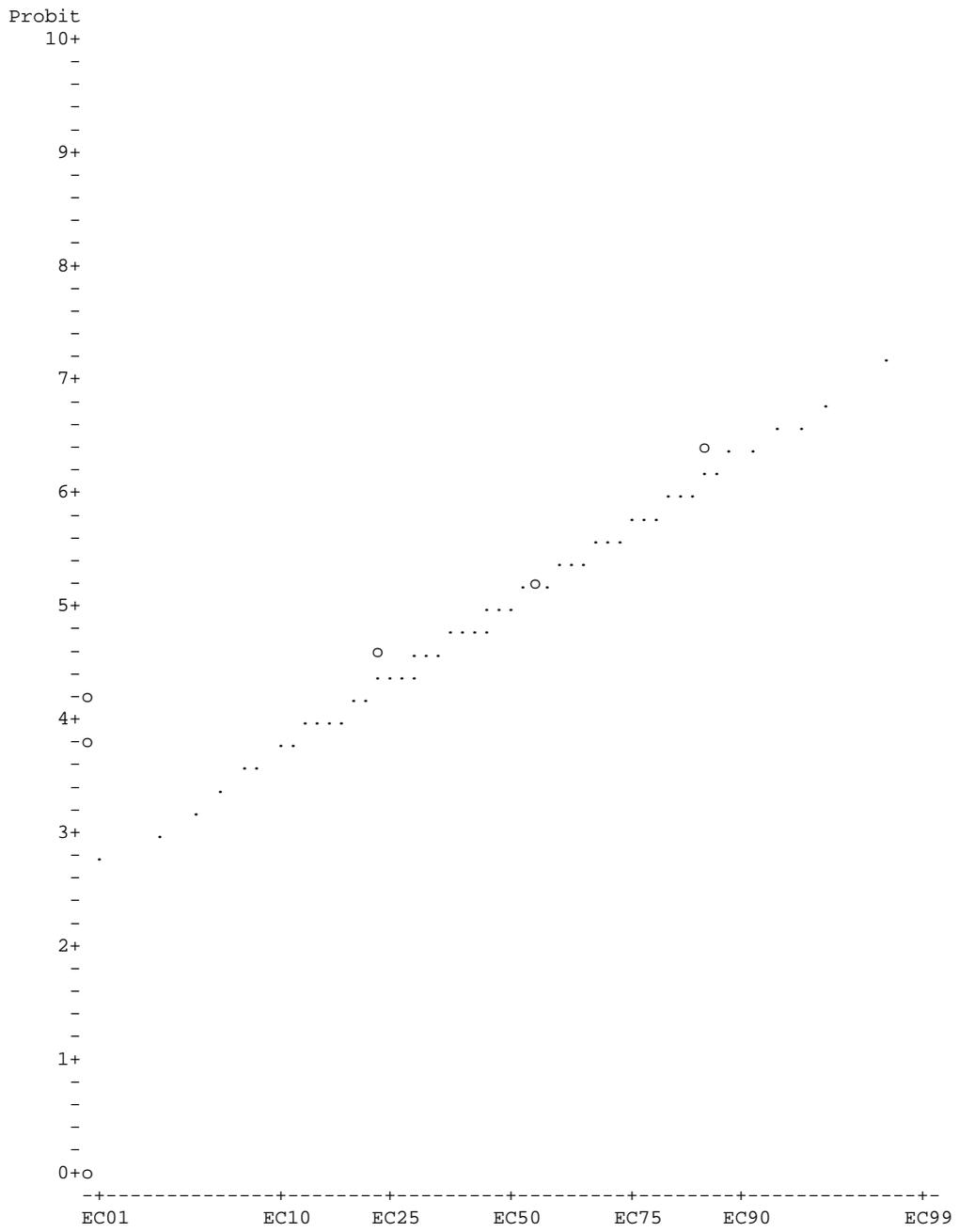
Parameter	Estimate	Std. Err.	95% Confidence Limits	
Intercept	5.223761	0.180531	(4.869920,	5.577601)
Slope	3.074542	0.830733	(1.446306,	4.702778)
Spontaneous Response Rate	0.351985	0.033222	(0.286870,	0.417100)

Experiment 3 + 6

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	95% Confidence Limits	
		Lower	Upper
LC/EC 1.00	0.148	0.017	0.296
LC/EC 5.00	0.247	0.049	0.418
LC/EC 10.00	0.324	0.087	0.505
LC/EC 15.00	0.389	0.127	0.575
LC/EC 50.00	0.846	0.570	1.106
LC/EC 85.00	1.838	1.350	4.043
LC/EC 90.00	2.208	1.551	5.860
LC/EC 95.00	2.899	1.887	10.264
LC/EC 99.00	4.829	2.682	29.823

PLOT OF ADJUSTED PROBITS AND PREDICTED REGRESSION LINE



15.3.3 Linear interpolation

15.3.3.1 Experiment 3: Output from ICPIN.EXE

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	.01	.05	.1	.5	1
Response 1	6	9	5	4	4	4
Response 2	8	6	7	5	6	3
Response 3	8	7	7	5	4	3

*** Inhibition Concentration Percentage Estimate ***

Toxicant/Effluent: LAB
 Test Start Date: Test Ending Date:
 Test Species: C. elegans
 Test Duration: 24h
 DATA FILE: 3.icp
 OUTPUT FILE: 3.i50

Conc. ID	Number Replicates	Concentration % dw	Response Means	Std. Dev.	Pooled Response Means
1	3	0.000	7.333	1.155	7.333
2	3	0.010	7.333	1.528	7.333
3	3	0.050	6.333	1.155	6.333
4	3	0.100	4.667	0.577	4.667
5	3	0.500	4.667	1.155	4.667
6	3	1.000	3.333	0.577	3.333

The Linear Interpolation Estimate: 0.8750 Entered P Value: 50

Number of Resamplings: 80 Those resamples not used had estimates above the highest concentration/ %Effluent.

The Bootstrap Estimates Mean: 0.7732 Standard Deviation: 0.1531

No Confidence Limits can be produced since the number of resamples generated is not a multiple of 40.

Resampling time in Seconds: 0.00 Random_Seed: 74293626

15.3.3.2 Experiment 6: Output from ICPIN.EXE

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	.05	.1	.5	1	2
Response 1	8	7	5	7	2	1
Response 2	7	5	9	3	2	1
Response 3	6	7	5	5	3	0
Response 4	9	7	5	5	3	0

*** Inhibition Concentration Percentage Estimate ***

Toxicant/Effluent: LAB
 Test Start Date: Test Ending Date:
 Test Species: C. elegans
 Test Duration: 24h
 DATA FILE: 6.icp
 OUTPUT FILE: 6.i50

Conc. ID	Number Replicates	Concentration % dw	Response Means	Std. Dev.	Pooled Response Means
1	4	0.000	7.500	1.291	7.500
2	4	0.050	6.500	1.000	6.500
3	4	0.100	6.000	2.000	6.000
4	4	0.500	5.000	1.633	5.000
5	4	1.000	2.500	0.577	2.500

```

6          4          2.000          0.500          0.577          0.500
-----
The Linear Interpolation Estimate:      0.7500   Entered P Value: 50
-----
Number of Resamplings:      80
The Bootstrap Estimates Mean:  0.7155 Standard Deviation:      0.1172
Original Confidence Limits:  Lower:      0.4500 Upper:      0.8712
Expanded Confidence Limits:  Lower:      0.2700 Upper:      0.9439
Resampling time in Seconds:      0.06   Random_Seed: -575367110

```

15.3.3.3 Experiment 3 + 6: Output from ICPIN.EXE

```

Conc. ID      1      2      3      4      5      6      7
-----
Conc. Tested  0      .01     .05     .1     .5     1     2
-----
Response 1    8          7     5     7     2     1
Response 2    7          5     9     3     2     1
Response 3    6          7     5     5     3     0
Response 4    6     9     5     4     4     4
Response 5    8     6     7     5     6     3
Response 6    8     7     7     5     4     3
-----

```

```

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: LAB
Test Start Date:      Test Ending Date:
Test Species: C. elegans
Test Duration:                24h
DATA FILE: 3_and_6.icp
OUTPUT FILE: 3_and_6.i50
-----

```

Conc. ID	Number Replicates	Concentration % dw	Response Means	Std. Dev.	Pooled Response Means
1	6	0.000	7.167	0.983	7.222
2	3	0.010	7.333	1.528	7.222
3	6	0.050	6.333	1.033	6.333
4	6	0.100	5.500	1.761	5.500
5	6	0.500	4.833	1.472	4.833
6	6	1.000	2.833	0.753	2.833
7	3	2.000	0.667	0.577	0.667

```

-----
The Linear Interpolation Estimate:      0.8056   Entered P Value: 50
-----
Number of Resamplings:      80
The Bootstrap Estimates Mean:  0.7777 Standard Deviation:      0.0878
Original Confidence Limits:  Lower:      0.5741 Upper:      0.9259
Expanded Confidence Limits:  Lower:      0.5046 Upper:      0.9620
Resampling time in Seconds:      0.00   Random_Seed: -187657378

```

15.4 Publications

Portions of the literature review and some results have been published in journals, conference proceedings and posters. They are reproduced here for information.

In addition to these, a manuscript based closely on Chapter 6 has been submitted to *Chemosphere* and it is intended that the data from Chapter 5 will form the basis of a short submission to *Environmental Toxicology*.

15.4.1 Land Contamination and Reclamation, 2001

Parts of the present literature review, and results from the MSc thesis submitted in 2000 have been published as Johnson, S.J., Barry, D.A., Christofi, N., and Patel, D. (2001) *Potential for anaerobic biodegradation of linear alkylbenzene cable oils: Literature review and preliminary investigation*. *Land Contamination & Reclamation* **9**, 279-291.

Potential for Anaerobic Biodegradation of Linear Alkylbenzene Cable Oils: Literature Review and Preliminary Investigation

S.J. Johnson, D.A. Barry, N. Christofi and D. Patel

Abstract

Linear alkylbenzene (LAB) cable oils are used for the electrical insulation of high-voltage underground power cables. Due to thermal movement of the cables, leaks can occur at the joints, resulting in cable oil leaking into the surrounding environment. A review of the literature indicates that relatively little is known about the fate of LAB as a bulk pollutant in soil. To investigate this, a physical model of a cable joint bay was constructed and contaminated with cable oil. Fluorometry confirmed that the cable oil became localised to the upper regions of the saturated zone where dissolved oxygen, pH, and oxidation-reduction measurements indicated that conditions were predominantly anaerobic, with evidence for sulphate reduction. Mathematical modelling indicates that these conditions were not due solely to the geochemistry of the system. Mesocosm experiments suggest that LAB may be degraded naturally under anaerobic conditions at rates high enough to justify the use of monitored natural attenuation as a remediation strategy. The mechanisms involved in anaerobic degradation are not currently well understood and more research is required to clarify these and identify the microorganisms involved.

Key words: natural attenuation, aerobic/anaerobic degradation, groundwater, bioremediation, physical model, geochemical model, PHREEQC, mesocosm experiment

INTRODUCTION

The electricity transmission system in England and Wales uses a combination of overhead power lines and underground power cables. The power cables consist of a copper conductor, surrounded by electrical insulation in the form of lapped paper tapes impregnated with insulating oil. This core is contained within a metallic

sheath for oil containment, as the oil has to be maintained at a positive pressure. A polymeric oversheath is applied for mechanical protection. The original insulating medium was mineral oil. Since the 1970s, a mixture of linear alkylbenzenes (LABs), colloquially known as dodecylbenzene (DDB) has been used. In general, cable oil leaks can occur through one of two mechanisms: (1) deterioration of the joints through thermal movement over a long period of time, or (2) third-party damage. At present, cable oil leaks are detected, located and repaired if the leak rate is above 40 L month⁻¹ (based on the detection/location limits using currently available methods). The backfill material surrounding the cable is removed and sent to a licensed landfill. The soil surrounding the immediate joint bay area typically cannot be excavated due to the nature of the sites, which are often in urban areas where access to the surrounding soil is restricted by other services, which could be damaged if the soil was excavated.

Characterisation of a batch of commercially available cable oil using gas chromatography – mass spec-

Received February 2001; accepted May 2001

Authors

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trometry (GC-MS) has shown it to contain 18 major components, ranging from decyl- to tridecyl-benzenes (Rowland 1996). However, there is some variation between batches. LABs are also present in small quantities in the environment as residues from the manufacture and use of linear alkylbenzene sulphonate detergents (Eganhouse 1986). Physical properties are summarised in Table 1. It is likely that the low aqueous solubility will be the ultimate limiting factor in the rate of biodegradation. The low density and poor aqueous solubility make it likely that the oil will partition at the water table.

Table 1. Physical properties of linear alkylbenzene (LAB) cable oil

Property	Value
Appearance	Clear colourless liquid ⁽¹⁾
Density at 20°C	0.86 kg L ⁻¹ ⁽²⁾
Boiling point	≥ 260°C ⁽¹⁾ 726°C ⁽²⁾
Kinematic viscosity at 20°C	7.5 – 8.5 mm ² s ⁻¹ ⁽¹⁾ 8.1 mm ² s ⁻¹ ⁽²⁾
Flashpoint	≥ 130 °C ⁽¹⁾ 150°C ⁽²⁾
Aqueous solubility	Immiscible ⁽¹⁾ < 0.01 mg L ⁻¹ ⁽²⁾ 0.41 mg L ⁻¹ ⁽³⁾ 4 – 7 nmol L ⁻¹ ⁽⁴⁾
Vapour pressure at 25°C	4.9 × 10 ⁻⁴ mmHg ⁽³⁾ 0.038 – 0.067 Pa ⁽⁴⁾
Henry's Law constant	7.1 × 10 ² torr L mol ⁻¹ ⁽³⁾
Soil partition coefficient, K _{oc}	2.2 × 10 ⁴ ⁽³⁾
Log octanol:water partition coefficient, K _{ow}	5.72 – 5.75 ⁽³⁾ 4.97 – 5.08 ⁽⁴⁾

Sources:

- 1 – BICC Cables safety data sheet, July 1994
- 2 – Shell health, safety and environment data sheets
- 3 – Gledhill *et al.* (1991)
- 4 – Sherblom *et al.* (1992)

This paper reviews current knowledge of the occurrence and fate of LAB in the environment. While there is much literature on the occurrence of LAB in aquatic sediments, this has been directed mainly towards using LAB as a marker for LAS detergents and hence of domestic wastewater contamination. Relatively little has been published about the fate of bulk LAB in the terrestrial subsurface. Data is presented that suggests that anaerobic conditions may prevail in cable oil-contaminated subsoil, that biogenic sulphate reduction

occurs, and that the cable oil is the probable carbon source for actively respiring anaerobes.

LITERATURE REVIEW

Linear alkylbenzenes (LABs) are produced synthetically for use in the manufacture of linear alkylbenzene sulphonate (LAS) detergents, varnishes and, significantly for this study, as electrical transmission cable oil. Recently, they have been demonstrated to occur naturally in crude oil (Dutta and Harayama 2001).

A variety of nomenclatures have been applied to LABs. Some authors name them according to the length of the longest aliphatic chain, while others treat them as fundamentally aromatic molecules with a single functional substitution consisting of unbranched (in the case of terminal isomers) or single-branched (at the carbon nearest the attachment to the benzene ring) alkyl chains. Thus, the isomer of C₁₈H₃₀ depicted in Figure 1 may be referred to in the literature as dodecyl-2-benzene, 2-phenyldodecane or (1-methylundecyl)-benzene. The latter, indicating a branched molecule, is the current IUPAC name. Nevertheless, the term LAB is still in common usage to distinguish these molecules from more highly-branched species. Some authors reserve the title LAB for terminal isomers, including toluene and ethylbenzene. It is common to use the shorthand *nCm* where *n* = position of the benzene ring and *m* = number of carbon atoms in the longest aliphatic chain. The isomer in Figure 1 would be referred to as 2C12 in this nomenclature. This leads to a situation in which each of the ~20 isomers may have as many as four designators. There is also variable use of, e.g. dodecylbenzene (DDB) to mean all C₁₈H₃₀ monoalkylated phenyls; only the terminal substitution of *n*-dodecane with a phenyl group or, colloquially, the entire mixture of synthetic LAB. Since there is ambiguity in the use of some of the terminologies, it is difficult to identify all the literature on the subject.

LABs used as precursors in the manufacture of LAS detergents are present in small amounts in the finished product. LABs are more persistent than LAS in riverine, estuary and marine sediments, which are often the receptors for waste effluents containing these detergents and so are a more conservative marker of long-term exposure to domestic wastewater. Sites at which this has been investigated include Tokyo Bay (Bayona *et al.* 1986), the Mersey Estuary (Preston and Raymundo 1993) and Santa Monica Bay (Zeng and Yu 1996; Zeng *et al.* 1997).

Existing studies of the biochemistry of LAB have largely been on terminal isomers (commercially available monoisomeric standards are all terminal isomers). It is unclear what the relevance of these studies is to

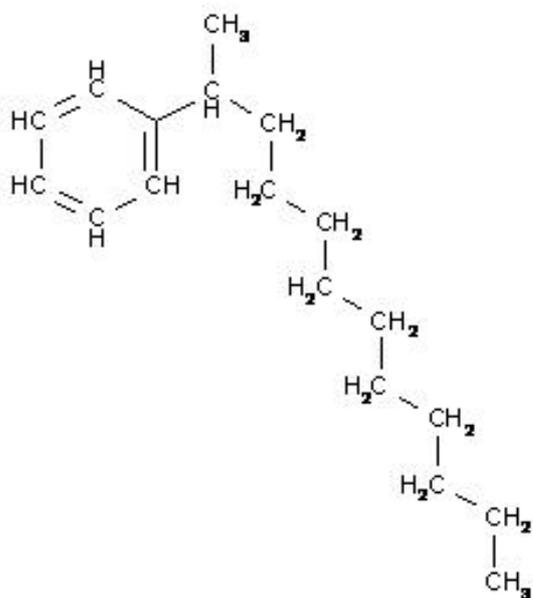


Figure 1. Single isomer of $C_{18}H_{30}$ linear alkylbenzene

LABs in the environment since commercially synthesised LABs do not include these isomers (Eganhouse 1986).

Aerobic biodegradation of 1-phenylalkanes has been demonstrated in *Nocardia* (Sariaslani *et al.* 1974), *Acinetobacter* (Amund and Higgins 1985) and *Pseudomonas* (Bayona *et al.* 1986; Smith and Ratledge 1989). Dodecylbenzene is considered to be readily biodegradable according to OECD guidelines 301D (1992). Indeed, cable oil is thought to act as a carbon source in contaminated soil (Reid *et al.* 2000). Studies on biodegradation rates of hydrocarbons indicate a hierarchy of ease of biodegradation in which *n*-alkanes > branched alkanes > low molecular weight aromatics > cycloalkanes (Leahy and Colwell 1990). The existence of many different isomers means that the trend is not so obvious for higher molecular weight aromatic compounds (Huesmann 1995). Nevertheless, this hierarchy is visible in features of LAB degradation that have been noted in the literature: there have been a number of studies of the isomeric composition of LAB in sediments from Tokyo Bay (Takada and Ishiwatari 1991; Takada and Ishiwatari 1990; Chalaux *et al.* 1995). These indicated preferential degradation of certain isomers. Other studies, of LAB in soils amended with anaerobically digested sewage sludge (Holt and Bernstein 1992; Mangas *et al.* 1998) and of LAB exposed to *Nocardia amarae* isolated from crude oil and petroleum-contaminated soils (Bhatia and Singh 1996), found similar patterns of isomeric enrichment. They all found that external isomers of LABs are degraded in preference to internal isomers. This was

also observed by Bayona *et al.* (1986) and Angley *et al.* (1992), both of whom found better biodegradation if the chain was long. This suggests that access to a free end of the alkane chain is significant in the initial degradation of LABs. Smith and Ratledge (1989) and Smith (1990) elucidated aerobic biodegradation routes for C1-C7 terminal alkylbenzenes, 1-phenyldodecane and 1-phenyltridecane by *Pseudomonas* sp. In the compounds with shorter chain lengths (up to seven carbons) the initial attack on the molecule was an oxidative cleavage of the benzyl ring. With longer chain lengths initial attack was via ω - or β -oxidation of the methyl terminus of the alkyl chain. It is possible that both methyl termini are attacked in non-terminal isomers.

Bhatia and Singh (1996) examined the aerobic biodegradation of commercial LAB by *Nocardia amarae*. They found that the position of the benzene ring affected ease of degradation, with external isomers being more readily degraded than internal ones. They examined the breakdown products and found that the *cis*, *cis*-muconic acid pathway was most significant, with the phenyl acetic acid pathway also being significant where the alkyl chain was an odd number of carbons long and the phenyl substitution was at an even carbon. In these cases *trans*-cinnamic acid formation provided a minor pathway.

Recently, work has been undertaken into the fate of cable oil in soil. Terrestrial subsoils are generally composed of chemically weathered minerals and thus have a lower organic carbon content than most aquatic sediments. Redox conditions vary over longer distances and the microbial flora is significantly different, especially when compared to marine environments. However, studies have produced promising results for cable oil bioremediation. Cheston (1997) and Tebbutt (1998) found that uncontaminated soils contained aerobic organisms that could degrade cable oil at low concentrations. Koussia (1999) found removal rates of $0.15 \mu\text{L ml}^{-1} \text{ week}^{-1}$ in unamended soil, with a 50% increase in rate when nutrients were added.

To date, much of the work done assumes that attenuation of LAB in the environment is due to a combination of physical process and aerobic biodegradation. However, oxygen is not always available and other terminal electron acceptors may be available in soil. For instance, electron acceptors are used preferentially by microbial communities according to availability in a BTEX plume ($\text{O}_2 > \text{NO}_3^- > \text{Fe(III)} > \text{SO}_4^{2-}$). Hence, it is important to consider potential degradation routes under a variety of redox regimes to understand whether degradation will continue to occur as each electron acceptor is depleted, or whether it is necessary to manipulate conditions to maintain a particular set of conditions.

Little is known about anaerobic degradation of the oil. However, where information on the degradation of a specific compound is unavailable, it may be possible to predict the likelihood that it will be degraded under particular conditions by referring to known degradation of other compounds with similar structures (Wackett and Ellis 1999). LABs share a number of structural features with BTEX compounds and with *n*-alkanes so it is useful to review research on a variety of compounds and electron acceptors. Until about a decade ago, it was generally considered that hydrocarbons were recalcitrant under anaerobic conditions. This is now changing and anaerobic biodegradation of hydrocarbons is beginning to be considered an accepted remedial option in some cases (Coates and Anderson 2000). Unlike aerobic metabolism, where there are relatively few pathways available for degradation of a compound, the exact pathways of anaerobic breakdown of hydrocarbons are usually novel. There is a general pattern, however (see Figure 2). Anaerobic degradation of BTEX compounds often proceeds via the formation of benzoyl-CoA.

Anaerobic degradation is generally slower than aerobic degradation of the same compound. Ethylbenzene degradation has been shown to be slow in anaerobic conditions (Borden *et al.* 1995). Degradation of some compounds may be inhibited if other, more readily

degraded carbon/energy sources are available: for instance, benzene degradation is thought to be inhibited in the presence of other hydrocarbons (Krumholz *et al.* 1996). Residual LAB in sediments has been attributed to the anaerobic conditions found there (Takada and Ishiwatari 1990). However, Herbath (2001) has demonstrated anaerobic degradation of cable oil with evidence of sulphate reduction in anaerobic soil mesocosm experiments, and cultured anaerobic cable-oil degrading microorganisms.

Nitrate-, sulphate- and iron-reduction have all been shown to play a part in degradation of numerous hydrocarbons (Aronson and Howard 1997). Long-chain alkanes seem to be most often degraded in sulphate reducing conditions while, on the whole, BTEX is more susceptible to nitrate reducing conditions. Fe(III) reduction has been shown to be significant in the degradation of a number of BTEX compounds (Lovley and Anderson 2000).

Aerobic degradation of alkane chains can be likened to fatty acid degradation by β -oxidation. However, β -oxidation requires molecular oxygen and so is unlikely to occur in strictly anaerobic conditions. Aekersberg *et al.* (1991) were the first to show that hexadecane and other long chain alkanes could be degraded to CO_2 by a bacterial strain under sulphate-reducing conditions. There was some evidence that this strain pro-

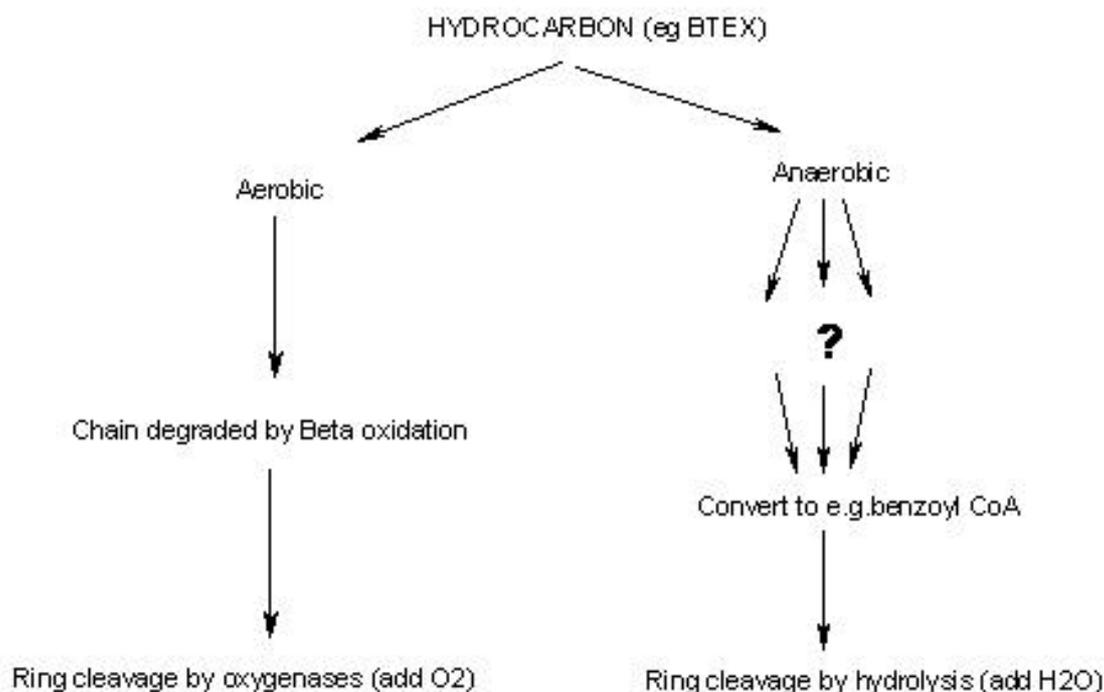


Figure 2. Routes to hydrocarbon biodegradation

duced membrane lipids with an odd number of carbon atoms when fed alkanes with an even number of carbon atoms. This suggests that the alkane chain undergoes the removal or addition of an odd number of carbon atoms in this organism, which contrasts with the strictly even removal of β -oxidation. This even-to-odd transformation has not been seen in subsequently identified alkane-degrading anaerobes and each species is specific to a limited range of chain lengths, indicating that a range of novel pathways are used (Heider *et al.* 1999).

There is evidence that short-chain alkylbenzenes are degraded anaerobically: Toluene (methylbenzene) is degraded under methanogenic conditions by organisms isolated from landfill sites (Wang and Barlaz 1998). A review article by Heider *et al.* (1999) describes catabolism of toluene to benzoyl CoA (a central intermediate in anaerobic catabolism of aromatic compounds) under sulphate-reducing, denitrifying and iron-reducing conditions. They describe catabolism of ethylbenzene by denitrifying bacteria, but note that the pathways for toluene and ethylbenzene are different, and indeed that most anaerobic hydrocarbon degradation pathways are quite novel. Catabolism of propylbenzene by sulphate-reducing bacteria was also reported. Denitrifying bacteria have been shown to possess the CoA ligases required for the formation of acyl-CoA (Villemur 1995). A number of alternative pathways for toluene have been proposed (Chee-Sanford *et al.* 1996; Heider *et al.* 1999) and toluene has been shown to be attacked by hydrolysis of the benzene ring (Grbic-Galic 1991). Anderson and Lovley (2000) showed that benzene can be degraded under sulphate-reducing conditions. Recent work on toluene, ethylbenzene and xylenes in aquifers (Elshahed *et al.* 2001) indicate that all are degraded to benzoate, with the production of methane, under sulphate-reducing conditions. It was suggested that the presence of intermediates from these pathways in the environment might be used to confirm that the contaminants are being degraded *in situ*. Other studies have recently led to the isolation of enzymes from denitrifying bacteria capable of further conversion of benzoyl CoA under anaerobic conditions (Boll *et al.* 2000). It has been shown that oxygenases involved in toluene degradation may be expressed, and function, at low DO (Costura and Alvarez 2000). A minimum DO is required for the oxygenation reaction itself, but it does indicate that at least some enzymes associated with aerobic degradation may be functional in an environment where (aerotolerant) anaerobic respiration prevails. At lower redox values, oxygenases are unlikely to function, and breakdown is more likely to be via hydrolysis. Studies on the degradation of benzoate by the phototropic bacterium *Rhodospseudomonas palustris* and the denitrifiers *Thauera aromatica* (formerly *Pseu-*

domonas sp strain K172) and *Azoarcus evansii* (formerly *Pseudomonas* sp strain K740) have led to the partial elucidation of a number of pathways (Harwood and Gibson 1997; Koch *et al.* 1993). The studies, using nuclear magnetic resonance (NMR) have demonstrated the sequence of breakdown products, though the enzymes involved have yet to be confirmed. There have not been any reports of higher alkylbenzenes being degraded anaerobically prior to Herbath (2001).

PRELIMINARY INVESTIGATION

A model of an underground cable joint bay ('mini pit') was constructed to provide data on the conditions that develop following a leak, for comparison with other studies (Cheston 1997; Tebbutt 1998; Koussia 1999; Herbath 2001). It also allowed the assessment of commercially available sensors and control technology (Johnson 2000).

The soil used in this study was a Horizon B soil of the DeBathe series, as used in related studies (Koussia 1999; Herbath 2001). It is characterised in Table 2. The original choice of this soil was based on its common occurrence in association with underground cables and its easy availability. The low organic carbon content was an advantage since any appreciable biological activity would probably be due to the added carbon source in the presence of adequate nitrogen and other nutrients. The soil was packed in the 'mini pit' (see below) according to a protocol devised for a larger model system (Macdonald 2000) such that the structure of the soil would be retained and the creation of preferential ('short-circuit') pathways avoided.

The mini pit (Johnson 2000) was constructed from a high-density poly(ethylene) (HDPE) container (C1400 4-way entry tank from Mailbox Mouldings International), with an internal volume of approximately 1 m³. Injection and extraction wells were assembled from drilled PVC drainpipe. Time domain reflectometry (TDR) probes were placed in the soil as it was loaded to allow monitoring of water content. TDR has become a recognised method of measuring water content of soil (Davis and Chudobiak 1975; Davis and Annan 1977; Topp *et al.* 1980). The experimental facility had no temperature control. The mini pit was contaminated with 5 L of cable oil, added as a single bolus through the injection well. It was inoculated with microorganisms cultured with cable oil as a sole carbon source, taken from a larger model system which had been contaminated with cable oil some seven months previously (Herbath 2001). Nutrients were added to provide final concentrations of 100 mg N kg⁻¹ soil and 45 mg P kg⁻¹ soil (Margesin and Schinner 1999).

Table 2. Analysis of soil prior to contamination, from Andrews (1999) except* (Lovelace 1999)

Parameter	Value
Dry matter content of air dried soil	98.5%
Water content of air-dried soil	1.6%
Particle size distribution:	
Sand (63 μm – 2 mm)	79.62%
Silt (2 μm – 63 μm)	12.63%
Clay (< 2 μm)	7.75%
Organic carbon	0.1 mg kg ⁻¹
pH _{1:5 soil:water extract}	5.7
Manganese _{1:5 soil:water extract}	0.3 mg kg ⁻¹
Calcium _{1:5 soil:water extract}	0.5 mg kg ⁻¹
Copper _{1:5 soil:water extract}	< 0.05 mg kg ⁻¹
Nickel _{1:5 soil:water extract}	0.3 mg kg ⁻¹
Ammonium _{1:5 soil:water extract}	65.9 mg kg ⁻¹
Nitrate _{1:5 soil:water extract}	34.1 mg kg ⁻¹
Phosphate _{1:5 soil:water extract}	< 0.05 mg kg ⁻¹
Sulphate _{1:5 soil:water extract}	3.8 mg kg ⁻¹
Carbonate _{1:5 soil:water extract}	< 0.05 mg kg ⁻¹
Bicarbonate _{1:5 soil:water extract}	164.8 mg kg ⁻¹
Bulk density	1.3 g cm ⁻³ 1.51 g cm ^{-3*}
Particle density	2.7 g cm ⁻³
Water holding capacity (WHC):	
Maximum	30.7%
0.05 bar	17.2%
01. bar	14.0%
0.4 bar	11.3%
2 bar	9.2%
15 bar	5.8%
Saturated hydraulic conductivity *	1.54 m day ⁻¹
Gravimetric moisture content at saturation *	23.39%
Porosity *	42.83%
Volumetric moisture content at saturation *	35.30%

Groundwater was recirculated at a rate of about 5 L day⁻¹ via an overhead reservoir, which allowed access for measurement of physico-chemical parameters. The reservoir was open to the atmosphere to allow oxygen to diffuse into the water prior to reinjection. Temperature, pH, oxidation-reduction potential (ORP) and dissolved oxygen (DO) were recorded from the points of water input and extraction. In order to measure conditions in the water being extracted from the mini pit without exposure to atmosphere, a sealed flow-through cell was used (Herbath 2001). For comparison, a math-

ematical model of the soil and groundwater geochemistry was constructed using PHREEQC (Parkhurst and Appelo 1999), a computer modelling application provided by the United States Geological Survey.

Samples of soil from the mini pit were cultured on nutrient agar and on a selective agar containing Bushnell-Haas salts (Atlas 1993) with 0.05% cable oil as the sole carbon source (DDB agar). A fluorometric technique (Fu *et al.* 2000) was used to assess the level and distribution of cable oil in the soil.

RESULTS AND DISCUSSION

Following contamination of the soil in the mini pit with cable oil, the oil became localised to the upper regions of the saturated zone. In the field, it is likely that this region can alternate between aerobic and anaerobic conditions and hence LAB may be exposed to a range of redox states. The mini pit system exhibited anaerobic conditions in the upper saturated zone, as evidenced by pH, oxidation-reduction potential and dissolved oxygen (see below). The monitored data show three major perturbations. These were caused by pump failures and the removal of water from the reservoir to aid nutrient addition.

The pH of water in the reservoir (IN) and flow-through cell (OUT) are shown in Figure 3. The pH of the water being pumped from the soil was consistently lower, at approximately 6.0, compared to that in the reservoir (6.5 – 7.0). The groundwater pH was thus slightly lower than the suggested optimum levels for bioremediation of 7.8 (Dibble and Bartha 1979) or 6.5 – 8.5 (Ritter and Scarborough 1995). An increase in pH upon exposure to the atmosphere was noted. This may be due to the loss of the buffering effect of dissolved bicarbonate, which escaped into the atmosphere from the water surface as CO₂, or it may be that aerobic microorganisms in the reservoir removed acidic products of anaerobic microbial metabolism.

The variation in oxidation-reduction potential (ORP) is shown in Figure 4. The instruments used to record ORP were only able to read down to about +5 mV, which translated to a Standard Potential of about -200 mV when corrected for the Ag/AgCl reference electrode. This gives an artificial 'plateau' effect in the trace of ORP OUT. To account partially for this, occasional spot checks were made using a bench pH meter connected to the same ORP electrode. These are plotted as single points (filled triangles in Figure 4) and indicate that the ORP in the groundwater was approximately -600 mV for most of the investigation. The soil analysis (Table 2) indicated that nitrate, sulphate and bicarbonate were present and thus potentially available for use as terminal electron acceptors by soil microor-

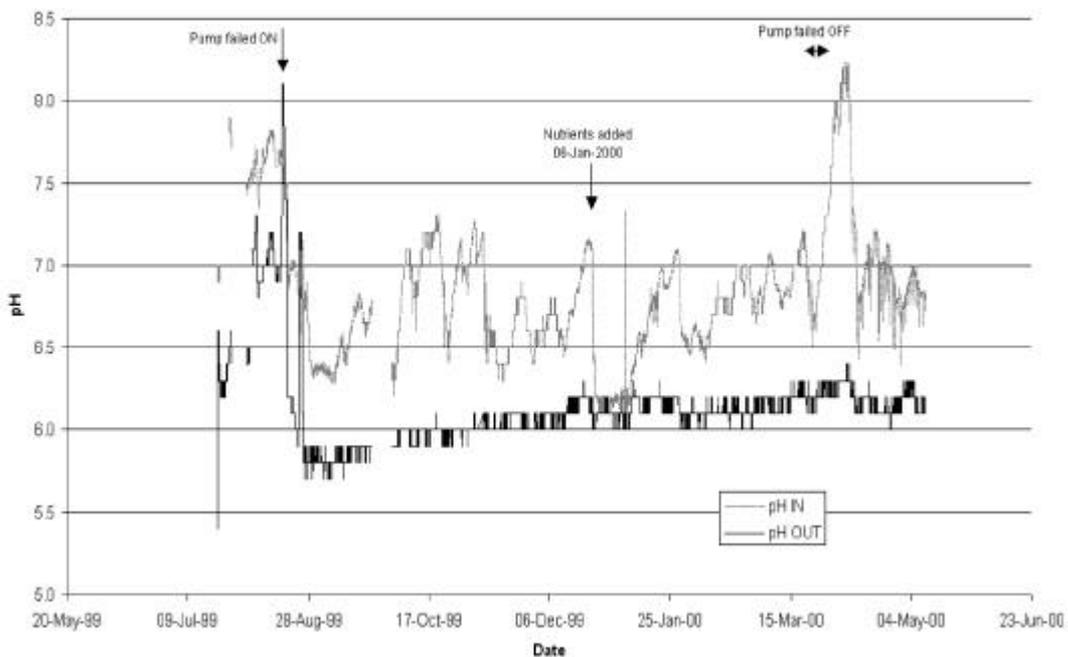


Figure 3. Variation of groundwater pH

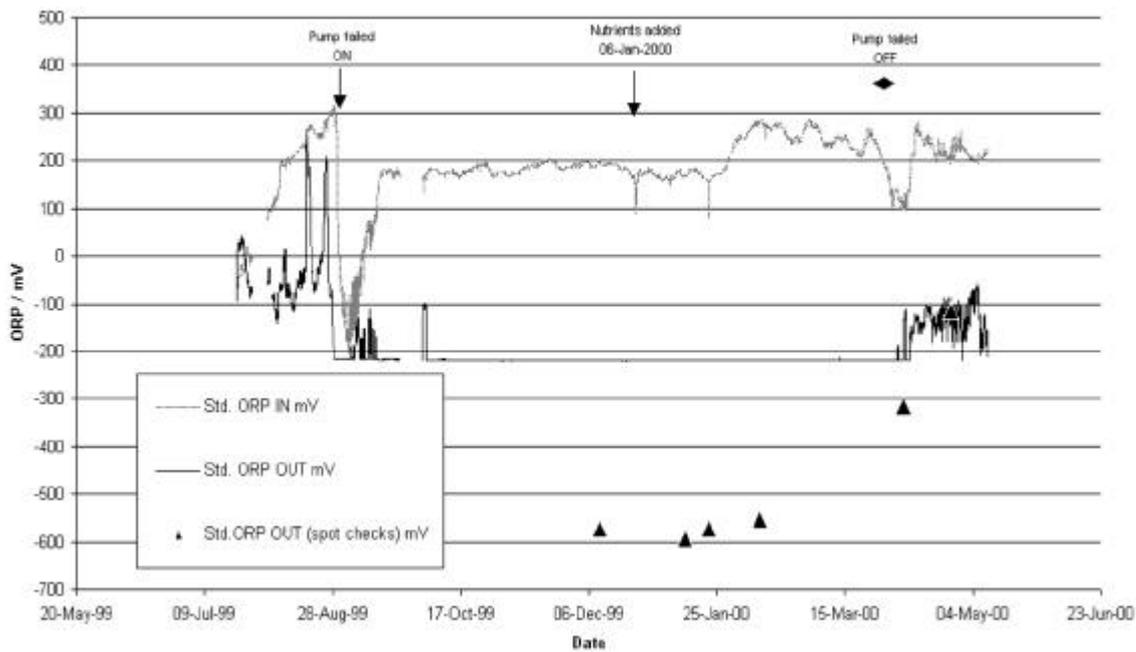


Figure 4. Variation of groundwater oxidation-reduction potential

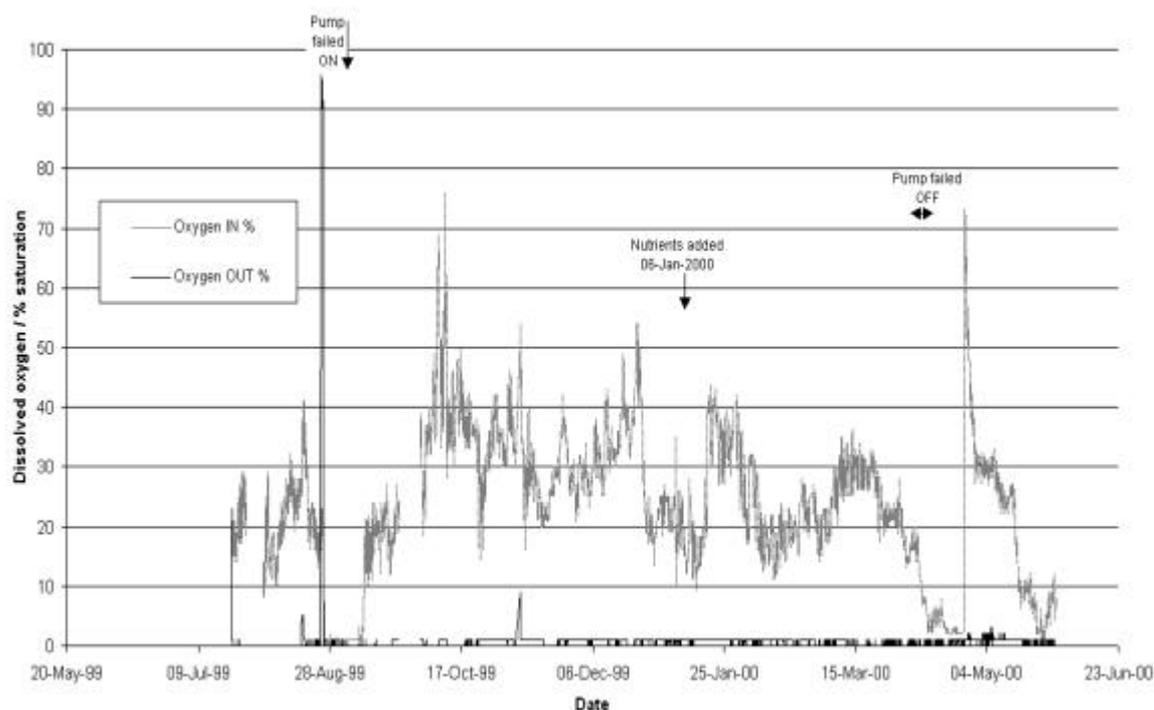


Figure 5. Variation of groundwater dissolved oxygen

ganisms. The low ORP observed in the groundwater is consistent with reduction of sulphate to sulphide. Further evidence of this reduction was the odour of hydrogen sulphide, noted when the settlement tank became anaerobic after the pumping system failed in the ON position. These signs suggest that microbial flora in the groundwater included sulphate-reducing organisms. A similar odour was reported by Herbath (2001) on dismantling anaerobic tanks used to investigate the removal of cable oil from soil under a variety of nutritional and an/aerobic regimes. In summary, the combination of low ORP, known availability of sulphate in the soil and the distinctive odour indicate that sulphate reduction played a significant part in the chemistry of the mini pit. It is highly likely that this was due to anaerobic biological activity, probably associated with cable oil degradation, since this was the only significant carbon source.

Figure 5 shows the temporal changes in dissolved oxygen (DO) in the groundwater. The events that were observed to perturb the pH also affected these readings. With the exception of the first pump failure, the DO reading from the flow-through cell did not depart appreciably from zero. The water being pumped out of

the soil contained no measurable oxygen, this suggests that there were aerobic processes occurring. These may have been biological but not necessarily so. If conditions in the soil were already strongly reduced, the oxygen could be consumed by chemical oxidation of the reduced products of anaerobic respiration. Since there were only two points of DO monitoring, there is no way of knowing how quickly the oxygen was used up but it is likely that any available oxygen was depleted near the injection well.

Figure 6 shows the distribution of aerobically cultured soil microorganisms within a vertical soil core from the mini pit. There are enhanced numbers of microorganisms near the water table (~ 600 mm) and below, where there is more water and a carbon source in the form of cable oil. Microorganisms capable of using cable oil as a sole carbon source (cable oil-degrading microorganisms, CDMs) were found to grow under aerobic conditions. This had previously been found by Cheston (1997) and Tebbutt (1998). The organisms isolated from DDB agar were all Gram positive and some were observed to form hyphae and spore-like structures. These are all features of the actinomycetes. Members of this group include the genus

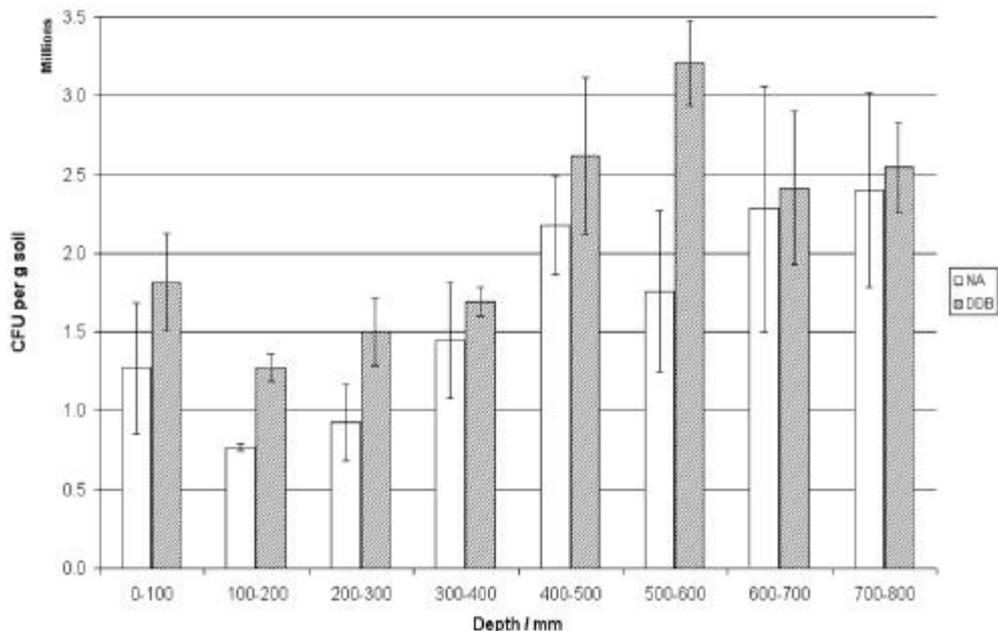


Figure 6. Vertical distribution of aerobic soil microorganisms (Error bars = 1 Std. dev., n = 3, NA = Nutrient agar, DDB = 0.5% cable oil (dodecylbenzene) agar)

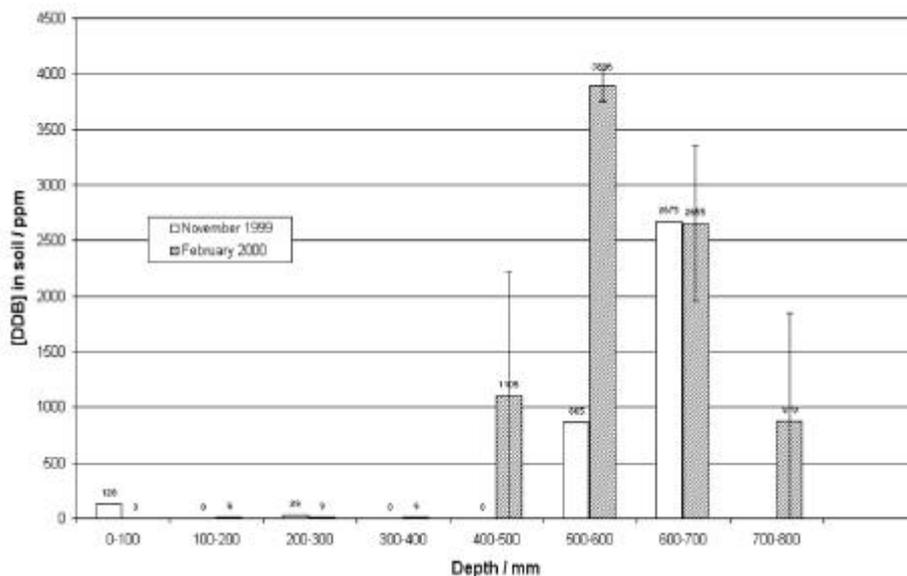


Figure 7. Cable oil distribution (Error bars on data from February 2000 = 1 Std. dev., n = 2, water table ~ 600 mm below soil surface)

Nocardia, which has been demonstrated to degrade LABs (Bhatia and Singh 1996). Many aerobic microorganisms are able to persist in a dormant form under anaerobic conditions. Hence, the existence of colony-forming units of cable oil-degrading aerobes in the soil, even where there is cable oil, is not evidence that biodegradation by the particular microorganisms isolated is taking place. It is also possible that a proportion of these organisms may be facultative anaerobes, able to grow under nitrate reducing (possibly denitrifying) conditions. Herbath (2001) demonstrated anaerobic soil organisms capable of survival on cable oil and mineral salts alone. She reported a significant reduction in cable oil concentrations within six months in soil under anaerobic conditions at 8°C, suggesting that natural attenuation of cable oil in anaerobic groundwater may be significant.

Soil cores were analysed for cable oil content (Figure 7). The data confirms that the cable oil was concentrated at or near the water table. This localisation of the oil was consistent with there being free oil partitioned at the water table (~ 600 mm depth, confirmed by TDR readings) and adsorbed onto soil particles, with only a very small proportion in solution. The data do not indicate that oil concentrations decreased with time, but since the sampling was destructive, the positions of each core were different. The apparent increase in oil concentrations was probably due to spatial inhomogeneity in the oil distribution (Macdonald 2000).

PHREEQC (Parkhurst and Appelo 1999) was used to construct a mathematical model of the soil and groundwater geochemistry using soil analysis data from Table 2 and including the added nutrients. The results of running the model (details not shown here) clearly indicate that the soil chemistry was unable to account for the highly reduced conditions and odour of hydrogen sulphide, suggesting that biologically-mediated mineralization of organic matter and nutrients must have occurred.

CONCLUSIONS

It appeared that the oil added to the soil was strongly localised to the upper saturated zone, where both aerobic and anaerobic conditions may occur. Previous investigations into cable oil degradation in soil assumed that conditions would be aerobic (Cheston 1997; Tebbutt 1998; Koussia 1999), and all of the degradative pathways so far described are aerobic. However, this study indicates that this is not necessarily the case. LABs are easily degraded under aerobic conditions, but appear to be less susceptible to anaerobic breakdown, possibly due to differences in the available degradative pathways of different isomers. However,

there is strong evidence from this work that biological activity can occur in soil under anaerobic conditions, with cable oil as the only significant carbon source. This is in agreement with Herbath (2001), who found that anaerobic removal of cable oil in soil does occur. While anaerobic degradation of LAB has not been demonstrated previously, the last decade or so has seen great advances in the understanding of anaerobic catabolism of hydrocarbons. LAB shares structural features with *n*-alkanes and with BTEX compounds. Examples of these compounds have been shown to be degraded under a variety of redox regimes (Aronson and Howard 1997; Harwood and Gibson 1997; Heider *et al.* 1999). It is not unreasonable to surmise whether LABs may be vulnerable to the same processes. The most likely route for anaerobic degradation of LABs will probably be an initial attack on the alkyl chain(s) to form an acyl CoA, followed by a hydrolytic ring cleavage.

This suggests that, in the case of oil contamination in urban areas, places with restricted access or larger areas of low-level contamination where excavation of the soil would be difficult, monitored natural attenuation (MNA) may provide a practical solution to dealing with cable oil contamination. If degradation rates were demonstrated to be high enough, MNA would likely be the preferred method. This strategy would require a fuller understanding of the microbiology and biochemistry of anaerobic degradation since it may be necessary to use enhanced natural attenuation – adding appropriate nutrients and electron acceptors – in some scenarios. In addition, the critical degradation components would need to be identified to determine the rate of degradation and provide a measure of the level of contamination remaining. Aerobic cable oil degrading microorganisms have been demonstrated in anaerobic regions of the soil, possibly as inactive spores that are able to grow once oxygen is available. If natural attenuation in these conditions proves to be too slow, there remains the option, for localised areas of contamination, of manipulating conditions to provide an environment suitable for the faster metabolism of aerobic microorganisms.

Further investigations into the fate of cable oil under a range of redox conditions are under way, using GC-MS to examine isomeric composition of the oil and any associated degradation products.

ACKNOWLEDGEMENTS

Experimental work was carried out as part of an MSc project (Johnson 2000) at Cranfield Institute of Bio-Science and Technology and was funded by the National Grid Company plc. Stephen Johnson would particularly like to thank Dr David Weston, Dr David

Aldred and Ms Yolande Herbath for their invaluable advice and assistance.

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15.4.2 European Geophysical Society XXVII General Assembly, 2002

Preliminary results were presented in Johnson, S.J., Christofi, N and Barry, D.A. Changes in isomeric composition of LAB cable oil associated with a range of terminal electron acceptors. Poster presented at European Geophysical Society XXVII General Assembly, Nice, France. April 2002.

Abstract

Synthetic linear alkylbenzenes (LABs) are used as insulating oils in underground electricity transmission cables. Following accidental damage to the cables or a failure of the cable system, oil may leak into the surrounding soil. The cable is normally repaired quickly and the majority of the oil is recovered, but access to soil containing residual contamination may be hampered by proximity to other buried services, so minimally invasive techniques for remediation are sought. Laboratory experiments have been designed to investigate LAB degradation under anaerobic conditions, as these are known to pertain in the field. Preliminary results indicate, for the first time, that microbial growth can occur with nitrate as a terminal electron acceptor (TEA) and LAB as the sole carbon source. LAB degradation was not evident under sulphate-reducing and methanogenic conditions. GC-MS data show that the isomeric composition of oil degraded under nitrate-reducing conditions is altered in a way that differs significantly from oil that has been degraded by aerobic microorganisms. Ongoing work, aimed at confirming these findings, includes repetition of the flask culture experiments, greater replication and the inclusion of an internal GC standard to allow the degradation to be fully quantified. Cable-oil degrading microorganisms (CDMs) will be isolated and identified, and the pathways of degradation delineated.

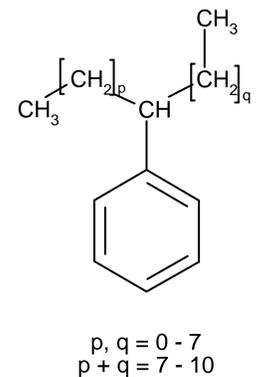


Figure 1. Generalized structure of LAB in cable oil.

Methods

Samples of unused cable oil supplied by National Grid Company plc. were exposed to microorganisms from an LAB-contaminated soil with a variety of terminal electron acceptors. Cable oil is a synthetic mixture of about 20 LAB isomers (not including stereoisomers) with the general structure shown in Figure 1.

Flasks containing 20ml Bushnell-Haas medium plus 0.1ml LAB as the sole carbon source were inoculated with organisms cultured from cable oil-contaminated soil (Johnson *et al* 2001). Aerobic (AER) and killed control (CON) flasks were incubated aerobically in a shaker/incubator. Other cultures containing either no additional TEA (MET), nitrate (NIT), sulphate (SUL) or both nitrate and sulphate (ANA) were incubated anaerobically under a 80% N₂/10% CO₂/5% H₂ atmosphere.

Three flasks from each treatment were harvested at intervals and the LAB extracted by shaking in *n*-hexane. 5µl of 1C12 LAB was added immediately prior to the extraction step to serve as an internal GC standard. The organic fraction was analysed by GC-MS:

HP5890 Series II Gas Chromatograph. Column: HP5 (5% polysiloxane), 0.32 mm i.d.; 0.25µm film thickness; 30 m length. **Carrier gas:** He. **Injection:** 1µl; splitless; 250°C. **Oven:** 50°C for 2min, increase at 10°C/min, 100°C for 2 min, increase at 2.5°C/min, 150°C for 2 min, increase at 5°C/min, 300°C for 1 min. **HP5972 Mass Selective Detector:** 280°C; scan m/z 35-500 at ~1 s⁻¹.

Typical chromatograms from a preliminary experiment (annotated with alkyl chain lengths and position of the phenyl group) without the addition of a standard are shown in Figures 2 & 3. The standard is, conveniently, eluted in the gap between 3C13 and 2C13, and thus does not interfere with any of the peaks of interest.

Peaks in the chromatograms of fresh LAB and CON flask extracts were identified as being identical by a library search, and by examining the mass spectra directly. Peaks in the experimental flasks were identified by comparison to the CON chromatograms, with particular attention to retention times.

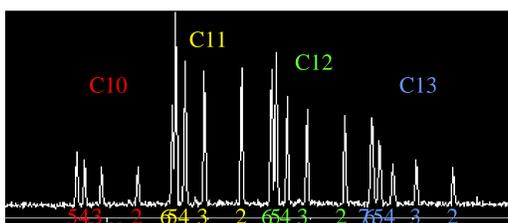


Figure 2. Typical GC of fresh LAB cable oil. Annotated with alkyl chain length and position of phenyl group.

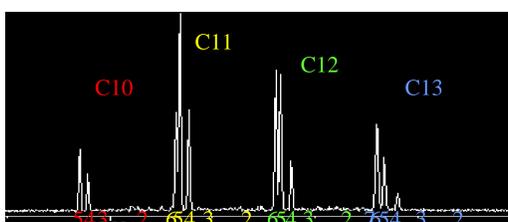


Figure 3. GC of LAB cable oil exposed to nitrate as a TEA (after 13 weeks).

Results and Discussion

Many, though not all, flasks in each of the anaerobic treatments displayed microbial growth. This, with the long lag phase, suggests that the inoculum contained rather low numbers of viable cable-oil degrading organisms. Growth took the form of a cream-coloured pellicle at the oil/water interface. The SUL and MET flasks showed similar, though less marked growth than the ANA and NIT treatments.

As expected, the AER flasks all showed growth, but there was not the distinct pellicle seen in the anaerobic cultures. Rather, the oil dispersed into sub-millimetre globules. It is likely that this was a result of the production of biosurfactants by aerobic CDMs, since the oil in the CON flasks, exposed to the same shaking regime, but not inoculated, did not become dispersed.

The volume of oil recovered from the flasks is plotted in Figure 4. The rather low recovery rate from the CON flasks may be due to volatilisation, or sorption mechanisms.

The most commonly used indicator of LAB degradation in environmental samples (e.g. for LAB residues in aquatic sediments, used as a marker for contamination with LAS detergents) is the ratio between "internal" and "external" isomers of C12 LAB (Equation 1), and this has been adopted for this study. The variation in I:E ratio in flasks from each treatment is shown in Figure 5.

$$I : E = \frac{[6C12 + 5C12]}{[4C12 + 3C12 + 2C12]}$$

Equation 1. Definition of I:E isomeric ratio (Naming convention nC_m, where n = position of benzene ring; m = length of alkyl chain).

The LAB and I:E data represent the mean of three flasks from each treatment harvested per time point. No distinction was made between flasks with visible growth and those without, so there is a relatively large error (not shown). However, the data are consistent with biodegradation of LAB in the AER, ANA and NIT flasks.

Nitrate data (not shown) are inconclusive, but nitrite analysis indicates that nitrite is produced in the NIT flasks. Interestingly, the same is not true in the ANA flasks and it has been suggested that molybdate, added to the NIT flasks to inhibit sulphate reduction, may interfere with the reduction of nitrite to gaseous nitrogen.

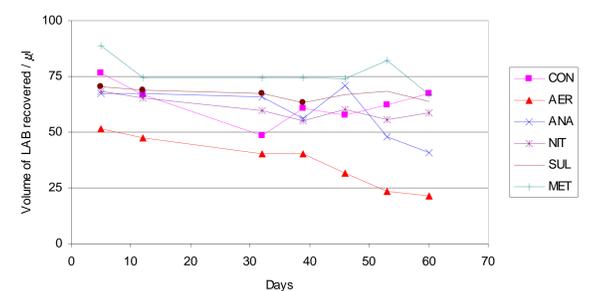


Figure 4. Volume of LAB recovered from flasks containing different TEAs.

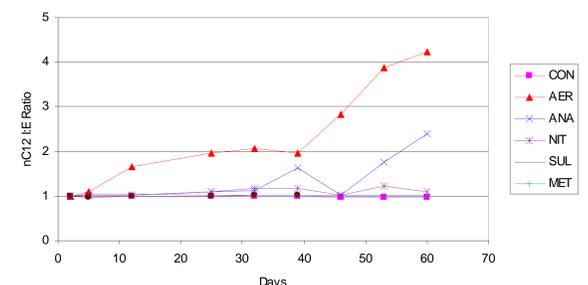


Figure 5. Variation in nC12 I:E ratio in LAB exposed to different TEAs.

It is likely that growth in the SUL and MET flasks uses nitrate present in the basic Bushnell-Haas medium as a TEA, though sulphate reduction and methanogenesis have not been ruled out.

No breakdown products have yet been identified. This may be because the LAB is degraded within single cells, or that breakdown products are water-soluble and so not extracted into the *n*-hexane. Samples of the aqueous phase have been retained for further study.

The experiment continues, but the data so far are strongly suggestive of LAB utilisation as a carbon and energy source by facultative anaerobes, using nitrate as a terminal electron acceptor.

Acknowledgements

This work is funded by the National Grid Company plc.

Reference

Johnson, S.J., Barry, D.A., Christofi, N., and Patel, D. (2001) Potential for anaerobic biodegradation of linear alkylbenzene cable oils: Literature review and preliminary investigation. *Land Contamination & Reclamation* 9, 269-291.

15.4.3 2nd European Biodegradation Conference, 2003

Results from the aqueous cultures and the data concerning the relationship between degradation and isomeric composition were presented in poster form at the 2nd European Bioremediation Conference in Chania, Crete from June 30 to July 4 2003.

It was published as a short paper in the proceedings of the conference as Johnson, S.J., Christofi, N and Barry, D.A. Anaerobic biodegradation of linear alkylbenzenes. Presented at the 2nd European Biodegradation Conference, Chania, Crete. 30 June - 4 July 2003.



Anaerobic Biodegradation of Linear Alkylbenzenes

S. J. Johnson, N. Christofi & D. A. Barry



Napier University

Abstract

Linear alkylbenzenes (LAB) are commonly found in the environment due to their use as a precursor in the manufacture of linear alkylbenzene sulphonate (LAS) detergents, in which they remain as trace contaminants. Other uses include insulating oils in buried electricity transmission cables, from where they may enter soil and groundwater in the event of damage to the cable sheath or joints. They are readily biodegraded under aerobic conditions but may be released into anoxic environments where they can persist for some time. Laboratory experiments show that LAB can be degraded under nitrate-reducing conditions, and isomeric analysis of the remaining LAB is good indicator of the degree of degradation. Work is continuing to identify changes in microbial diversity using denaturing gradient gel electrophoresis of rDNA polymerase chain reaction (PCR) products.

Oil-filled cables

Most high tension electricity transmission is via overhead lines suspended from pylons. This is an efficient and cost-effective solution. Where overhead lines are not appropriate, transmission may be via buried cables. Approximately 600 km of underground electricity cable is in use in England and Wales, at potentials of 275 and 400 kV. The majority of these cables (Fig. 1) are oil-insulated in many cases the insulating oil is a mixture of synthetic linear alkylbenzenes (Fig. 2).

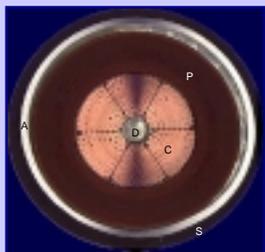


Figure 1. Section of oil-filled cable. The cable consists of a shaped copper conductor (C) with a central oil duct (D). The conductor is wrapped with paper tape (P) which is impregnated with cable oil. An aluminium casing (A) provides mechanical strength and an outer polythene sheath (S) prevents the ingress of water. Image courtesy National Grid Company plc.

The cables are occasionally subject to mechanical damage and failure of the joints between sections. This can result in the release of LAB into the surrounding soil and groundwater. Most of the oil is removed when the soil is excavated to allow the cable to be repaired, but residual oil may remain.

LAB is a light, non-aqueous phase liquid and so will partition at the water table where conditions are likely to be anaerobic. It is known to degrade rapidly in aerobic conditions but it is considered to be persistent in anaerobic sediments.

Aqueous cultures of microorganisms from a contaminated soil with LAB as the sole carbon and energy source indicate that it may be degraded under nitrate-reducing conditions

Measuring biodegradation

Gas chromatography – mass spectrometry is a sensitive method for both identification and quantifying hydrocarbons. Individual homologues of LAB, extracted by shaking in hexane, were separated and quantified by reference to an internal standard ($1C_{12}$ LAB – a terminal isomer, not present in the synthetic mixture).

External isomers, where the phenyl group is towards one end of the alkane chain, are more readily degraded than internal isomers under aerobic conditions. Cultures were incubated with a variety of terminal electron acceptors to investigate whether this was true under different redox conditions (Figs. 3 & 4).

A commonly used indicator of biodegradation of LAB is the ratio of internal to external isomers of nC_{12} LAB. This can be calculated from the peak areas obtained from GC-MS (Eq. 1)

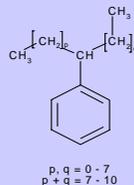


Figure 2. Generalized structure of LAB in cable oil.

$$I : E = \frac{6C_{12} + 5C_{12}}{4C_{12} + 3C_{12} + 2C_{12}}$$

Equation 1. Definition of I:E isomeric ratio (Naming convention nC_m , where n = position of benzene ring; m = length of alkyl chain).

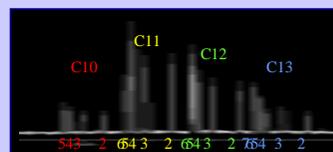


Figure 3. Typical GC of fresh LAB cable oil. Annotated with alkyl chain length and position of phenyl group.



Figure 4. GC of LAB cable oil exposed to nitrate as a TEA (after 13 weeks).

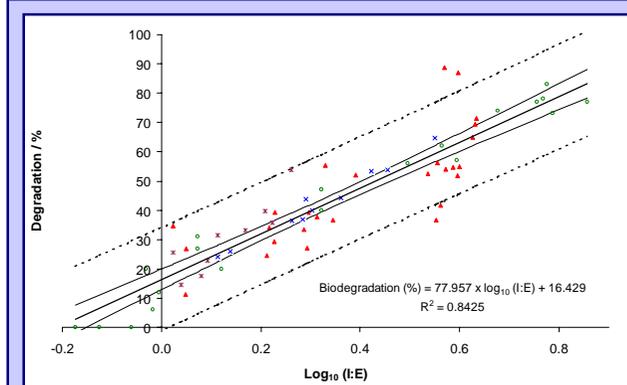


Figure 5. Plot of % biodegradation of LAB vs. \log_{10} internal:external (I:E) nC_{12} LAB isomer ratio with linear regression (heavy) line, 95% confidence (light) and 95% prediction intervals (broken lines). Data points are from individual bottles: aerobic (\circ), anaerobic containing multiple TEAs (\cdot) or nitrate alone (\blacktriangle) and aerobic data from Takada & Ishiwatari (\times). Thanks to Hiroshige Takada for providing raw data.

I:E ratio indicates biodegradation

Plots of % biodegradation against I:E ratio for a variety of conditions are not statistically different ($p < 0.05$) from each other, or from published aerobic data (Takada & Ishiwatari, 1990 *Environ. Sci. Technol.* **24**, 86-91).

The data are combined to give a common regression line (Fig. 5) that may be used to indicate the degree of biodegradation in contaminated sites where neither the original concentration, nor the redox history are known.

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Prepared for the 2nd European Bioremediation Conference, Chania, Crete, Greece 30 June – 4 July 2003

ANAEROBIC BIODEGRADATION OF LINEAR ALKYL BENZENE

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ABSTRACT

Linear alkylbenzenes (LAB) are commonly found in the environment due to their use as a precursor in the manufacture of linear alkylbenzene sulphonate (LAS) detergents, in which they remain as trace contaminants. Other uses include insulating oils in buried electricity transmission cables, from where they may enter soil and groundwater in the event of damage to the cable casing or joints. They are readily biodegraded under aerobic conditions but may be released into anoxic environments where they can persist for some time. Laboratory experiments show that LAB can be degraded under nitrate-reducing conditions, and isomeric analysis of the remaining LAB is good indicator of the degree of degradation. Work is continuing to identify changes in microbial diversity using denaturing gradient gel electrophoresis of rDNA polymerase chain reaction (PCR) products.

1 INTRODUCTION

Linear alkylbenzenes (LAB) have been produced commercially since the early 1960s for use in a variety of industries. The synthetic production pathway results in a mixture of isomers with a variety of alkyl chain lengths, dependent on the feedstock, with the phenyl group in any but the terminal position [22]. Conventionally, these isomers are described using the form nC_m LAB, where n = position of the benzene ring and m = number of carbon atoms in the longest aliphatic chain. For instance, using this convention, (1-methyl, undecyl)-benzene (also known as 2-phenyldodecane or dodecyl-2-benzene) is $2C_{12}$ LAB. Mixtures with an alkyl chain length of C_{10-13} are used to insulate buried electrical transmission cables. Leakage of this cable oil may occur due to deterioration of cable components or through mechanical damage. When a leak is detected, contaminated soil is excavated to effect a repair to the cable, but oil that has migrated away from the immediate vicinity of the cable may be inaccessible, e.g. due to surface infrastructure or other buried services. Monitored natural attenuation is an attractive, and perhaps the only financially viable, strategy in such circumstances. Similar mixtures of $C_{10-13/14}$ LAB are also used in the manufacture of linear alkylbenzenesulfonate (LAS) detergents. Trace amounts remain in the detergent and thus are found in aquatic sediments where LAS contamination occurs. For the same reason, LAB is also seen in sewage sludge used to amend soils [17]. LAB was used for a period in the early 1990s as a major component of drilling oil [16; 21], and has also been identified as occurring naturally in the n -hexane fraction of some crude oils [4; 8]. Alkylbenzenes were reported in sewage extracts as early as 1974 [18], and LAB was identified as an environmental contaminant by Crisp *et al.* [2], who suggested that they may be derived from LAS detergents, either as unreacted feedstock or by microbial desulfonation. Eganhouse *et al.* identified LAB in marine sediments [6]. This was supported by Ishiwatari *et al.* [13], who found them associated with LAS detergent contamination in sediments in Tokyo Bay. However, LAB has not previously been studied as a bulk contaminant in its own right.

The relative amounts of different homologues of LAB in environmental samples can be used as an indicator of biodegradation. A commonly used predictor is the ratio of internal (I) to external (E) isomers of C_{12} LAB [24; 25], calculated as $I:E = (6C_{12} \text{ LAB} + 5C_{12} \text{ LAB}) / (4C_{12} \text{ LAB} + 3C_{12} \text{ LAB} + 2C_{12} \text{ LAB})$. It has been assumed that significant degradation only occurs under aerobic conditions [11]. However, indications that anaerobic degradation of LAB may occur [7; 14] have prompted investigations of cable oil biodegradation where the LAB is exposed to a variety of terminal electron acceptors (TEAs). Data from aqueous cultures of soil micro-organisms containing LAB cable oil as the sole carbon and energy source with a variety of electron acceptors is presented, and the linear regressions are compared using statistical methods.

2 MATERIALS AND METHODS

2.1 Batch cultures

Aqueous cultures consisting of universal bottles containing 20 ml of Bushnell-Haas (B-H) broth [1] with 100 μ l LAB cable oil as the sole carbon source were inoculated with 100 μ l of a suspension of organisms cultured from

cable oil-contaminated soil [14]. Aerobic (AER) and control (CON) bottles were incubated in a shaker/incubator at 25°C. Other cultures containing either no additional TEA (MET), nitrate (NIT), sulfate (SUL) or both nitrate and sulfate (ANA) were loosely capped and incubated at 25°C under an 85% N₂/10% CO₂/5% H₂ atmosphere. Added TEAs were calculated to be stoichiometrically 3-4 times more than that required for complete oxidation of the hydrocarbon. Sodium molybdate (1.0×10^{-2} mol l⁻¹) was added to the NIT cultures to inhibit sulfate-reduction [19]. Similarly, 1.0×10^{-2} mol l⁻¹ sodium chlorate was added to inhibit nitrate reduction [12] in the SUL cultures. The NIT and SUL cultures contained 2.5×10^{-2} mol l⁻¹ 2-bromoethanesulfonic acid (BES) to inhibit methanogenesis [15]. The CON bottles contained both additional nitrate and sulphate, but no inhibitors.

Three vials from each treatment were harvested and destructively analyzed at ten points over a period of 97 days. The contents of each universal bottle were poured into a 100 ml conical flask and LAB was extracted by shaking vigorously for 20 min in two volumes of *n*-hexane with 5 µl of pure 1C₁₂ LAB a terminal isomer (and therefore absent from the synthetic mixture) as an internal GC standard to allow the peak areas to be quantified. A 2 ml aliquot of the organic fraction was transferred to a GC vial and analyzed by GC-MS:

2.2 GC-MS.

Separation was carried out on a Hewlett-Packard (Agilent) HP5890 Series II gas chromatograph fitted with an HP6890 autoinjector. The samples were applied as 1 µl splitless injections at 250°C to an HP5 (5% polysiloxane), 0.32 mm i.d., 0.25 mm film thickness, 30 m capillary column with He as the carrier gas. The column was held at 50°C for 2 min, increased at 10°C min⁻¹, held at 100°C for 2 min, increased at 2.5°C min⁻¹, held at 150°C for 2 min, increased at 5°C min⁻¹, and finally held at 300°C for 1 min. The GC effluent was fed to an HP5972 mass-selective detector at 280°C with *m/z* range of 35-500 scanned at ~1 s⁻¹. Peaks in the total ion current (TIC) chromatograms of fresh LAB and control bottle extracts were identified as being identical by searching against the Wiley138 mass spectrum library, and by examining the mass spectra directly. Peaks in the experimental bottles were identified by comparison to the control bottle chromatograms, with particular attention to retention times. The volume of LAB recovered was calculated by summing the TIC peak areas due to LAB isomers and comparing to the 5 µl 1C₁₂ standard. The equation used was: cable oil volume = $\Sigma(\text{cable oil peak area})/1\text{C}_{12}$ peak area \times 1C₁₂ volume.

Data from the experimental bottles were compared to the control bottles to discount removal of LAB via physical and chemical routes (sorption, volatilization, etc.). Loss from the control (CON) bottles, which were open to the atmosphere, was higher than anticipated, rendering them unsuitable for comparing to the sealed, anaerobic treatments. Anaerobic bottles (MET) containing no additional TEA, which were included to investigate whether methanogenic biodegradation occurred, showed no appreciable biodegradation and so were used as anaerobic controls.

3 RESULTS AND DISCUSSION

A variety of analytical methods have been used to analyze LAB degradation [5; 10; 23]. GC-MS has proved particularly sensitive [10] and the efficiency of the method is not heavily dependent on the details of extraction and GC methods. For soil, shaking in hexane has been shown to be up to 90% efficient, in terms of LAB extraction, over a range of soil types and moisture contents [9; 20]. In any event, because a ratio is being measured, rather than an absolute amount, the extraction efficiency is not critical, so long as the extraction method is not selective for different isomers. Extraction efficiency, being the volume of LAB recovered from the anaerobic control (MET) bottles divided by the volume of LAB added at the start of the experiment, was 74% (n = 33, standard deviation = 12.3%).

Many of the anaerobic treatments failed to show any degradation at all. Microbial growth was not quantified, but where it occurred it was visible as a pellicle at the LAB:water interface. No growth was observed in any of the bottles containing sulfate as the sole additional TEA. This confirms that the activity in the ANA bottles was due to nitrate-, rather than sulfate-reduction and is supported by the depletion of total oxidised nitrogen compared to the CON bottle in both NIT and ANA cultures (data not shown). This was to be expected since the original enrichments also failed to display measurable activity under these conditions and the inoculum was prepared using nitrate-reducing enrichments.

Anaerobic biodegradation was associated with dissimilatory nitrate reduction, with no degradation seen under sulfate-reducing or methanogenic conditions. The ratio of internal to external C₁₂ LAB isomers was calculated and the log₁₀ of this value regressed against % biodegradation of LAB cable oil for each of the experimental conditions. Since the intention was to detect any relationship between isomeric composition and degree of degradation, data points from anaerobic bottles with less than 10% degradation or a log₁₀ (I:E) of less than 0.02 were discarded. Data from Takada and Ishiwatari [24] were similarly analyzed. The linear regression data have similar slopes and intercepts and are summarized in Table 1.

Table 1. Linear regression of % biodegradation of LAB vs. \log_{10} internal:external (I:E) nC_{12} LAB isomer ratio with different terminal electron acceptors

Terminal electron acceptor	Slope	Intercept	R ² (%)
Aerobic (O ₂)	73.0	17.4	61.0
Multiple TEAs (anaerobic – nitrate and sulphate)	92.1	13.0	97.6
Nitrate (plus molybdate to inhibit sulphate reduction)	126.9	13.4	79.0
Aerobic (O ₂) data from Takada and Ishiwatari [24]	81.0	15.0	96.0
Composite regression using all data	77.9	16.4	84.3

The linear fit obtained using all the data was found to be significant ($p < 0.001$). The regression lines for aerobic and anaerobic biodegradation were checked to see whether they were statistically different by comparing them to this regression as described by Draper and Smith [3], and the null hypothesis - that the slopes and intercepts of the individual regressions were identical to the composite line - was accepted in every case ($p < 0.05$). All the available degradation data were plotted (Figure 1) to yield a regression equation that may be applied to field data where the redox history is unknown.

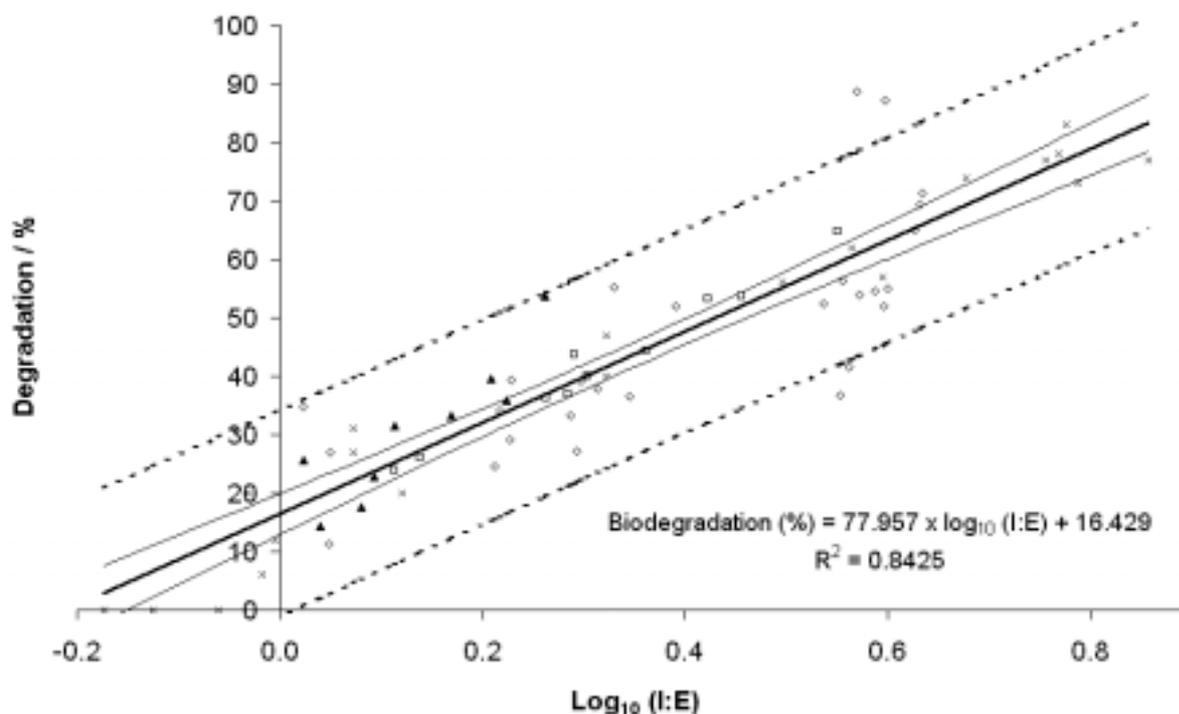


Figure 1. Plot of % biodegradation of LAB vs. \log_{10} internal:external (I:E) nC_{12} LAB isomer ratio with linear regression (heavy) line, 95% confidence (light) and 95 % prediction intervals (broken lines). Data points are from individual bottles: aerobic (\diamond), anaerobic containing multiple TEAs (\square) or nitrate alone (\blacktriangle) and aerobic data from Takada & Ishiwatari [24](\times)

LAB is biodegraded in liquid culture under both aerobic and nitrate-reducing conditions. That the aerobic degradation was so rapid using anaerobically cultured inoculum suggests that facultative anaerobes may play a significant role. Differential degradation of internal and external isomers follows similar patterns under a range of

conditions, while physical and chemical removal does not significantly favor any isomer. The ratio of internal to external nC₁₂ isomers of LAB in the n-hexane fraction may provide a robust indicator of the degree of biological degradation in soils that have been contaminated with LAB, even where the redox history of the site is unknown.

4 ACKNOWLEDGMENTS

This work is funded by National Grid Transco plc. The authors thank Hideshige Takada of Tokyo University of Agriculture & Technology for providing raw data used in the statistical analysis.

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15.4.4 Engineering Geology, 2003

Portions of the literature review were used in a review paper presented at the Geoenvironmental Impact Conference held in Edinburgh from 17-19 September 2001

The original paper appeared in the conference proceedings as Johnson, S. J., Woolhouse, K. J., Prommer, H., Barry, D. A., and Christofi, N. Contribution of anaerobic microbial activity to natural attenuation of the "B" in BTEX. Yong, R. N. and Thomas, H. R. Geoenvironmental Impact Management. 2001. London, Thomas Telford Publishing.

The following is an expanded version, published as Johnson, S. J., Woolhouse, K. J., Prommer, H., Barry, D. A., and Christofi, N. (2003). *Contribution of anaerobic microbial activity to natural attenuation benzene in groundwater*. Engineering Geology **70**, 343-349



Contribution of anaerobic microbial activity to natural attenuation of benzene in groundwater

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Abstract

Anaerobic biodegradation of hydrocarbons, using a variety of terminal electron acceptors (TEAs), is increasingly being reported both in laboratory studies and in the field. Of all the petroleum hydrocarbons, benzene is considered the most problematical due to its high toxicity and relatively high aqueous solubility. These, combined with its peculiarly stable structure, mean that it has long been considered recalcitrant in all but aerobic conditions. There is now a small, but growing, literature to suggest that this may not in fact be the case. We present an assessment of the field, encompassing reviews up to 1997 and original papers published since then. It appears that benzene is indeed degraded anaerobically, but that organisms capable of doing so are not ubiquitous. In addition, benzene degradation may be competitively inhibited by the presence of more readily degraded compounds such as toluene. Certainly, the occurrence and rate of benzene attenuation under anaerobic conditions is far more site-specific than for other benzene, toluene, ethylbenzene and xylenes (BTEX) compounds. We discuss a mathematical method for modelling redox-dependent, differential degradation rates.

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Keywords: Benzene; BTEX; Anaerobic degradation; Nitrate-reduction; Iron-reduction; Sulphate reduction; Methanogenesis; Modelling

1. Introduction

Petroleum contains, in addition to many other hydrocarbon constituents, benzene, toluene, ethylbenzene and xylenes (BTEX). These are the most significant components in terms of pollution potential as they are the most soluble. Leaks of petroleum, leading to contamination of soil and groundwater by BTEX compounds, are widespread. Thus, dissolved BTEX compounds in the subsurface environment are candidates for removal via naturally occurring pro-

cesses, whereby redox reactions mediated by autochthonous microorganisms result in the production of less harmful, even benign, products (Wiedemeier et al., 1996).

Benzene typically makes up less than 2% of petroleum (Irwin, 1997), but is important since it is considered the most toxic and persistent of all petroleum components. Its solubility in water is only 1.78 g l^{-1} (Stephen and Stephen, 1963), yet it is the most soluble of petroleum hydrocarbons (Alexander, 1999). In addition, its structure and shape make it difficult to oxidise and degrade. Reasonable evidence exists showing that the TEX compounds all degrade naturally in groundwater systems (Aronson and Howard, 1997), whereas for benzene the picture is mixed.

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Indeed, it is thought that benzene degradation may be inhibited in the presence of other hydrocarbons, such as toluene (Krumholz et al., 1996), though the mechanism for this is unclear.

BTEX degradation occurs most rapidly under aerobic conditions. However, aquifers are often anoxic. In the absence of dissolved oxygen in groundwater, benzene degradation rates decrease or can stop altogether. The aerobic degradation of benzene, via catechol, is well established (Aronson and Howard, 1997) and will not be further considered here. The application of oxygen to anoxic soil, sediments and groundwater is possible by a variety of means (biopiles, injection of O₂/air/aerated water/hydrogen peroxide or the injection of chlorite, which is degraded by perchlorate-reducing bacteria to yield oxygen in situ (Coates et al., 1998)). These are intrusive and, therefore, relatively expensive measures. Hence, where feasible, monitored natural attenuation (intrinsic bioremediation) is likely to remain the most widespread remediation technique for BTEX-contaminated aquifers.

Natural attenuation encompasses a host of physical processes (e.g. dispersion, dilution, sorption and volatilisation) as well as chemical and biological degradation. Biodegradation depends on microbial activity that varies with hydrogeological site characteristics and aquifer geochemistry (Allard and Neilson, 1997). Here, anaerobic benzene biodegradation is examined, considering evidence from both laboratory and the field. Literature surveys reveal conflicting evidence on conditions required for its degradation (Harwood and Gibson, 1997; Aronson and Howard, 1997; Heider et al., 1999). Thus, it is difficult to predict a priori the occurrence/rate of benzene removal without a detailed understanding of aquifer conditions.

A 1997 review of likely mechanisms (Harwood and Gibson, 1997) suggested that benzene could be degraded via benzoate under a range of conditions. No single microbial species had been shown to degrade completely the compound under anaerobic conditions, although stable benzene-degrading enrichment cultures were known. Toluene-degrading organisms, however, had been identified, and included members of the nitrate-reducing genera *Azoarcus* and *Thauera*, and the iron-reducing *Geobacter metallireducens* as well as a variety of unnamed sulphate-reducers. The

only organisms known to degrade BTEX compounds anaerobically are bacteria, but it has been suggested that the currently poorly studied anaerobic fungi might prove to be involved.

Aronson and Howard (1997) reviewed and tabulated a large number of laboratory and field investigations. The majority of published studies failed to demonstrate anaerobic benzene degradation. Those that did indicated that benzene was degraded under nitrate-, Fe(III)- and manganese-reducing, and sometimes under methanogenic conditions. Reinhard et al. (1997) examined BTEX removal from groundwater in the presence of nitrate and sulphate and showed that benzene and *o*-xylene were the most recalcitrant. Under nitrate-reducing conditions, ethylbenzene, toluene and *m*-xylene were removed within 6 days. In the presence of sulphate, toluene and xylenes were removed after 60 days. Many authors attributed the lack of degradation to insufficient residence time. Others suggested that since benzene degradation appears to be inhibited in the presence of other carbon sources, it might be that the degradation seen in the field was due to aerobic degradation at the plume periphery.

Since these reviews, a number of pertinent papers have been published. Space does not allow for the comprehensive review of papers published prior to 1997. We therefore suggest this paper should be read in conjunction with the earlier reviews (Aronson and Howard, 1997; Harwood and Gibson, 1997; Heider et al., 1999).

2. Benzene degradation under different redox regimes

Cellular respiration, the process by which living cells obtain energy to support metabolic processes, comprises a chain of oxidation–reduction couples, whereby energy is extracted via a stepwise oxidation (i.e., removal of electrons) of organic and inorganic molecules. In order to proceed, there needs to be a relatively more oxidised chemical species available at each step to prevent the accumulation of electrons that would hinder the reaction kinetics. The compounds that provide a “sink” for the electrons expelled at the end of the chain of reactions are known as terminal electron acceptors (TEAs). In

aerobic respiration, the TEA is molecular oxygen, but in the absence of oxygen, a number of less highly oxidised compounds may serve, assuming organisms capable of making use of them are present. Available TEAs are generally used in the environment in decreasing order of oxidation–reduction potential. Possible TEAs include NO_3^- , Fe(III), Mn(IV), SO_4^{2-} and CO_2 . Lovley (2000) noted that benzene degradation had been reported with all these common electron acceptors.

2.1. Nitrate-reducing conditions

Benzene has long been considered recalcitrant in the field under nitrate-reducing conditions. Where it has been seen, it has been much slower than under aerobic conditions and it appears that O_2 is still required as a substrate for the oxygenases that mediate the oxidative cleavage of the aromatic ring, even if it is not used as a TEA (Anid et al., 1993; Durant et al., 1999). Benzoate is often considered to be a central metabolite in the degradation of monoaromatic hydrocarbons, and it has been shown to be degraded under denitrifying conditions (Harwood and Gibson, 1997) though some workers still point to the apparent inability of nitrate-reducers to degrade benzene (Kao and Borden, 1997). Nales et al. (1998) demonstrated benzene degradation under nitrate-reducing conditions, but found that TEX substrates competitively inhibited its degradation. They also demonstrated benzene degradation under sulphate- and Fe(III)-reducing conditions, but not with methanogenesis. Burland and Edwards (1999) link benzene degradation to reduction of nitrate to nitrite (but not to conversion to gaseous nitrogen).

The most noteworthy paper in this field in recent years described benzene oxidation by two strains of the genus *Dechloromonas* with nitrate as the sole electron acceptor (Coates et al., 2001), the first time this has been shown in a single organism, rather than in enrichment cultures or sediment studies. Other than the ability to degrade benzene, the two strains were isolated on the basis of very different metabolic capabilities—one was isolated by its ability to reduce (per)chlorate, the other on its ability to oxidise humic matter. This, along with the demonstrated ubiquity of members of the genus, is pointed to by the authors as an indication that they hold

potential for treatment of benzene-contaminated environments.

2.2. Iron-reducing conditions

Iron is considered to be especially significant in hydrocarbon degradation in marine sediments, with Fe(III), chelated to a variety of compounds, shown to stimulate benzene oxidation in anaerobic sediment (Lovley et al., 1996; Caldwell et al., 1999). Kazumi et al. (1997) showed that benzene was degraded in methanogenic, sulphate-reducing and iron-reducing conditions. Benzene loss also occurred in the presence of Fe(III) in sediments from freshwater environments. Heider et al. (1999) noted that benzene was degraded under iron-reducing conditions but that no single benzene-degrading organism had been isolated. A community including members of the genus *Geobacter* was implicated. Many primitive benzene-degrading bacteria (hyperthermophiles), previously thought to require SO_4^{2-} , have been shown to grow using Fe(III) as an electron acceptor (Anderson et al., 1998; Rooney-Varga et al., 1999). Caldwell and Suflita (2000) found evolution of phenol and benzoate under a range of conditions (Fe(III)- and sulphate-reduction, and with methanogenesis), supporting the theory that benzoate is a central metabolite in anaerobic degradation of aromatic compounds.

3. Sulphate-reducing conditions

Several workers have demonstrated benzene degradation under sulphate-reducing conditions in soil collected from contaminated sites (e.g. Phelps et al., 1996). Chaudhuri and Wiesmann (1995) showed that degradation was via benzoate. Benzene degradation was comprehensively demonstrated in sulphate-reducing sediments from San Diego Bay (Lovley et al., 1995). Reinhard et al. (1997) investigated BTEX degradation under a range of redox conditions, but benzene degradation was only associated with sulphate reduction. Enrichment of aquatic sediments with known benzene-degraders leads to degradation of benzene and growth of benzene-degrading organisms, suggesting that the lack of benzene degradation in some aquifers is due to failure of appropriate

organisms to colonise the aquifer, rather than adverse environmental conditions (Weiner and Lovley, 1998a). A sulphate-reducing consortium was found to remain relatively complex despite the culture's long exposure to benzene as the only carbon and energy source (over 3 years) and repeated dilutions of the original enrichment (Phelps et al., 1998). Conversely, complete mineralisation of benzene to CO₂ has been demonstrated, apparently within single cells, in microcosms (Lovley et al., 1995), and more recently in a contaminated aquifer (Anderson and Lovley, 2000).

3.1. Methanogenesis

Where no electron acceptors other than CO₂ are present, it is suggested that benzene might be degraded to CO₂ and methane. In such a situation, it would not be possible to mineralise all the benzene. However, in the absence of any other TEA, this might play a role in limiting the extent of the contaminant plume. Benzene has been shown to be converted to CH₄ and CO₂ with no lag phase in the absence of other electron acceptors (Grbic-Galic and Vogel, 1987; Kazumi et al., 1997; Weiner and Lovley, 1998b).

4. Modelling differential degradation rates

Modelling of the complex biogeochemical interactions is a valuable means of quantifying the varied and complex interactions between contaminants, the local hydrogeology (groundwater flow) and the local hydrogeochemistry and, ultimately, making predictions of (i) the viability of natural attenuation as a remediation technique for BTEX compounds in aquifers and (ii) the feasibility/efficiency of enhanced remediation schemes. Here, we provide a modelling approach for microbial degradation of organic contaminants. The approach could be used in conjunction with flow, transport and geochemical modelling to describe the fate of such contaminants in groundwater systems (Barry et al., 2002). For the quantification of hydrocarbon compounds, models of different levels of complexity exist. Probably the most common mathematical formulation is based on linking the removal of

organic compounds to the microbial growth rate. For a single organic compound, single microbial species (or a consortium that is not differentiated into different species) and single TEA, this growth rate can be expressed by

$$\frac{dX_{\text{growth}}}{dt} = v_{\text{max}} \frac{C_{\text{org}}}{K_{\text{org}} + C_{\text{org}}} \frac{C_{\text{ea}}}{K_{\text{ea}} + C_{\text{ea}}} X, \quad (1)$$

where t is time, X is the local microbial concentration (subject to both growth and decay), v_{max} is an asymptotic maximum specific uptake rate, C_{org} and C_{ea} are the aqueous concentrations of the organic compound (substrate) and TEA, respectively. K_{org} and K_{ea} are the half-saturation constants for the organic compound and the TEA, respectively. For the complete mass-balance of the bacterial group, microbial decay needs to be considered, leading to

$$\frac{dX}{dt} = \frac{dX_{\text{growth}}}{dt} + \frac{dX_{\text{decay}}}{dt}, \quad (2)$$

with

$$\frac{dX_{\text{decay}}}{dt} = -v_{\text{dec}} X, \quad (3)$$

where v_{dec} is a decay rate constant (which could be replaced with an experimentally derived function). During microbial growth, both organic substrate and TEA are consumed at rates that are proportional to v_{max} . Thus, for a known reaction stoichiometry, the degradation rate can be easily determined from

$$\frac{dC_{\text{org}}}{dt} = Y_{\text{org}} \frac{dX_{\text{growth}}}{dt}, \quad (4)$$

for the substrate and, similarly, for the TEA,

$$\frac{dC_{\text{ea}}}{dt} = Y_{\text{ea}} \frac{dX_{\text{growth}}}{dt}, \quad (5)$$

where Y is an appropriate stoichiometric factor. As written, the above formulation treats multiple hydrocarbon compounds as one single compound with similar physico-chemical properties and, consequently cannot mimic the above-discussed differential degradation (or recalcitrance) of compounds

under varying redox conditions. To simulate this, Eq. (1) needs to be modified to

$$\frac{dX}{dt} = \left[\left(\sum_{n=1, n_{org}} \frac{dX_n}{dt} \right) - v_{dec} \right] X, \quad (6)$$

where each of the growth terms dX_n/dt is derived from a compound-specific term similar to Eq. (1) where the uptake rates v_{max} can then differ between different substrates. The decay rate, however, is not expected to vary, and so appears only once in Eq. (6). If more than one TEA is involved in the degradation process, this will typically require simulation of concentrations of multiple microbial groups, each associated with a particular TEA. To inhibit growth of bacteria in the presence of a thermodynamically more favourable TEA, one or more inhibition terms of the form

$$I_{inh,ea} = \frac{K_{inh,ea}}{K_{inh,ea} + C_{inh,ea}} \quad (7)$$

can be included as a factor in Eq. (1) and/or Eq. (6), where $C_{inh,ea}$ is the concentration of a more favourable TEA and $K_{inh,ea}$ is an inhibition constant that needs to be much smaller than typical concentrations of the more favourable TEA. The Monod-type inhibition term $I_{inh,ea}$ will then remain near 0 as long as the more favourable TEA is present in significant amounts but reaches its maximum value (of unity) as soon as the more favourable TEA is depleted (no growth-inhibition).

Finally, it is possible to take into account the release of nutrients and metabolic products from decaying cells using:

$$\frac{dC_{prod}}{dt} = Y_{prod} \frac{dX_{dec}}{dt}. \quad (8)$$

If initial X , C_{org} , C_{ea} and C_{prod} are known, the system of ordinary differential equations given in this section may be solved by standard numerical methods, e.g. the Runge–Kutta method (Abramowitz and Stegun, 1972). With the above equations incorporated into a numerical flow and transport model, e.g. Prommer et al. (1999, 2000a,b), it is then possible to simulate redox-dependent, differential degradation rates, e.g. benzene might degrade at the same or faster

rate as toluene under aerobic conditions whereas under selected anaerobic conditions it might degrade more slowly or not at all.

5. Discussion and conclusions

It is evident from a number of studies that the prevailing redox, physicochemical and biological conditions all play a part in determining the rate and extent of benzene degradation. Where degradation of hydrocarbons has been seen under anaerobic conditions, it appears that often the reaction pathway is unique to the organisms, compound and TEA, as summarized in Fig. 1.

Heider et al. (1999) noted in their review paper that the enzymes required for the anaerobic metabolism of hydrocarbons are substrate-induced, i.e., they are produced by the organism in response to the presence of the compound. Cells grown on toluene, for instance, will produce toluene-degrading enzymes, cells grown on another carbon/energy source will not. Compared to aerobic metabolism, it appears that enzymes involved in anaerobic metabolism are more substrate-specific.

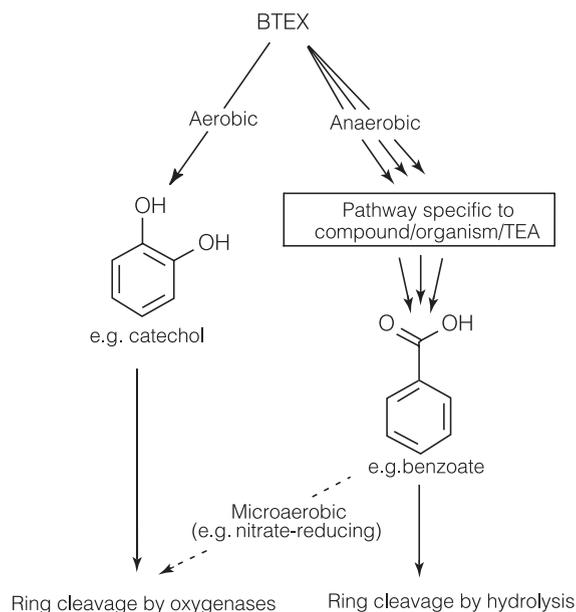


Fig. 1. Generalised BTEX biodegradation pathways.

The overall picture of anaerobic benzene degradation is that it can and does occur in a variety of conditions, but that organisms capable of utilising benzene anaerobically are by no means ubiquitous. There are also contrasting data concerning the use of TEAs in degradation, with studies by Nales et al. (1998) showing benzene degradation under NO_3^- , SO_4^{2-} and Fe(III)-reducing conditions and not under methanogenic conditions, while Kazumi et al. (1997) report degradation under methanogenic, SO_4^{2-} and Fe(III)-reducing conditions only. Where rapid benzene degradation is seen in the field, it is often associated with shallow aquifers and it is suggested that most of this degradation is aerobic, along the margins of contaminant plumes, with a limited amount occurring anaerobically within the plume body (Aronson and Howard, 1997). Anaerobic degradation of benzene is clearly far more site-specific than for the remaining TEX compounds, its extremely stable structure making it less susceptible to microbial attack and thus highly dependant on both biotic and abiotic factors. A high organic fraction has been shown to inhibit anaerobic benzene degradation (Nales et al., 1998), as has the presence of alternative energy sources (Corseuil et al., 1998). In the presence of high concentrations of BTEX compounds, it may be that removal of the more easily degraded TEX component and associated TEA may be responsible for the persistence of benzene in the environment. The use of enrichment cultures may favour faster-growing species at the expense of slower-growing microorganisms which are capable of degrading benzene over longer incubation periods/residence times (Rabus et al., 1999). It is likely that a combination of laboratory experiments and modelling of hydrogeology and hydrogeochemistry will aid in determining the potential for natural attenuation at a given site. While still poorly understood, anaerobic biodegradation is acknowledged as a factor in the natural attenuation of a variety of compounds, including some, such as benzene, once considered to be recalcitrant in all but aerobic conditions.

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