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Bioremediation of poly-aromatic hydrocarbon (PAH) contaminated soil by co-composting

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Bioremediation of Poly-Aromatic Hydrocarbon (PAH) Contaminated Soil by Co-composting

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SUMMARY

Bio-augmentation (addition of micro-organisms to contaminated materials and sites) and bio-stimulation (modification of contaminated materials and sites to enhance the growth of indigenous micro-organisms) are two approaches to treat contaminated soil. While good reasons exist for both methods having the potential to be successful bioremediation strategies, different studies showed seemingly contradicting results and there is still a need to generalise findings so that advice can be given on remediation strategies to use depending on the type of contamination and soil.

As poly-aromatic hydrocarbon (PAH) contaminated sites are typically of large size, economic concerns must not be ignored when looking for remediation techniques. Composting is an easy to implement and inexpensive method that has been found to be effective in removing contaminants from soil under different conditions. Results from different studies are, however, difficult to compare due to differing conditions they were carried out under as well as due to different soil types and contamination levels.

The objective of this study was to establish the suitability of bacterial and fungal additives in a composting process for bio-remediation of soil with a high clay content contaminated with mainly large and more difficult to access PAHs. During co-composting of contaminated soil microbial communities, physical and chemical conditions, and contaminant concentration and availability change constantly and influence each other.

Changes in temperature are characteristic for a composting process and influence several other factors such as microbial community development and contaminant availability. Temperature-profiles were applied and changes in process characteristics were analysed by monitoring different physico-chemical characteristics; microbial community changes were monitored using phospholipid-fatty-acid (PLFA) analysis; GC-MS analysis was used to investigate changes in solvent-extractable PAH concentrations and PAH volatilisation.

Firstly different types of manures were chosen as bacteria dominated inocula to be used as co-composting agents. Results showed differences in microbial community composition as well as some differences in physico-chemical characteristics of the treatments, but no correlation with PAH removal could be detected. While horse and cattle manure addition was slightly more successful in removing low (LMW) and medium molecular weight (MMW) PAHs, high molecular weight (HMW) PAH concentrations showed increases, but were slightly decreased with chicken manure addition.

Secondly two different white-rot fungi (*Pleurotus ostreatus* and *Trametes versicolor*), inoculated in sawdust, were added to the contaminated soil both singly and in combination. While physico-chemical characteristics were mainly not affected, *P. ostreatus* removed all measured PAHs by a larger extent than the other treatments. It was also noted that the highest rate of PAH removal appeared at the beginning of the experiment when fungal biomass was high.

In a third experiment the hypothesis that a combination of fungi and bacteria is most suitable to remediate contaminated soil was tested. *P. ostreatus* inoculated sawdust was added to the contaminated soil and chicken manure was added at three different stages during the experiment. The results showed that manure addition at the beginning of the composting process delayed PAH removal, but PAH removal rates were greater in the fungus and manure amended treatments compared to the control treatment.

Additionally, the effect of ageing on the removal behaviour of PAHs was investigated by adding two deuterated PAHs to the soil (the LMW PAH Acenaphthene-D10 and the MMW PAH Benz[a]anthracene-D12). Results show no advantage of fungus and manure addition. However, differences to the removal of the weathered PAHs Acenaphthene and Benz[a]anthracene show the importance of bioavailability and indicate that temperature might not play such an important role when dealing with a fresh contamination compared to one where the contaminated soil has aged.

The results of this study showed that addition of *P. ostreatus* inoculated sawdust enhanced the removal of PAHs from a soil where simple oxygen introduction did not show further PAH reduction. However additional amendment with chicken manure as a source of bacteria did not show further reductions in PAH concentrations.

TABLE OF CONTENTS

SUMMARY I
TABLE OF CONTENTS III
LIST OF FIGURES VIII
LIST OF TABLES XV
LIST OF EQUATIONS XVI
ABBREVIATIONS AND ACRONYMS XVII
ACKNOWLEDGEMENTS XVIII
AUTHORS DECLARATION XIX

CHAPTER 1 – Preface 1

CHAPTER 2 – Bioremediation of poly-aromatic hydrocarbon (PAH) contaminated soil by composting – a Review 7

2.1. ABSTRACT 7
2.2. INTRODUCTION 8
2.2.1. Treatment methods for contaminated soil 9
2.2.2. Characteristics of PAHs and their occurrence in the biosphere 10
2.2.3. PAH behaviour in soil: Bioavailability and the effect of ageing 12
2.3. CO-COMPOSTING OF HYDROCARBON CONTAMINATED SOIL 13
2.3.1. Different bioremediation techniques 15
2.3.2. Augmenting contaminated soil with different additives 16
2.3.3. Bio-augmentation and bio-stimulation 17
2.3.4. Physico-chemical effects 19
2.3.5. Surfactants 22
2.3.6. Effects of compost on contaminated soil 23
2.3.7. PAH extraction methods 24
2.3.8. PAH losses through volatilisation 25
2.4. PAH DEGRADATION PATHWAYS 26
2.4.1. Bacterial degradation pathways 27
2.4.2. Fungal degradation pathways 28
2.5. ORGANISMS DEGRADING HYDROCARBONS 29
2.5.1. Bacteria degrading PAHs 30
2.5.2. Genetically engineered organisms degrading PAHs 32
2.5.3. Fungi degrading PAHs 33
2.5.4. Comparison of micro-organisms that degrade PAHs 37
2.6. ANALYSIS OF MICROBIAL CONSORTIA 40
2.7. CONCLUSIONS 43

CHAPTER 3 – Materials and Methods 47

3.1. CONTAMINATED SOIL AND ADDITIVES 47
3.2. EXPERIMENTAL SETUP 48

3.3. SAMPLING AND PRE-TREATMENT OF SAMPLES **51**

3.4. ANALYSIS **51**

3.4.1. Physico-chemical analysis 51
3.4.2. PAH extraction from soil 53
3.4.3. Extraction of metabolic breakdown products from PAH degradation 54
3.4.3.1. Analysis of extracted PAH data 55
3.4.4. PAH volatilisation 58
3.4.5. Microbial community analysis 59
3.4.5.1. Groupings of PLFAs 60
3.4.6. GC-MS analysis 61
3.4.7. Statistical analysis 64

CHAPTER 4 – Comparison of the effects of different types of manure at different temperatures on the bioremediation of PAH contaminated soil **65**

4.1. ABSTRACT **65**

4.2. INTRODUCTION **66**

4.3. MATERIALS AND METHODS **68**

4.3.1. Additives 68
4.3.2. Treatments 68

4.4. RESULTS AND DISCUSSION **70**

4.4.1. Physico-chemical analysis 70
4.4.1.1. Total organic matter content 70
4.4.1.2. pH 70
4.4.1.3. Total N 71
4.4.1.4. Total C 71
4.4.1.5. Carbon to Nitrogen Ratio 73
4.4.1.6. Electrical conductivity 73
4.4.1.7. Ammonia emissions 74
4.4.1.8. Carbon dioxide emissions 75
4.4.2. Microbial community changes 75
4.4.2.1. Total microbial and bacterial PLFA and differences in the PLFA profiles of the manures added 75
4.4.2.1.1. Gram-negative and Gram-positive bacteria indicating PLFAs 77
4.4.2.1.2. Actinobacteria indicating PLFA 78
4.4.2.2. Fungi indicating PLFA 80
4.4.2.3. Stress indicating PLFAs 81
4.4.2.4. Influence of additives on the PLFA profile development 83
4.4.3. Changes of extractable PAH amounts 85
4.4.3.1. Changes in percentages of PAH concentrations remaining in the treatment mixtures over the 32 day experiment 86
4.4.3.1.1. Effects of treatments on extractable USEPA PAHs 86
4.4.3.1.2. Treatment effects of PAHs group into low, middle, and high molecular weight categories 92
4.4.3.2. Influence of incubation temperature on changes in PAH concentrations 96
4.4.3.3. Effects of extractable PAH concentrations 99
4.4.3.4. PAH volatilisation 100
4.4.4. Discussion of effects of different process characteristics on PAH removal 102
4.4.4.1. Interactions of microbial community changes and PAH contamination 102

4.5. CONCLUSIONS **104**

CHAPTER 5 – Comparing the effects of different fungal inocula at different temperatures to bioremediate PAH contaminated soil **107**

5.1. ABSTRACT **107**

5.2. INTRODUCTION **108**

5.3. MATERIALS AND METHODS **110**

5.3.1. Additives 110
5.3.2. Treatments 111

5.4. RESULTS AND DISCUSSION **112**

5.4.1. Physico-chemical analysis 112
5.4.1.1. Total organic matter content 112
5.4.1.2. pH 112
5.4.1.3. Total N 113
5.4.1.4. Total C 113
5.4.1.5. Carbon-to-nitrogen ratio 113
5.4.1.6. Electrical conductivity 114
5.4.1.7. Carbon dioxide emissions 114
5.4.2. Microbial community changes 116
5.4.2.1. Total microbial and bacterial PLFAs 116
5.4.2.1.1.Gram-negative and Gram-positive bacteria indicating PLFAs 117
5.4.2.2. Stress indicating ratio of iso-to-anteiso branched PLFAs 119
5.4.2.2.1. Actinobacteria indicating PLFA 120
5.4.2.3. Fungi indicating PLFA 121
5.4.2.4. Differences in the microbiological profile of the different treatments 122
5.4.3. Changes of extractable PAH amounts 125
5.4.3.1. Changes in percentages of PAH concentrations remaining in the treatment mixtures over the 48 day experiment 126
5.4.3.1.1. Effects of treatments on extractable USEPA PAHs 126
5.4.3.1.2. Treatment effects of PAHs group into low, middle, and high molecular weight categories 130
5.4.3.2. Influence of incubation temperature on changes in PAH concentrations 133
5.4.3.3. Effects of extractable PAH concentrations 136
5.4.3.4. PAH volatilisation 138
5.4.4. Discussion of effects of different process characteristics on PAH removal 140
5.4.4.1. Effects of the treatments on the survival and activity of the introduced fungi 141

5.5. CONCLUSIONS **144**

CHAPTER 6 – Effects of Pleurotus ostreatus and chicken manure addition at different temperatures/times on the bioremediation of PAH contaminated soil **146**

6.1. ABSTRACT **146**

6.2. INTRODUCTION **147**

6.3. MATERIALS AND METHODS **148**

6.3.1. Soil 148
6.3.2. Additives 149

6.3.3. Treatments 150
6.3.4. Statistical Analysis 151

6.4. RESULTS AND DISCUSSION 151

6.4.1. Physico-chemical analysis of the process 151
6.4.1.1. Total organic matter content 152
6.4.1.2. pH 152
6.4.1.3. Total N 153
6.4.1.4. Total C 153
6.4.1.5. C to N Ratio 154
6.4.1.6. Electrical conductivity 154
6.4.1.7. Ammonia emissions 155
6.4.1.8. Carbon dioxide emissions 156
6.4.2. Microbial community changes 157
6.4.2.1. Total microbial and bacterial PLFAs 157
6.4.2.2. Gram-negative and Gram-positive bacteria indicating PLFAs 158
6.4.2.3. Stress indicating PLFAs 160
6.4.2.4. Actinobacteria indicating PLFA 161
6.4.2.5. Fungi indicating PLFA 163
6.4.2.6. Influence of additives on the PLFA profile development 165
6.4.3. Changes in extractable weathered PAH amounts 169
6.4.3.1. Changes in percentages of PAH concentrations remaining in the treatment mixtures over the 91 day experiment 169
6.4.3.1.1. Effects of treatments on extractable weathered USEPA PAHs 170
6.4.3.1.2. Effects of treatments on extractable grouped LMW PAHs 175
6.4.3.1.3. Effects of treatments on extractable grouped MMW and HMW PAHs 176
6.4.3.2. Influence of incubation temperature on changes in PAH concentrations 178
6.4.3.3. Effects of extractable PAH concentrations 180
6.4.3.4. Combined influences of the different treatments and temperature(time) on the behaviour of grouped PAHs 182
6.4.3.5. PAH volatilisation 183
6.4.4. Discussion of effects of different process characteristics on PAH removal 185
6.4.4.1. The role of physico-chemical characteristics and microbial communities 186
6.4.4.2. Advantages of different treatments 187

6.5. CONCLUSIONS 188

CHAPTER 7 – Differences in degradation behaviour between a fresh and aged source of contamination 191

7.1. ABSTRACT 191

7.2. INTRODUCTION 191

7.3. MATERIALS AND METHODS 193

7.3.1. Soil 193
7.3.2. Additives and Treatments 194
7.3.3. Statistical Analysis 194

7.4. RESULTS 195

7.4.1. Comparison of changes of extractable deuterated and weathered PAH concentrations 195
7.4.1.1. Total amounts and concentrations over the 91 day experiment 195

7.4.1.2. Degradation rates of weathered and deuterated PAHs during each temperature step 197

7.5. DISCUSSION 202

7.5.1. Ageing - the importance of bioavailability 202
7.5.1.1. The influence of adsorption 202
7.5.1.2. Temperature effects 203
7.5.2. Treatment effects 204

7.6. CONCLUSIONS 204

CHAPTER 8 – Synopsis and outlook 206

8.1. ABSTRACT 206

8.2. INTRODUCTION 207

8.3. INFLUENCE OF DIFFERENT TEMPERATURE REGIMES ON PAH REMOVAL 208

8.4. THE EFFECT OF AGEING ON PAH BIOAVAILABILITY 210

8.5. SUMMARY AND COMPARISON OF THE DIFFERENT EXPERIMENTS CONCERNING PAH REMOVAL 211

8.5.1. Manure amendment 211
8.5.2. Fungi addition 212
8.5.3. Combination of fungi and manure addition 212
8.5.4. Overall comparison of the most effective PAH removal treatments 213
8.5.5. Comparison of the fungi-amended treatments 214
8.5.5.1. PAH removal 214
8.5.5.2. Inoculum size 216

8.6. CONCLUSIONS AND OUTLOOK 217

APPENDIX 1 (Studies on co-composting) **221**

APPENDIX 2 (Bacteria and Fungi degrading PAHs and PAH-metabolites) **233**

APPENDIX 3 (Work presented at seminars and conferences) **239**

REFERENCES 251

LIST OF FIGURES

Chapter 1 – Preface

Figure 1.1: Outline of the study showing the initial situation and the experiments performed to address the problem. 2

Chapter 2 - Literature Review

Figure 2.1: Two-dimensional structure and molecular weight of 16 priority PAHs identified by the U.S. EPA. (after Sander and Wise, 1997) 11

Figure 2.2: Pathways of PAH degradation by bacteria (after Bamforth and Singleton, 2005 and Cerniglia, 1992) 27

Figure 2.3: Pathways for the fungal metabolism of PAHs (after Cerniglia, 1997) 28

Figure 2.4: generalised structure of a PLFA with glycerol backbone (red), fatty acid chains (black), and phosphate (blue) 41

Figure 2.5: Summary of the processes governing PAH behaviour in soil. For explanation see text. 44

Figure 2.6: Schematic showing the main factors influencing a co-composting process of PAH contaminated soil. TOM = total organic matter 45

Chapter 3 - Materials and Methods

Figure 3.1: Experimental setup showing the waterbath and aeration system. The insert shows filled composting vessels at the beginning of the experiment. 50

Figure 3.2.: Schematic representation of the experimental setup for one pilot-scale vessel. All experimental vessels were placed in the same waterbath but had individual aeration systems. 50

Chapter 4 - Manure Addition

Figure 4.1: Mean values of different physico-chemical analyses with the different treatments during the different stages of the 32 day experiment. Treatments are labelled as Horse manure = blue, Cattle manure = green, Chicken manure = yellow, TC = pink. The light blue background represents the applied temperature profile. Error bars represent mean ±S.E.; n=3 72

Figure 4.2: Mean values of CO2 and NH3 analyses with the different treatments during the different stages of the 32 day experiment. Treatments are labelled as Horse manure = blue, Cattle manure = green, Chicken manure = yellow, TC = pink. The light blue background represents the applied temperature profile. Error bars represent mean ±S.E.; n=3 74

Figure 4.3: Mean concentrations of total bacterial PLFAs in nmol per g dry sample detected in the different treatments over the different stages of the 32 day experimental period. Error bars represent mean ±S.E.; n=3 76

Figure 4.4: Mean values of the ratio of Gram-negative-to-Gram-positive bacteria indicating PLFAs detected in the different treatments during the different stages of the 32 day experimental period. Error bars represent mean ±S.E.; n=3 77

Figure 4.5: Mean values of the concentration of Gram-negative and Gram-positive bacteria indicating PLFAs in nmol per g dry sample detected in the different treatments during the different stages of the 32 day experimental period. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 78

Figure 4.6a/b: Mean ratio of Actinobacteria indicating PLFAs in % of bacterial PLFAs (a) and concentration of Actinobacteria indicating PLFA in nmol per g dry sample detected in the different treatments over the 32 day experimental period. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 79

Figure 4.7a/b: Mean ratio of fungal-to-bacterial PLFAs (a) and concentration of fungal PLFA in nmol per g dry sample detected in the different treatments over the 32 day experimental period. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 81

Figure 4.8: Mean ratio of iso-to-anteiso branched PLFAs detected in the different treatments over the 32 day experimental period. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 82

Figure 4.9: Diagnostic Biplots showing associations of PLFAs with the different treatments at each sampling point. The treatments are indicated as follows: 1 = horse manure, 2 = cattle manure, 3 = chicken manure, C = TC, a-c indicate replicate vessels. 84

Figure 4.10: Mean percentage of total PAHs remaining (%) in the different treatments at the end of different temperature steps of the 32 d experiment. The red dotted line connects the percentage of the remaining total PAHs detected with the untreated soil after 32 days with the 100% in the beginning of the experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 86

Figure 4.11a: Mean concentrations of individual USEPA PAHs remaining (%) in the Horse and Cattle manure treatments at the end of different time periods steps during the 32 day experiment in % from concentrations measured at the beginning of the experiment. Error bars represent ±S.E.; n=3 89

Figure 4.11b: Mean concentrations of individual USEPA PAHs remaining (%) in the Chicken manure and TC treatments at the end of different time periods steps during the 32 day experiment in % from concentrations measured at the beginning of the experiment. Error bars represent ±S.E.; n=3 90

Figure 4.12: Mean concentration of LMW PAHs in the different treatments at the end of different temperature steps of the 32 day experiment. The red dotted line connects the percentage of the remaining grouped LMW PAHs detected with the untreated soil after 32 days with the 100% in the beginning of the experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 92

Figure 4.13: Mean concentration of MMW PAHs in the different treatments at the end of different temperature steps of the 32 day experiment. The red dotted line connects the percentage of the remaining grouped LMW PAHs detected with the untreated soil after 32 days with the 100% in the beginning of the experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 93

Figure 4.14: Mean concentration of HMW PAHs in the different treatments at the end of different temperature steps of the 32 day experiment. The red dotted line connects the percentage of the remaining grouped LMW PAHs detected with the untreated soil after 32 days with the 100% in the beginning of the experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 94

Figure 4.15: Mean rate of degradation of grouped LMW and MMW PAHs during the different temperature steps of the 32 day experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 97

Figure 4.16: Mean rate of degradation of grouped HMW PAHs during the different temperature steps of the 32 day experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 98

Figure 4.17: Amounts of volatilised grouped LMW and MMW PAHs with the different treatments during the different temperature steps of the 32 day experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3 ...101

Chapter 5 - Fungal Inocula

Figure 5.1: White-rot fungi *Pleurotus ostreatus* (a) and *Trametes versicolor* (b) growing on decaying wood. ...110

Figure 5.2: Mean values of the different physico-chemical analyses with the different treatments during the different stages of the 48 d experiment. Treatments are labelled as P.ost = blue, T.vers = green, P&T-Mix = yellow, TC = pink. The light blue background represents the applied temperature profile. Error bars represent mean ±S.E.; n=3 ...115

Figure 5.3: Mean values of total measured microbial PLFAs in nmol per g dry sample detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3 ...116

Figure 5.4: Mean values of the ratio of Gram-negative-to-Gram-positive bacteria indicating PLFAs detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3 ...117

Figure 5.5: Mean values of the concentration of Gram-negative and Gram-positive bacteria indicating PLFAs in nmol per g dry sample detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3 ...118

Figure 5.6: Mean values of the ratio of iso-to-anteiso branched PLFAs detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3 ...119

Figure 5.7: Mean values of the ratio of total Actinobacteria indicating PLFAs in % of total microbial PLFAs detected in the different treatments over the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3 ...120

Figure 5.8: Mean values of the amount of fungi indicating PLFAs in nmol per g dry sample with the different treatments over the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3 ...121

Figure 5.9: Mean values of the ratio of fungal-to bacterial PLFAs detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3 ...122

Figure 5.10: Diagnostic Biplots showing associations of PLFAs with the different treatments at each sampling point. The treatments are indicated as follows: P = P.ost, T = T.vers, M = P&T-Mix, C = TC, a-c indicate replicate vessels. ...124

Figure 5.11: Mean percentages of concentrations of total USEPA PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining total PAHs detected in the untreated soil after 48 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=3 ...126

Figure 5.12a: Mean percentages of concentrations of individual PAHs remaining in the P.ost and T.vers treatments by the end of the different experimental periods. Error bars represent mean ±S.E.; n=3 ...128

Figure 5.12b: Mean percentages of concentrations of individual PAHs remaining in the P&T-Mix and TC treatments by the end of the different experimental periods. Error bars represent mean ±S.E.; n=3 ...129

Figure 5.13: Mean percentages of concentrations of grouped LMW PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining LMW PAHs detected in the untreated soil after 48 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=3 ...131

Figure 5.14: Mean percentages of concentrations of grouped MMW PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining MMW PAHs detected in the untreated soil after 48 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=3 ...131

Figure 5.15: Mean percentages of concentrations of grouped HMW PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining HMW PAHs detected in the untreated soil after 48 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=3 ...132

Figure 5.16: Mean rates of degradation of grouped LMW PAHs with the different treatments during the different temperature steps of the 48 day experiment. Error bars represent mean ±S.E.; n=3 ...134

Figure 5.17: Mean rates of degradation of grouped MMW PAHs with the different treatments during the different temperature steps of the 48 day experiment. Error bars represent mean ±S.E.; n=3 ...135

Figure 5.18: Mean rates of degradation of grouped HMW PAHs with the different treatments during the different temperature steps of the 48 day experiment. Error bars represent mean ±S.E.; n=3 ...136

Figure 5.19: Mean rates of volatilised LMW and MMW PAHs with the different treatments during the different temperature steps of the 48 day experiment. Error bars represent mean ±S.E.; n=3 ...139

Chapter 6 - Fungi and Manure Addition

Figure 6.1: Mean values of different physico-chemical analyses with the different treatments during the different stages of the 91 d experiment. Treatments are labelled as T-d01 = blue, T-d21 = green, T-d35 = yellow, TC = pink. The light blue background represents the applied temperature profile. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...155

Figure 6.2: Mean values of CO2 and NH3 emissions with the different treatments during the different stages of the 91 d experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...156

Figure 6.3: Mean values of the amount of total measured microbial PLFAs in nmol per g dry sample detected with the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E. .; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...158

Figure 6.4: Mean values of the ratio of Gram-negative-to-Gram-positive bacteria indicating PLFAs in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...159

Figure 6.5: Mean values of the concentrations of Gram-negative and Gram-positive bacteria indicating PLFAs in nmol per g dry sample detected in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...159

Figure 6.6: Mean values of the ratio of iso-to-anteiso branched PLFAs in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...161

Figure 6.7: Mean values of the amounts of total Actinobacteria indicating PLFAs in % of the total amounts of bacterial PLFAs detected in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...161

Figure 6.8: Mean values of the amounts of total Actinobacteria indicating PLFAs in nmol per g dry sample in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...162

Figure 6.9: Mean values of the amounts of total Actinobacteria indicating PLFAs in nmol per g dry sample in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...163

Figure 6.10: Mean values of the ratio of fungi-to-bacteria indicating PLFAs in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...164

Figure 6.11: Diagnostic Biplot showing association of PLFAs with the different treatments at day 07. T1=T-d01, T2=T-d21, T3=T-d35, C=TC, a-c indicating replicate vessel. ...166

Figure 6.12: Diagnostic Biplot showing association of PLFAs with the different treatments at day 28. T1=T-d01, T2=T-d21, T3=T-d35, C=TC, a-c indicating replicate vessel. ...167

Figure 6.13: Diagnostic Biplot showing association of PLFAs with the different treatments at day 42. T1=T-d01, T2=T-d21, T3=T-d35, C=TC, a-c indicating replicate vessel. ...168

Figure 6.14: Mean percentages of concentrations of total aged USEPA PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining total PAHs detected in the untreated soil after 91 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...170

Figure 6.15a: Concentrations of measured PAHs per g ash (% of start amounts) at the end of different temperature steps of treatments T-d01 and T-d21 during the 91 day experiment. Bars represent ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3. The behaviour of the deuterated PAHs Acenaphthene-D10 and Benz[a]anthracene-D12 are discussed in chapter 7. ...173

Figure 6.15b: concentrations of measured PAHs per g ash (% of start amounts) at the end of different temperature steps of treatments T-d35 and TC during the 91 day experiment. Bars represent ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3. The behaviour of the deuterated PAHs Acenaphthene-D10 and Benz[a]anthracene-D12 are discussed in chapter 7. ...174

Figure 6.16: Mean percentages of concentrations of grouped weathered LMW PAHs remaining by the end of different temperature steps. The red dotted line connects the percentage of the remaining LMW PAHs detected with the untreated soil after 91 days with the 100% at the beginning of the experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...175

Figure 6.17: Mean percentages of concentrations of grouped weathered MMW PAHs remaining by the end of different temperature steps. The red dotted line connects the percentage of the remaining MMW PAHs detected with the untreated soil after 91 days with the 100% at the beginning of the experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...176

Figure 6.18: Mean percentages of concentrations of grouped weathered HMW PAHs remaining by the end of different temperature steps. The red dotted line connects the percentage of the remaining HMW PAHs detected with the untreated soil after 91 days with the 100% at the beginning of the experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...177

Figure 6.19: Mean rates of degradation of grouped MMW and HMW PAHs with the different treatments during the different temperature steps of the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...178

Figure 6.20: Mean rates of degradation of grouped MMW and HMW PAHs with the different treatments during the different temperature steps of the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...179

Figure 6.21: Mean volatilisation rates of the amounts of PAHs measured in relation to the amount of those PAHs detected in the soil in the vessel at any time. Rates are given for grouped aged LMW and MMW PAHs during the different temperature steps with the different treatments during the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...184

Figure 6.22: Mean PAH concentrations measured in ng per litre air taken from the headspace of the treatment vessels. Concentrations are given for grouped weathered LMW and MMW PAHs during the different temperature steps with the different treatments during the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...184

Chapter 7 - Fresh and Aged Contamination

Figure 7.1: Mean percentages of the added deuterated PAHs Acenaphthene-D10 and Benz[a]anthracene-D12 and their weathered counterparts Acenaphthene and Benz[a]anthracene remaining in the different treatments by the end of different temperature steps of the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3 ...196

Figure 7.2a: Analysis of the average removal per day of Acenaphthalene-D10 and its aged counterpart Acenaphthene with the different treatments over the 91 day experiment. *r* represents average removal rate per day, subscripts indicate h= heating phase (dashed line, day 0 to 42), c=cooling phase (day 42 to 91), h1=heating phase before manure addition, h2=heating phase after manure addition. ...200

Figure 7.2b: Analysis of the average removal per day of Benz[a]anthracene-D12 and its aged counterpart Benz[a]anthracene with the different treatments over the 91 day experiment. *r* represents average removal rate per day, subscripts indicate h= heating phase (day 0 to 42), c=cooling phase (day 42 to 91), h1=heating phase before manure addition, h2=heating phase after manure addition. ...201

Chapter 8 - Synopsis and Outlook

Figure 8.1: Mean PAH concentrations remaining in the temperature control (TC) treatments found at the end of the implied temperature steps in % of the initial concentrations with the different experiments (TC1 = TC "manure experiment" Chapter 4; TC2 = TC "fungi experiment" Chapter 5; TC3 = TC "fungi/manure experiment" Chapter 6). The light grey background additionally represents the applied temperatures as given on the x-axis during the experiment. Error bars represent mean ±S.D.; n=3 ...209

Figure 8.2: Mean concentrations of grouped PAHs in µg per g ash measured in the treatment mixtures during the two experiments involving the addition of fungi-inoculated sawdust over 7 (P.ost) and 13 (T-d21, T-d35) weeks. Week 7/9 corresponds to week 7 for the P.ost treatment and week 9 for the T-d21 and T-d35 treatments. Error bars represent mean ±S.D.; n=3 for P.ost, n=6 for T-d21/T-d35 weeks 0-3, n=3 for T-d21 weeks 4-13, n=3 for T-d35 weeks 4-13. ...215

Figure 8.3: Mean total fungal PLFA amounts in nmol per g dry sample (a) and mean ratio of fungal-to-bacterial PLFAs (b) of the fungal-inoculated treatments at the end of each temperature step/week of the 7 (P.ost) and 13 (T-d21, T-d35) week experiments. Week 7/9 corresponds to week 7 for the P.ost treatment and week 9 for the T-d21 and T-d35 treatments. Error bars represent mean ±S.D.; n=3 for P.ost, n=6 for T-d21/T-d35 weeks 0-3, n=3 for T-d21 weeks 4-13, n=3 for T-d35 weeks 4-13. ...216

LIST OF TABLES

Chapter 3 - Materials and Methods

Table 3.1. Physico-chemical characteristics and microbial community profiles of the soil and sawdust used for the experiments. Values represent mean ±S.D. where applicable; n=3 49

Table 3.2: GC-MS settings for the different PAH and PLFA analyses 63

Chapter 4 - Manure Addition

Table 4.1. Physico-chemical characteristics and microbial community profiles of the manure types used for the experiment (± S.D.) if applicable. 69

Table 4.2. Percentage of PAHs remaining at the end of the experiment. 87

Table 4.3. Mean concentrations of individual and grouped PAHs (µg g^{-1}) at the beginning (day 0) and end (day 32) of the experiment. Values are concentrations in µg per g ash ±S.D., n=3 99

Chapter 5 - Fungal Inocula

Table 5.1: physico-chemical characteristics and microbial community profiles of the sawdust inoculated with the two different fungi used for the experiment (± S.D.) if applicable. ...111

Table 5.2: Percentage of individual PAHs remaining at the end of the 48 d experiment. ...127

Table 5.3: PAH concentrations of 15 detected USEPA PAHs at days 0 and 48 of the experiment after mixing the soil with the different additives. Values are averages (µg per g ash) of the amounts measured in the replicate vessels ±S.D. ...137

Chapter 6 - Fungi and Manure Addition

Table 6.1. Physico-chemical characteristics and microbial community profiles of the *P. ostreatus*-inoculated sawdust and the chicken manure used for this experiment. Values represent mean ± S.D. where applicable. ...149

Table 6.2.: Percentage of individual PAHs remaining in the treatment mixtures at the end of the experiment. ...172

Table 6.3: PAH concentrations of 15 detected USEPA PAHs at day 0 of the experiment after mixing the soil with the different additives. Values are averages (µg per g ash) of the amounts measured in the replicate vessels ±S.D.; n=6 for T-d21/T-d35 day 0, else n=3 ...181

Chapter 7 - Fresh and Aged Contamination

Table 7.1: Initial concentrations (µg g^{-1} ash) of the added deuterated PAHs and their weathered counterparts present in the treatments. Letters a,b,c represent the replicate vessels. ...195

Table 7.2: Factor of PAH removal (*r*) and R^2 values obtained by linear regression to calculate average reduction of extractable PAH concentrations for the added deuterated PAHs and their aged counterparts. ...198

Chapter 8 - Synopsis and Outlook

Table 8.1: Applied temperature profiles of the three experiments. ...208

Table 8.2: Comparison of mean PAH concentrations (µg/g) and ratio of PAH removal (%) ±S.D.; n=3, at the end of the experiments in the most promising treatments. ...213

LIST OF EQUATIONS

Chapter 3 - Materials and Methods

E.3.1 Determination of moisture contents 52

E.3.2 Determination of ash contents 52

E.3.3 Determination of TOM contents 52

E.3.4 Calculation of the percentage of PAHs remaining in the soil-mixtures 56

E.3.5 Determination of PAH degradation rates during single temperature steps 57

Chapter 7 - Fresh and Aged Contamination

E.7.1 General form of an exponential equation ...198

E.7.2 Linear integrated from of the exponential equation ...198

ABBREVIATIONS AND ACRONYMS

°C	Degree Centigrade
ANOVA	Analysis of Variance
C	Carbon
cm	Centimetre(s)
CO_2	Carbon dioxide
d	Day
df	Degrees of freedom
EC	Electrical conductivity
g	Gram
GC-MS	Gas-chromatography-mass-spectrometry
HMW	High molecular weight
kg	Kilogram(s)
l	Litre
LMW	Low molecular weight
m	Metre
M	Molar
mg	Milligram(s)
min	Minute
ml	Millilitre(s)
mm	Millimetre(s)
MMW	Medium molecular weight
MW	Molecular weight
N	Nitrogen
n	Sample size
ng	Nanogram(s)
NH_3	Ammonia
nmol	Nanomol
P. ostreatus	*Pleurotus ostreatus*
PAH	Poly-aromatic hydrocarbon
PCB	Poly-chlorinated Biphenyls
PLFA	Phospholipid fatty acid
ppb	Parts per billion
ppm	Parts per million
ppmv	Part per million by volume
rpm	Rounds per minute
s	Second
S.D.	Standard deviation
S.E.	Standard error
T. versicolor	*Trametes versicolor*
TOM	Total organic matter
TPH	Total petroleum hydrocarbon
USEPA	United States Environmental Protection Agency
v/v	Volume by volume
µg	Microgram(s)
µS	Micro-Siemens

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Chapter 1

Preface

This thesis is concerned with the scientific evaluation and identification of effective treatment methods for the remediation of soil contaminated with aged poly-aromatic hydrocarbons (PAHs). The key challenge was to find an easy-to-implement and cost-effective way of treating highly PAH contaminated soil from a former refinery site. Prior to the remediation trials detailed in this thesis, the soil had been excavated and treated in windrows (elongated stockpiles measuring about 100 m long, 5 m wide at the base and up to 1.5 m tall with a triangular cross section) with regular turning and irrigation for 2 months. After this 2 month period 70% of the total measured PAHs identified as priority pollutants by the US Environmental Protection Agency (USEPA) had been degraded, however, the soil was still deemed highly contaminated (total USEPA PAH amounts ranging from 1800 to 2000 mg kg^{-1} soil). The PAHs remaining were considered the less available and more difficult to degrade PAHs and consisted of a higher proportion of high and medium molecular weight PAHs.

Composting has been shown to be very effective in breaking down relatively recalcitrant organic matter as it promotes higher rates of enzymatic-mediated degradation due to the intrinsically aerobic conditions, higher temperatures, and shifts in microbial community structure. Additionally, composting is one of the cheapest methods to treat contaminated soil and it has been shown to improve soil quality through the addition of organic matter.

The effects of co-composting processes on PAH degradation have been investigated in several studies under a range of conditions and with different kinds of contaminated materials (see Chapter 2 - Literature Review). A review of the literature indicated that the wide range of remediation conditions, alongside the different preconditions, make it difficult to compare individual studies. Consequently, this has significantly hampered the adoption of the technology within industry.

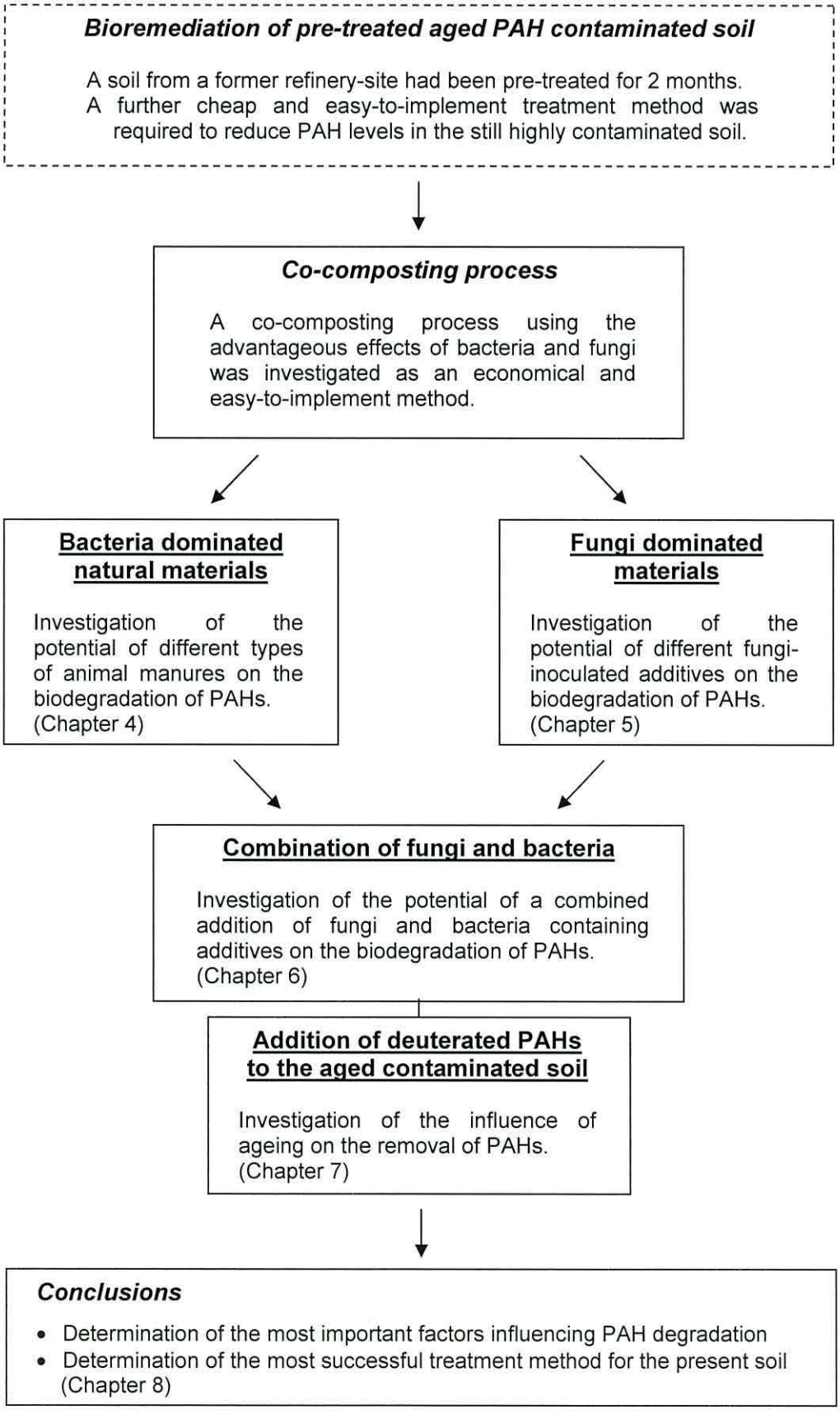

Figure 1.1: Outline of the study showing the initial situation and the experiments performed to address the problem.

Composting processes rely on micro-organisms transforming the feedstock. Those micro-organisms belong to the kingdoms of Bacteria or Fungi. Both microbial groups have been shown to have advantageous effects in degradation processes. Typically, in comparison to bacteria, fungal rates of PAH degradation are slow and inefficient, but fungi can reach and degrade PAHs that are physically and chemically outside the range of bacterial degradation. The ecological role of fungi could therefore be significant as they have the ability to hydroxylate a wider variety of PAHs, and the resulting polar intermediates can be mineralised by soil bacteria or detoxified to innocuous components.

Most studies have investigated the use of bacteria or fungi and only a few have looked at the combined use of organisms of both kingdoms. Also, when deciding which materials to use as co-composting agents, results of different studies are difficult or impossible to compare due to different experimental conditions (fungal strains, levels and type of contamination, physical matrix, nutrient additives etc).

The present study addresses these difficulties by investigating the potential of different bacteria and fungi dominated co-composting additives under the same conditions, as outlined in Figure 1.1.

HYPOTHESES

The overall hypotheses of this study are that:

- The addition of a combination of both fungal and bacterial inocula to contaminated soil enhances PAH removal.
- The time-point at which fungal and bacterial inocula are added determines the effectiveness of the micro-organisms and therefore the success of the remediation process.

THESIS AIMS

Addressing the hypotheses, the overall aims of this thesis are:

1) To identify the effect of manures with different characteristics on the removal of PAHs from aged contaminated soil.

2) To examine the effects of white-rot fungi on PAH removal from aged contaminated soil.

3) To [a] evaluate a complementary effect of fungus and manure addition on PAH removal from an aged contaminated soil and to [b] determine the importance of the time-point at which manure is added.

4) To assess the influence of "ageing" on PAH availability and removal from contaminated soil.

5) To investigate the influence of temperatures that are characteristic in a composting process on PAH removal from aged contaminated soil.

The above aims will be addressed by carrying out experiments with different animal derived manures and white-rot fungi mixed with aged PAH contaminated soil and treated under aerobic conditions with changing temperatures. Results of the investigated treatments will be compared and the most successful manure and fungal treatments will be selected. The selected additives will be used to investigate the effects of a combined amendment on PAH removal from the aged contaminated soil. To determine the influence of the time at which manure is added to the fungi amended soil, manure will be added at different stages of the experiment. Deuterated PAHs will be added to the aged contaminated soil to represent a fresh contamination. To assess the influence of ageing on removal of PAHs from the contaminated soil the behaviour of the deuterated PAHs will be compared to that of their aged counterparts present in the soil.

THESIS OUTLINE

The subsequent parts of this thesis are laid-out as follows:

Chapter 2 – Literature review

The literature review provides a critical review of existing studies investigating the co-composting of PAH contaminated soil and the role of bacteria and fungi during the co-composting processes. Current findings are discussed and the future research needs are identified.

Chapter 3 – Materials and methods

In this chapter, the materials and methods used throughout the thesis are described. This includes the experimental setup as well as the statistical analysis procedures.

Chapter 4 – Manure experiment

This chapter investigates the effects of animal manures, as a readily-available source of bacteria, on PAH degradation during the co-composting of PAH contaminated soil. Influences on microbial community profiles as detected using phospholipid fatty acid (PLFA) analysis and extractable PAH concentrations are analysed. (Thesis aim 1,(5))

Chapter 5 – Fungi experiment

This chapter investigates the effects of two white rot fungi, added to the contaminated soil with pre-inoculated sawdust, on PAH degradation during the co-composting of PAH contaminated soil. Influences on microbial community profiles as detected using PLFA analysis and extractable PAH concentrations are analysed. (Thesis aim 2,(5))

Chapter 6 – Combination of fungi and manure

In this chapter, a combination of the most promising additives is investigated. The hypothesis is that a combination of fungal and bacterial additives would enhance biodegradation even of a soil contaminated with aged PAHs where remediation is notoriously difficult. The experiment determined the effect of a fungus-inoculated sawdust and manure on PAH removal from contaminated soil. (Thesis aims 3 [a/b],(5))

Chapter 7 – Fresh and aged contamination

As an extension of chapter 6, this Chapter investigates the influence ageing has on the removal rates of PAHs. The soil used in the experiment, described in Chapter 6, had been additionally spiked with two deuterated-PAHs representing a fresh source of contamination. Removal behaviour of the freshly added PAHs is compared to those of their weathered PAH counterparts. (Thesis aim 4,(5))

Chapter 8 – Synopsis and outlook

This chapter collates, assimilates and summarises the findings of the different experiments and gives an overall evaluation of the suitability of the investigated processes to bio-remediate an aged PAH contaminated soil. This final chapter also identifies future research needs.

Chapter 2

Bioremediation of poly-aromatic hydrocarbon (PAH) contaminated soil by composting – a Review

(Part of the content of this Chapter is reproduced from (Loick *et al.* 2009))

2.1. ABSTRACT

This chapter presents a comprehensive and critical review of the research on different co-composting approaches to bioremediate hydrocarbon contaminated soil, organisms that have been found to degrade PAHs, and PAH breakdown products. Advantages and limitations of using certain groups of organisms and recommended areas of further research are identified. Studies investigating the use of composting techniques to treat contaminated soil are broad ranging and differ in many respects making comparisons of the different approaches very difficult. Many studies have investigated the use of specific bio-additives in the form of bacteria or fungi with the aim of accelerating contaminant removal, however, few have employed microbial consortia containing organisms from both kingdoms despite knowledge suggesting that synergistic relationships exist between them in contaminant removal. Recommendations suggest further studies should attempt to systemise the investigations of composting approaches to bio-remediate PAH-contaminated soil, to focus on harnessing the biodegradative capacity of both bacteria and fungi to create a cooperative environment for PAH degradation, and to further investigate the array of PAHs that can be lost during the composting process by either leaching or volatilisation.

2.2. INTRODUCTION

Sites contaminated with industrially-derived pollutants represent a significant threat to human health as well as to the integrity of the wider environment (Samanta *et al.* 2002; Wong *et al.* 2002). Furthermore, these sites also bear economic disadvantages to landowners as the land cannot be used in any convenient way until the contamination is effectively removed. There are three main remediation techniques for dealing with contaminated sites (Hackenbush 2004): the first involves the complete removal of the contaminated soil implying that the excavated soil will have to be stored (still contaminated) at a municipal waste site; the second option involves the containment of the contaminants within the soil. This *in-situ* containment has the obvious disadvantage that the future usage options of the site will be severely restricted; and the third option involves the removal of the contaminant by biological, chemical, or physical treatment which is inevitably either more labour or time intensive than the former two options.

Since 1975, the European Union (EU) has introduced a series of new waste management legislation. One of the major introductions is the Landfill Directive (Council Directive 1999/31/EC) which sets targets to reduce landfill of biodegradable municipal waste to 75% of 1995 levels by 2006, 50% by 2009 and 35% by 2016. Even if countries heavily dependent on landfill such as the UK, will be able to claim derogations (i.e. exemptions) to delay meeting the targets by four years, which means 2020, there is still an urgent requirement to develop alternative methods for waste treatment (European Commission 1999; Karl and Ranne 1999). Additionally, the need to manage recalcitrant and persistent materials, both in terms of environmental protection and human health, is of great importance when dealing with contaminated material. Finding new and effective ways to convert and metabolise potentially harmful substances into innocuous and potentially re-useable material is therefore a primary objective for both industry and government agencies.

2.2.1. Treatment methods for contaminated soil

Out of the three main categories of treatment methods for contaminated soil, namely [1] the removal of contaminated soil, [2] the containment of the contaminant, and [3] the removal of the contaminant, only the last strategy resembles a real attempt to solve the problem of contamination whereas the other two methods only move the problem to another time or place.

Ultimately, all organic molecules are biodegradable in geological time; however, some man-made chemicals remain particularly persistent in the environment when viewed in a human context. Although there is significant variability in the resistance of organic pollutants to degradation, the list of compounds which are considered to be completely recalcitrant in human terms is becoming smaller as the ability to detect and measure small conversion rates is becoming more advanced (Leisinger and Brunner 1986).

However, new materials are constantly being generated in the laboratory and industry that contain structures foreign to nature (i.e. xenobiotics). These compounds are often characterised by long half-lives in the environment. Most of them are synthetic organochlorines, anthropogenically introduced into the environment as industrial chemicals, agrochemicals, or unintentional by-products of industrial processes. Examples include dichlorodiphenyltrichlorethane (DDT), polychlorinated biphenyls (PCBs), heptachlor, dioxins, toxaphene, technical chlordane. Often these and other naturally occurring pollutants not foreign to nature such as polycyclic aromatic hydrocarbons (PAHs), do not biodegrade to any great extent in anaerobic landfills (e.g. plastics) and they also have a tendency to bioaccumulate in the food chain (e.g. DDT) (Lee *et al.* 2003). Consequently, different methods continue to be found to safely handle and effectively treat such materials.

After recycling, the next best treatment option for biodegradable materials is to treat the material by composting, which is defined as a managed process of bio-oxidation of a solid heterogeneous organic substrate including a thermophilic phase (Composting Council of Canada). Composting has been shown to be very effective at breaking down relatively recalcitrant

organic matter (Amir *et al.* 2005). As the composting process is very effective in degrading different organic materials, emphasis has been placed on finding composting processes that can also degrade persistent chemicals such as PAHs. In the case of contaminated sites, the composting process is also relatively easy to implement in many environmental situations.

2.2.2. Characteristics of PAHs and their occurrence in the biosphere

Polycyclic aromatic hydrocarbons (PAHs) are an important group of organic contaminants which are commonly found in industrially contaminated environments (Warshawsky 1999). They are a group of hydrocarbons made up from multiple interconnected 5- or 6-carbon (benzene) rings, which in nature are mainly formed by incomplete combustion of carbon containing fuels (Jacob *et al.* 1986; Freeman and Cattell 1990). They are detected in air (Koeber *et al.* 1999; Lim *et al.* 1999), soil and sediment (Langworthy *et al.* 1998; Márquez-Rocha *et al.* 2000), surface water, groundwater and road runoff (Pitt *et al.* 1995; Boxall and Maltby 1997); are dispersed from the atmosphere to vegetation (Wagrowski and Hites 1997) and contaminate foods (Edwards 1983). As a result of their natural and anthropogenic sources, in combination with global transport phenomena, PAHs can be found in all regions of the world including extreme environments with minimal human impact such as Antarctica (Kanaly and Harayama 2000).

Point sources, which can originate from petroleum or diesel spills and from industrial processes such as gasification during coke production (Cerniglia 1984), represent the most significant causes of environmental release and concern. Even though the contaminated areas are relatively small in size the concentration of the contaminant at these sites is often high and associated with other noxious contaminants such as benzene, toluene, ethyl-benzene, and xylene (BTEX), heavy metals and aliphatic hydrocarbons which can hinder remediation efforts (Bamforth and Singleton 2005).

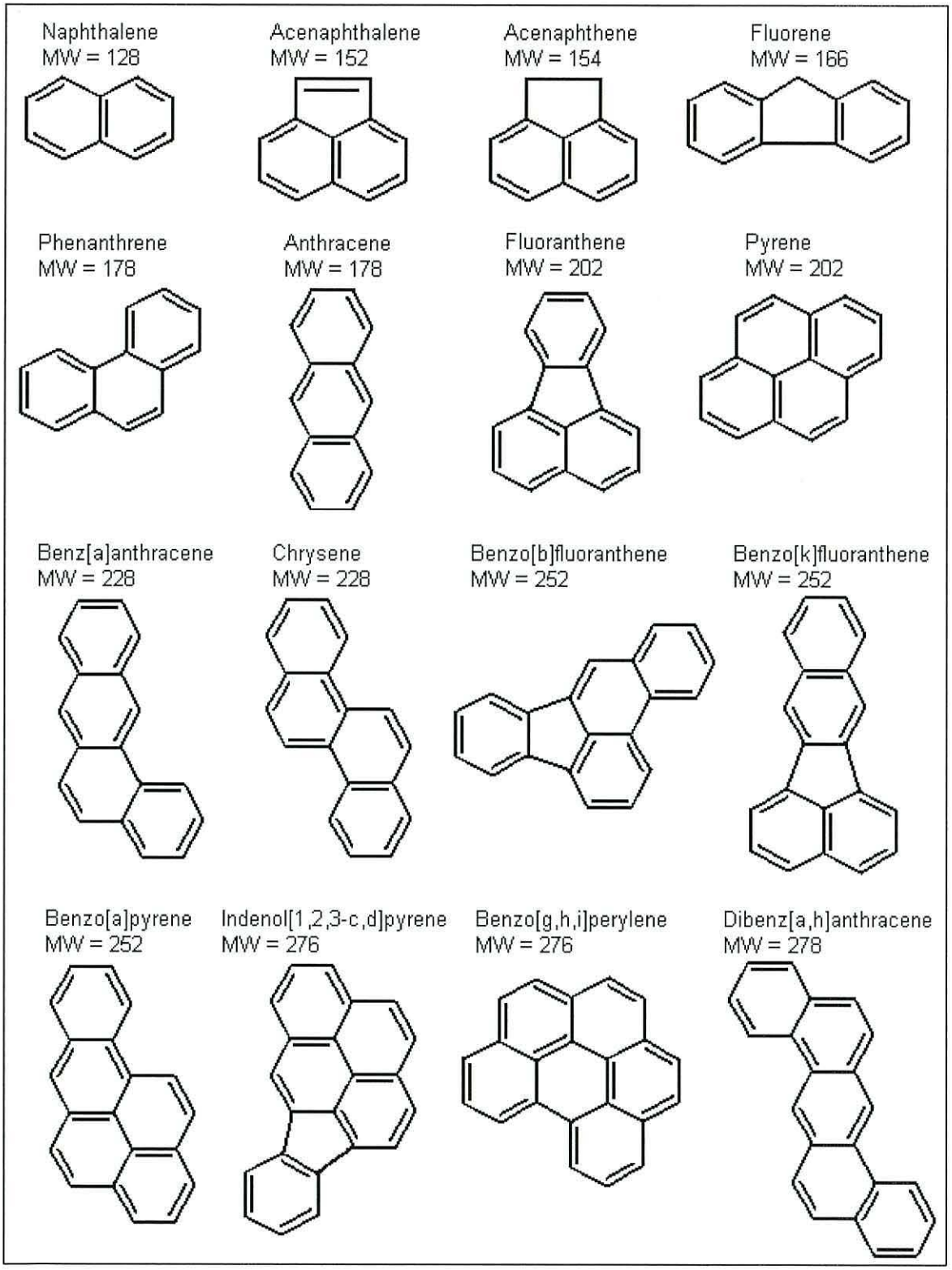

Figure 2.1: Two-dimensional structure and molecular weight of 16 priority PAHs identified by the U.S. EPA. (after Sander and Wise, 1997)

Depending on the source of contamination, soils can contain PAH concentrations ranging between 1 µg kg^{-1} and 300 g kg^{-1} total PAHs (Kanaly and Harayama 2000; Bamforth and Singleton 2005). Furthermore, as a

consequence of incomplete combustion of materials such as coal and wood, atmospheric levels of PAHs ranging from 60 mg m^{-3} and 3 mg m^{-3} air have been reported (Freeman and Cattell 1990). Therefore the development of practical bioremediation strategies for heavily impacted sites is urgently needed.

The chemical properties of individual PAHs are dependent in part upon their molecular size (i.e. their number of aromatic rings) as well as their molecule topology (i.e. their pattern of ring linkage) (Kanaly and Harayama 2000). An increase in the size and angularity of a PAH molecule generally results in an associated increase in hydrophobicity and electrochemical stability which contribute to its persistence (Kanaly and Harayama 2000). PAHs are also known to exhibit acutely toxic effects or possess mutagenic, teratogenic, or carcinogenic properties and as a consequence some are classified as priority pollutants by the U.S. Environmental Protection Agency (EPA) (Kanaly and Harayama 2000) (Figure 2.1).

Human exposure to PAHs occurs through skin-contact, inhalation of polluted air and tobacco smoke, and oral intake of smoked and other foods and polluted water (International Agency of Research on Cancer (IARC) 1983). Due to its common occurrence and high carcinogenicity in PAH mixtures, Benzo[a]pyrene (BaP) is often used as a marker for the carcinogenic potential of such mixtures (Sun *et al.* 1982; Naylor *et al.* 1990; Culp *et al.* 1998; Singh *et al.* 1998; Shimizu *et al.* 2000; Kazerouni *et al.* 2001; Saunders *et al.* 2001).

2.2.3. PAH behaviour in soil: Bioavailability and the effect of ageing

Soil represents a major sink for organic contaminants in the environment (Semple *et al.* 2003) and the longer an organic chemical is in contact with soil, the less bioavailable and extractable it becomes (Hatzinger and Alexander 1995; Reid *et al.* 2000; Semple *et al.* 2001). This phenomenon is described as "ageing" and the extent depends on the characteristics of the pollutant as well as the soil properties (Cerniglia 1992; Hatzinger and

Alexander 1995). It is widely accepted that sorption is the controlling factor in the ageing process. It can occur as sorption to soil organic matter and inorganic soil constituents. Another important factor is diffusion of compounds into spatially remote areas (soil macro and micro pores as well as within soil organic matter). As compound uptake by micro-organisms is far more extensive from fluid than from sorbed states it has been proposed that pollutant mass transfer governs bioavailability (Semple *et al.* 2003).

2.3. CO-COMPOSTING OF HYDROCARBON CONTAMINATED SOIL

The first reports of composting approaches being used as a bioremediation technology to treat contaminated wastes appeared in the 1980s (Antizar-Ladislao *et al.* 2004) and different bioremediation-methods (i.e. the usage of micro-organisms to degrade pollutants) and systems such as *in-situ*, on-site, and bioreactor treatments for soil contaminated with PAHs have been reviewed and compared by Wilson and Jones (1993).

The authors concluded that studies on *in-situ* treatment, which has generally consisted of recirculation and treatment of contaminated groundwater indicate that *in-situ* treatment is inefficient for the removal of many PAHs from contaminated soil within reasonable time periods. Inadequacies are due to strong absorption of PAHs to soil and low PAH aqueous solubility as well as temperature and soil type limitations.

Compared to *in-situ* treatment, on-site treatment of the same soil was found to increase the degradation of PAHs, as conditions for PAH degradation are more easily enhanced. Thus degradation of low-molecular-weight PAHs is generally significant during on-site treatment and therefore treatment of soil contaminated with PAHs containing mainly less than four aromatic rings (e.g. medium distillate fuels) is relatively successful (Song *et al.* 1990; Wang and Bartha 1990; Wilson and Jones 1993). The removal of high-molecular-weight (HMW) PAHs, however, is limited within a reasonable period of time. Additionally, for successful on-site treatment, careful soil

preparation is necessary and a number of limitations such as the presence of other materials toxic to micro-organisms can significantly reduce the effectiveness of the treatment.

Compared to on-site treatment of the same soil it was found that the rate of PAH degradation was increased when the soil was treated in bioreactors. Within bioreactors the optimum degradation conditions are easier to maintain. Additionally, good mixing of the soil with microbial populations and other additives such as nutrients and surfactants can be achieved more easily. However, running costs are generally higher than with *in-situ* or on-site treatment.

Since then, several more studies on the biodegradation of hydrocarbons such as fuel, solvents, and oils have been carried out. A table giving an overview of studies on treating contaminated soil using composting approaches can be found in Appendix 1 (Table A.1). Besides *in-situ*, on-site, and bioreactor techniques including chemical and physical treatments, co-composting contaminated soils with different materials has been used to accelerate the biodegradation of hydrocarbons in soil.

Hydrocarbons are also found in nature and are potentially biodegradable but the degrading micro-organisms usually have a small population size and are limited by environmental conditions (Leahy and Colwell 1990; Ringelberg *et al.* 2001; Wong *et al.* 2002; Antizar-Ladislao *et al.* 2005b).

The most effective remediation of PAHs is achieved when PAHs are completely mineralised by micro-organisms to CO_2 and not simply "lost" either by [1] being washed out or volatilised which would simply redistribute them to another place, nor [2] becoming bound ("locked-up") through sorption onto soils or organic matter where the PAHs will be unavailable for degradation. Even though solid phase binding of PAHs reduces the overall risk to the environment and human health, the long-term fate of the bound organic contaminants remains uncertain.

It is widely accepted that more than one species of micro-organism is needed for complete bioremediation of oil contamination and the rate of biodegradation of hydrocarbons in the environment was found to be

dependent on the type of hydrocarbons present, the environmental conditions, and the composition of the indigenous microbial community (Balba *et al.* 1998). Several studies have investigated composting as a bioremediation process for soil (see below). This was achieved by mixing contaminated soil with composting substrates and treating it in different ways.

2.3.1. Different bioremediation techniques

Using soil in an oil lake area, Balba *et al.* (1998) compared the bioremediation techniques of landfarming, windrow composting piles, and static bioventing piles. The contaminated soil was supplemented with inorganic fertilisers and amended with a mixture of compost and wood chips to enhance microbial growth and co-metabolic activities. After one year, a significant reduction in oil concentration was detected in all treatments. The total loss of alkanes was between 82-91% compared to 16-26% loss in untreated controls whereas the total loss of total petroleum hydrocarbons (TPH) was between 64-83% compared to a 13-20% loss in the untreated controls.

Research on the use of composting approaches for bioremediation of PAH-contaminated waste has recently been reviewed by Antizar-Ladislao *et al.* (2004) who showed that most investigations until then have focused on operational considerations rather than on the physical, chemical, and biological mechanisms that underpin bioremediation and composting technologies.

In three following studies, Antizar-Ladislao *et al.* (2005a; 2005b; 2006) investigated the degradation of PAHs in an aged coal tar contaminated soil under in-vessel composting conditions. In a first study, three different tests were performed to investigate the biodegradation of PAHs. Firstly the contaminated soil was composted with green waste. Secondly, microbial activity was $HgCl_2$-inhibited within the soil-green waste mixture, to evaluate abiotic losses, while in the third experiment only soil was incubated.

The results suggested that the main loss mechanism in the soil-green waste reactors was biological, whereas in the soil reactors it was chemical. In following studies they investigated the effects of different incubation temperatures, soil:green-waste ratios and moisture contents. Their results showed optimal degradation rates at the temperature of 38°C, a soil-to-green waste ratio of 0.8:1, and a moisture content of 60%. Under those conditions 77% of the total PAHs were removed after 98 days.

Comparing the biodegradation of PAHs via composting at a set temperature of 38°C to a temperature profile simulating a natural composting process (38°C for two weeks, followed by 55°C, for another two weeks, 70°C for one week, and 38°C for the final two weeks), they showed that composting with a temperature profile resulted in less PAH removal than keeping the composting temperature at 38°C.

Like most of the other studies the main focus was laid on operational parameters and the investigation of optimal physical conditions for optimal biodegradation.

2.3.2. Augmenting contaminated soil with different additives

The following studies investigated the effect of different organic and inorganic additives on the degradation of hydrocarbons in contaminated soils. Those additives consisted of different nutrients, organic matrices, and organisms and the creation of different chemical and physical conditions.

Kästner *et al.* (1995) added mature compost to soil, contaminated with ^{14}C-labeled Anthracene. They found that the addition of the compost stimulated the disappearance of the extractable fraction of Anthracene significantly. With the compost addition 23% of the Anthracene was mineralised and 42% fixed to the soil matrix, whereas without compost just 22% could not be recovered. In another experiment, Kästner *et al.* (1999) found, that the addition of compost increased the mineralisation of ^{14}C-labeled Anthracene from 43 to 67% while the amounts fixed to the soil and therefore not recoverable decreased from 45 to 21%.

Treating soil contaminated with petroleum hydrocarbons including PAHs, Wellmann *et al.* (2001) determined whether the degradation rate and amount of hydrocarbons in the soil was affected by adding inorganic fertiliser or animal manure to the soil. They found that treating the soil with 20% manure was most effective for the degradation of petroleum hydrocarbons as 81% were removed by day 41 whereas with $(NH_4)_2SO_4$ fertiliser only 54% were removed and within the control only 32% of the hydrocarbons were removed.

Wong *et al.* (2002) added pig manure at three different ratios (12.5%, 25%, 50%) to a soil spiked with 100 mg kg^{-1} each of the PAHs Phenanthrene, Anthracene, and Pyrene and investigated its effect on the degradation of these PAHs in a bench-scale composting system. They found that the manure could increase the populations of total thermophilic and mesophilic bacteria as well as PAH-degrading bacteria and enhances the amounts of soluble organic carbon, ammonia nitrogen, and soluble phosphorous in the composting mass in the early stages of the composting process. In their experiment a manure ratio of 25% was most effective and 90% of the initial PAHs were removed at the end of the composting process.

During the co-composting of highly PAH contaminated soil with poultry-manure, Atagana (2004) found that high nitrogen (N) content and high temperature had limited effects on microbial load and degradation capacity of the compost, but that the high microbial load of the poultry manure in connection with the high nutrient content probably was responsible for the adoption of micro-organisms to the carbon (C) source in this soil, leading to its degradation.

2.3.3. Bio-augmentation and bio-stimulation

Studies comparing the performance of bio-augmentation (addition of micro-organisms to contaminated materials and sites) and bio-stimulation (modification of contaminated materials and sites to enhance the growth of indigenous microbes) have suggested that nutrient addition alone had a

greater effect on oil biodegradation than did the addition of microbial products when oxygen (O_2) supply was not limited (Jobson *et al.* 1974; Venosa *et al.* 1996; Lee *et al.* 1997).

In a comparative study, Brook *et al.* (2001) investigated the effect of different nitrogen sources and concentrations on the degradation rate of PAH containing diesel fuel in nutrient limited soil at two C:N ratios. At a set temperature of 25°C they found highest degradation rates for ammonium sulphate at a C:N ratio of 20:1. The authors developed a degradation rate correlation as a function of nitrate and ammonium concentrations. This function suggested the occurrence of a nitrate inhibition at elevated nitrate concentrations.

Other studies found that, depending on the degrading organisms, additional nutrients can be important for the degradation of hydrocarbons as they may trigger the expression of certain enzymes.

Fungal peroxidase enzymes for example, are usually produced during secondary metabolism brought about by nutritional starvation. Nitrogen is one of the nutrients triggering ligninolytic enzymes production and thus pollutant degradation (such as PAH). The expression of ligninolytic enzymes under nutrient limited conditions is probably an adaptation of the white rot fungi to the low nutrient levels found in wood (Fernando and Aust 1994). The presence of excess nitrogen was expected to restore primary growth of all white rot fungi, but it suppresses both lignin peroxidase activity and polymer decomposition in some strains (Faison and Kirk 1985; Buswell 1991).

However, some studies suggest that high carbon or nitrogen concentrations induce the production of peroxidase enzymes in other white rot fungi, and can therefore positively influence the degradation rates (Kaal *et al.* 1993; Collins and Dobson 1995; Kaal *et al.* 1995). Additionally many studies proved that lignin degrading enzymes of *Pleurotus ostreatus* are not affected by the nitrogen content of the medium (Leatham and Kirk 1983; Boyle *et al.* 1992; Hatakka 1994).

Examining bacterial community dynamics and biodegradation processes in a highly creosote-contaminated soil undergoing different bioremediation treatments, Vinas *et al.* (2005) found a sharp increase in the size of the heterotrophic and PAH-degrading microbial populations coinciding with the highest rate of TPH and PAH biodegradation. This finding implies that specific bacterial phylotypes are associated both with different phases of PAH degradation and with nutrient addition in a pre-adapted PAH-contaminated soil. However, especially for Benzo[a]anthracene and Chrysene, the biodegradation values were higher when no nutrients were added. They found aeration and moisture content to be the key factors associated with PAH bioremediation and that neither bio-surfactant addition, bio-augmentation, nor ferric octate addition led to differences in PAH or TPH biodegradation, compared to biodegradation with nutrient treatment.

New approaches for bio-augmentation as a remediation technology have recently been reviewed by Gentry *et al.* (2004). These approaches include bio-augmentation with cells encapsulated in a carrier, gene bio-augmentation where the added inoculant can transfer remediation genes to indigenous micro-organisms, rhizosphere bio-augmentation where the microbial inoculant is added along with a plant that serves as a niche for the inoculants growth, and phyto-augmentation where the remediation genes are engineered directly into a plant for use in remediation without a microbial inoculum.

2.3.4. Physico-chemical effects

Physico-chemical factors affecting a composting process include moisture content, nutrient content (including N, C, and C:N ratio), NH_3, CO_2, pH, total organic matter (TOM), and electrical conductivity (EC).

As decomposition induced by micro-organisms occurs most rapidly in the thin liquid films found on the surfaces of the organic particles (Richard *et al.* 2002), the composting materials should maintain a moisture content of 40 to 65% (by weight) to support the metabolic activity of the micro-organisms.

Carbon is the most abundant element in any living cell and the basis of all functional biological molecules and comprises about 50% of the mass of microbial cells. Nitrogen is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents. Nitrogen (N_2) being abundant to nearly 79% in the atmosphere is unavailable for use by most organisms due to the triple bond between the two nitrogen atoms. In order for nitrogen to be used for growth it must be available in the form of ammonia (NH_3), ammonium (NH_4^+) or nitrate (NO_3^-) ions.

It was found, that the initial C:N ratio has an effect on the composition of material. For example, Eiland *et al.* (2001) showed that in composts with low initial C:N ratios microbial biomass and respiration was higher and caused a faster degradation of cellulose and hemicellulose, whereas in composts with high initial C:N ratios the microbial biomass and respiration was lower and resulted in a far lower degradation of these fibres.

The pH affects the availability of nutrients, particularly microelements and can be used to follow the process of decomposition, as during the initial stages of decomposition, organic acids are formed so that pH normally drops to 4.5-5.0. The acidic conditions are favourable for growth of fungi and breakdown of lignin and cellulose. Soon these acids are consumed and the pH begins to rise to around 8 (Williams 1996). Compost micro-organisms operate best under mild alkaline to acidic conditions, with pHs in the range of 5.5 to 8. As composting proceeds, the organic acids become neutralised. Although the natural buffering effect of the composting process lends itself to accepting material with a wide range of pH, the pH level should not exceed eight as at higher pH levels, more ammonia gas is generated and may be lost to the atmosphere (Williams 1996).

Electrical conductivity is a numerical expression of the ability of an aqueous solution to carry an electrical current. It depends on the presence of ions, their total concentration, mobility, and temperature and can therefore give an indication of the salt and nutrient content of the compost mixture (Yateem *et al.* 1998).

Besides the C:N ratio, the amount of organic amendments, and pH, the temperature profile has been shown to be another key-factor affecting composting of weathered hydrocarbon contaminated soils. Not only is the temperature important for regulating microbial activity within the compost but it also affects the behaviour of the PAHs. With increasing temperatures the solubility of PAHs increases, which increases their bioavailability. In addition, increasing temperatures also decreases the solubility of oxygen thereby reducing the metabolic activity of aerobic micro-organisms (Bamforth and Singleton 2005). The following studies investigated the effect of temperature on the degradation process.

In a 30-day experiment investigating the effects temperature has on the rate of hydrocarbon degradation in composts, Beaudin *et al.* (1999) found the highest rates of degradation were at 23°C (56% of the total hydrocarbons were lost) whilst at 40°C only 44% was lost. However, the rate of hydrocarbon loss then increased again at 50°C where a loss rate of 47% was apparent. They also found that the length of the thermophilic phase (50°C) correlated with the measured amount of hydrocarbon degradation, whereas Chung *et al.* (2000) showed that the maintenance of thermophilic temperatures is influenced by the concentration of the contaminant.

Amir *et al.* (2005) investigated the fate of PAHs during composting of lagooning sludge. Lagooning sewage sludge was mixed with straw and composted for 180 days. After this time a reduction of 88% of total extractable PAHs was measured. Using the temperature as an indicator for microbial activity the authors found that "during the stabilisation phase of composting, extensive microbial degradation of PAHs, mainly those with a low number of aromatic rings, was achieved following development of intense thermophilic communities". They came to the conclusion that due to their number of aromatic rings and their molecular structure different individual PAHs are more or less strongly absorbed and therefore more or less easily desorbed and available for degradation.

2.3.5. Surfactants

PAHs in soil can be adsorbed to mineral surfaces (i.e. clays) and organic matter (i.e. humic and fulvic acids). The sorption becomes more and more irreversible the longer the PAH remains in contact with the soil and the lower its biological and chemical extractability (Bamforth and Singleton 2005). Soil properties that alter the degradation rate of PAHs (e.g. soil structure, particle size, organic matter content) have been reviewed by Manilal and Alexander (1991), Sims *et al.* (1990), and Steiber *et al.* (1990).

To successfully bio-remediate PAH-contaminated soils the contaminant must be available for degradation. One way of achieving this is by adding surfactants, surface-active molecules that have both hydrophobic and hydrophilic domains and are capable of lowering the surface tension and the interfacial tension of the organisms in their growth medium, to the soil, i.e. they are providing a "bridge" between the hydrophobic PAH molecule and the hydrophilic microbial cell (Bamforth and Singleton 2005). Surfactants can also emulsify hydrophobic compounds, form stable emulsions and increase PAH solubility and consequently bioavailability in the environment (Cameotra and Bollag 2003).

In an examination of the effect of introducing bacteria and non-ionic surfactants on the degradation of PAHs in soil, Madsen and Kristensen (1997) found that although the inoculating freshly contaminated soil with Phenanthrene-utilising bacteria stimulated the mineralisation of ^{14}C-Phenanthrene, a large more diverse inoculum was required to establish full Phenanthrene mineralisation in the soil. The addition of the non-ionic surfactants alcohol ethoxylate and glycoside enhanced the degradation of freshly contaminated soil as well as a coal tar contaminated soil, where Pyrene, Benzo[b,j,k]fluoranthene, and Benzo[a]pyrene were found to be resistant without the addition of the surfactants. Their results also suggest that the use of surfactants that are mineralised at a moderate rate may be more applicable for increasing the availability of PAHs in soil, as the surfactant-related enhancement of the degradation of PAH contaminants was less convincing when a rapidly degradable glycoside surfactant was used.

When investigating the ability of the white-rot fungus *Bjerkandera* to degrade PAHs in soil, Grotenhuis *et al.* (1999) found a large increase in the degradation rate when the bioavailability of PAHs was increased by adding surfactants, as opposed to just a slight increase when the peroxidase activity was increased and the aeration optimised.

The use of surfactants under thermophilic conditions was also investigated by Wong *et al.* (2004). They reported that improved degradation was significant when surfactants produced by *Pseudomonas aeroginosa* were added. Similarly, Jacques *et al.* (2005) isolated two different *P. aeroginosa* strains and one *Pseudomonas citronellolis* strain able to degrade Anthracene. They showed a significant increase in Anthracene degradation due to the surfactants produced by *P. citronellolis.*

2.3.6. Effects of compost on contaminated soil

In 1996, Kästner and Mahro found that the presence of a solid organic matrix seemed to be essential for the enhanced microbial degradation of PAHs, however, this stimulatory effect was not linked to the release of nutrients from the compost nor the shift of soil pH brought about by the compost. In addition, their results showed that the decrease of soil PAH concentrations after compost addition were not caused by sorption to the newly introduced organic matter, but moreover to a stimulation of microbial activity. The results also suggest that the compost diminished the sorptive effect of the pure soil, i.e. by the occupation of sorptive sites, which made PAHs more available for biodegradation by the associated microbial community.

Additionally, it was noted by Vogtmann and Fricke (1992) and Chaney *et al.* (1996) that either mature or immature composts are generally low in xenobiotic organic compounds, suggesting that degradation of pesticides occurs early in the process. In 2002, Barker and Bryson also came to similar conclusions when they found that rapid degradation of xenobiotics commonly occurs during the first 30 days of the composting process.

2.3.7. PAH extraction methods

The soxhlet extraction has been the standard extraction method for the determination of non-ionic compounds for several decades (Knepper *et al.* 2003) and is considered a benchmark technique against which all other techniques are compared (Dean and Saim 1998). The PAH extraction method ISO 18287 used in this study is principally based on the extraction method described in ISO 13877 (International Organization for Standardization 2006). It has been found by Berset *et al.* (1999) that results obtained by this method are comparable to results obtained by soxhlet extraction.

Other methods such as supercritical fluid extraction and accelerated solvent extraction have been shown to be more efficient in extracting PAHs from an aged contaminated soil (Berset *et al.* 1999). However, it has also been found that the extraction efficiency is dependent on the sample matrix (e.g. soil type, total organic matter content) (Hatzinger and Alexander 1995; Macleod and Semple 2000; Belkessam *et al.* 2005). Overall no extraction method can guarantee that all present contaminants will be extracted from an aged contaminated soil.

As an alternative to try to extract the total amount of PAHs present, methods have been developed to determine only the bioavailable PAH fraction. This is the fraction which can be taken up or transformed by living organisms and can therefore be used to indicate toxicity-levels. However, because of the complexity of interactions between the soil, the contaminant, and the organisms present, there is no all-encompassing extractant to describe bioavailability (Semple *et al.* 2003) especially as bioavailability has been found to differ between organisms and species (Reid *et al.* 2000). Those differences and uncertainties are an additional factor making comparisons and assessment of toxicity difficult, especially when dealing with a complex medium that inhabits a large variety of different organisms.

2.3.8. PAH losses through volatilisation

Declining PAH concentrations in soil are not always solely down to mineralisation as PAH compounds can also be lost due to sorption of PAHs to the soil-matrix making them non-extractable, or by volatilisation of either the parent compound or their breakdown products. To date, only a few studies have looked at the effect volatilisation has on decreasing amounts of PAHs during different treatment methods of PAH contaminated soil.
Cousins and Jones (1998) investigated the air-soil exchange of semi-volatile organic compounds and observed statistically significant losses for the majority of PCBs and PAHs from spiked soil which was exposed to outdoor air during a nine month experiment. Especially for Acenaphthene and Fluorene, they found losses so large that the levels in the spiked soil were similar to the levels in the unspiked soil.

In another experiment studying structure-biodegradability relationships for ten PAHs in the presence of 1-phenyldecanes as a primary substrate Bossert and Batha (1984) found a significant loss of 3-ring PAHs through volatilisation.

In contrast to this Civilini (1994) found less than 10 % of total losses for all PAHs due to volatilisation with Acenaphthene being an exception as with this PAH loss through volatilisation accounted for approximately 54 %.

Similarly, by adding selected PAHs to two different uncontaminated soil types and incubating the spiked soil at 25°C for 2 days, Park *et al.* (1990) found that volatilisation accounted for approximately 30% of the total loss of Naphthalene and approximately 20% of 1-methylnaphthalene. However, volatilisation only accounted for 0.1% of the total for PAHs with more aromatic rings.

Guerin found no losses of low-molecular-weight PAHs through volatilisation while Kirchmann and Ewnetu (1998) found no significant losses of oil compounds through volatilisation from compost, during their experiment of co-composting oil wastes with horse manure for 4.5 months.

2.4. PAH DEGRADATION PATHWAYS

Several micro-organisms can degrade simple PAHs like Naphthalene, and some can even break down more toxic and complex ones, such as BaP e.g. in coal tar and cigarette smoke; (Kanaly and Harayama 2000). It has been shown that microbial degradation of oil occurs by attack on aliphatic or light aromatic fractions (Higgins and Gilbert 1978; Cerniglia 1984; Gibson and Subramanian 1984; Weissenfels *et al.* 1990a) whereas high molecular weight aromatics, resins, and asphaltenes are considered to be somehow recalcitrant or exhibit only low rates of biodegradation (Balba *et al.* 1998).

The amount of extractable PAHs can be decreased by degradation and mineralisation by micro-organisms, by binding to soil or other particles, and by volatilisation where PAHs evaporate into the atmosphere and are distributed to another place, which is why potential evaporation of PAHs during remediation processes should be monitored carefully.

Müller *et al.* (1998) isolated micro-organisms who were able to degrade Naphthalene, Phenanthrene, and Anthracene under thermophilic conditions. Their results indicate that metabolites produced differ significantly from those formed under mesophilic conditions indicating a different degradation pathway.

Likewise, Annweiler *et al.* (2000) showed that degradation of Naphthalene by a thermophilic *Bacillus thermoleovorans* (at 60°C) differs from the pathways known for mesophilic bacteria, as several new metabolites [apart from typical ones well known from Naphthalene degradation by mesophiles] were found. Tables A.2 and A.3 (see Appendix 2) provide an overview of metabolites found during PAH degradation. More detailed pathways have been proposed by Evans *et al.* (1965), Cerniglia *et al.* (1992), Grifoll (1995), and others (see reviews by Sutherland *et al.* (1995), Cerniglia (1997), Kanaly and Harayama (2000), Mrozik *et al.* (2003) and Bamforth and Singleton (2005)).

2.4.1. Bacterial degradation pathways

The first step in the aerobic catabolism of a PAH molecule by bacteria is the oxidation of the PAH to a dihydrodiol by a multi-component enzyme system incorporating both atoms of molecular oxygen into the PAH nucleus (Gibson *et al.* 1975) as shown in Figure 2.2. After oxidation, the dehydrated intermediates may be processed through either an *ortho* or a *meta* cleavage type of pathway leading to intermediates which are further converted to intermediates of the tricarboxylic acid cycle (Kanaly and Harayama 2000).

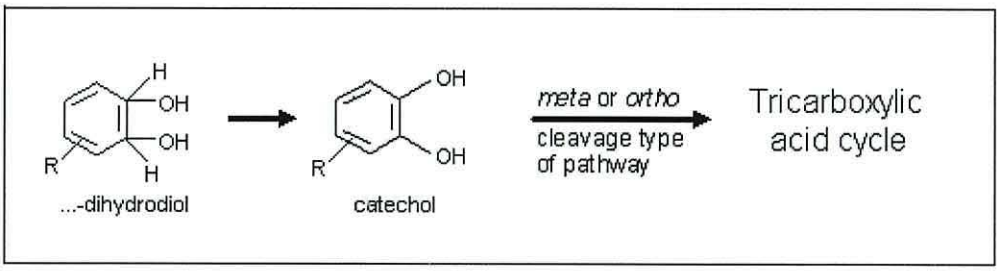

Figure 2.2: Pathways of PAH degradation by bacteria (after Bamforth and Singleton, 2005 and Cerniglia, 1992)

A study by Shimura *et al.* (1999) suggested the existence of two independent degradation pathways for a *Bacillus* sp. strain, as biphenyl grown cells of strain JF8 which degraded p-chlorobiphenyl barely degraded Naphthalene while Naphthalene grown cells did not degrade p-chlorobiphenyl.

Several enzymes being responsible for the degradation of hydrocarbons have been found encoded either on plasmids or chromosomally (Yen and Gunsalus 1982; Bosch *et al.* 1999; Mrozik *et al.* 2003). A more detailed review on the degradation pathways and metabolites of selected PAHs and the enzymes involved has been given by Mrozik *et al.* (2003).

In contrast to bacteria, mammals incorporate just one atom of molecular oxygen into the PAH to form arene oxides that can either undergo enzymatic hydration by epoxide hydrolase to form trans-dihydrodiols or else rearrange non-enzymatically to form phenols (Daly *et al.* 1972).

2.4.2. Fungal degradation pathways

The structures of PAHs are somewhat similar to that of lignin. White-rot fungi (Basidiomycetes) which are known to degrade lignocellulose (e.g. *Bjerkandera sp.)* also have the potential to oxidise PAHs via aspecific extracellular enzymes (Grotenhuis *et al.* 1999). A more detailed overview on the degradation and oxidation of PAHs by ligninolytic fungi and a list of metabolites produced from PAHs by fungi has been given by Cerniglia (1997). In this review it was also stated that ligninolytic fungi can co-metabolise PAHs to form *trans*-dihydrodiols, phenols, quinines, and dihydrodiol-epoxides (Figure 2.3).

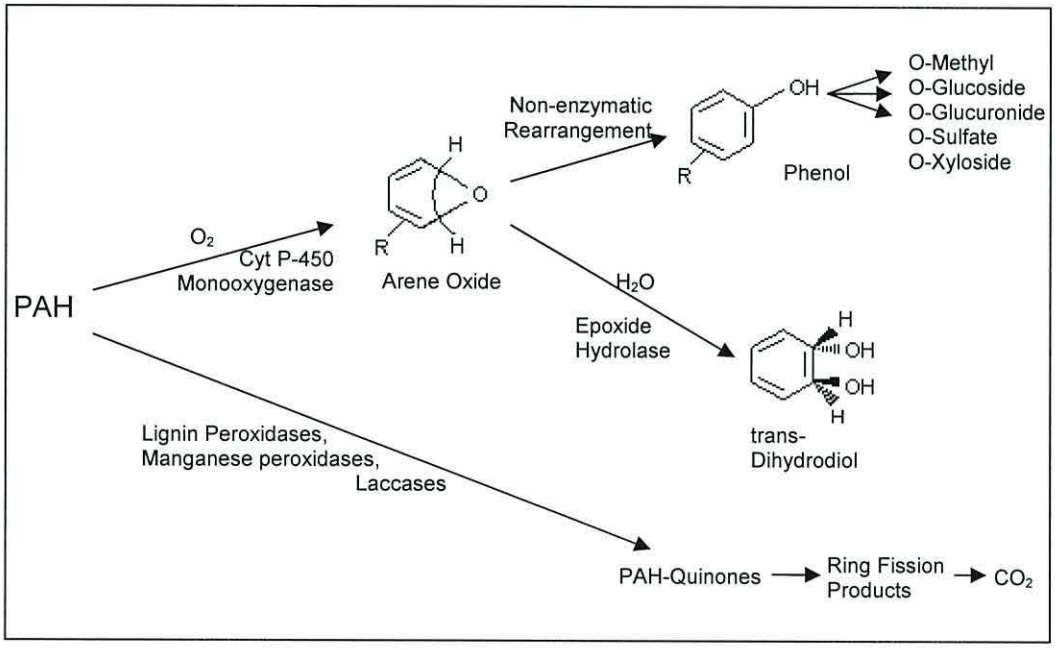

Figure 2.3: Pathways for the fungal metabolism of PAHs (after Cerniglia, 1997)

Different extracellular enzymes responsible for the degradation of lignin (e.g. lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccases) are believed to be involved in the degradation of PAHs (Harayama 1997). Not all white rot fungi, however, have the ability to produce all of these enzymes (e.g. laccases, lignin peroxidases, manganese peroxidases, aryl alcohol oxidase), as different fungal strains have different ligninolytic systems which distinguish them from each other (Hatakka 1994). Since

extracellular peroxidases are able to oxidise complex aromatic compounds, white rot fungi are expected to be the most efficient fungi capable of degrading poorly bioavailable PAHs (Field *et al.* 1992).

2.5. ORGANISMS DEGRADING HYDROCARBONS

That micro-organisms possess catabolic abilities that may be used for the biodegradation of PAH-contaminated waste and water has been shown since the 1970s (Antizar-Ladislao *et al.* 2004). Especially after the Gulf war in 1991, much interest has been shown in the use of hydrocarbon degrading micro-organisms to treat oil pollution in soils and sand at high temperatures and with a high salt concentration (Sorkhoh *et al.* 1992; Sorkhoh *et al.* 1993; Al-Daher *et al.* 1998; Balba *et al.* 1998; Al-Maghrabi *et al.* 1999).

The biodegradation of hydrocarbons in the environment is a complex process. The qualitative and quantitative aspects of this biodegradation process are dependent upon a range of factors including [1] the amount and nature of the hydrocarbons present, [2] the environmental conditions, [3] the composition and size of the microbial community, and [4] its response to the presence of hydrocarbons which is dependent on how favourable the conditions for the growth of those organisms are. When co-composting PAH contaminated soil, certain organisms can degrade PAHs faster under the more favourable conditions provided (e.g. aeration, temperature, moisture).

With bacteria assuming the dominant role in marine ecosystems and fungi becoming more important in freshwater and terrestrial environments those two groups of micro-organisms are the key agents for the degradation of hydrocarbons (Leahy and Colwell 1990). If communities are adapted, because of having been exposed to hydrocarbons previously, they typically exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination (Leahy and Colwell 1990).

2.5.1. Bacteria degrading PAHs

A wide variety of bacteria capable of metabolising PAHs has been reported, including *Achromobacter*, *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Comamonas*, *Corynebacterium*, *Flavobacterium*, *Microbacterium*, *Micrococcus*, *Moraxella*, *Mycobacterium*, *Neptunomonas*, *Nocardia*, *Paenibacillus*, *Porphyrobacter*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas*, *Streptomyces*, *Vibrio* and *Xanthomonas* (Atlas 1981; Bossert and Bartha 1984; Leahy and Colwell 1990; Sorkhoh *et al.* 1993; Bodour *et al.* 2003) and microbial degradation of hydrocarbons has been reviewed by Atlas (1981) and Leahy and Colwell (1990).

Since then, more bacterial species have been identified, such as organisms of the eight genera *Afipia*, *Janthinobacterium*, *Leptothrix*, *Massilia*, *Methylobacterium*, *Rhizobium*, *Sinorhizobium* and *Thiobacillus* which were all found to degrade Phenanthrene (Bodour *et al.* 2003). Furthermore the results of Bodour *et al.* indicate that the dominant phenanthrene-degrading population changed over the course of their 6-month experiment confirming earlier findings that a diverse community participates in Phenanthrene degradation in the environment, and also suggesting that the composition of this community is temporally variable.

Bacillus strains (*Bacillus* sp.) were found by Sorkoh *et al.* (1993) when investigating degrader organisms in desert samples from oil contaminated areas in Kuwait. Two strains belonging to the genus *Bacillus* degraded 80-89% (5 g l^{-1}) of crude oil within 5 days at their optimum growth temperature of 60°C.

A thermophilic *Bacillus* strain (*Bacillus sp.*) was also found to completely degrade phenol up to a concentration of 5 mM, whereas it was inhibited above a concentration of 10 mM. This strain was later found to also completely degrade all three isomers of 1 mM cresol, an active constituent of creosote, but did not utilise either toluene or xylene (Mutzel *et al.* 1996).

The Naphthalene metabolism of the thermophilic aerobic bacterium *Bacillus thermoleovorans* Hamburg 2 which grows at 60°C on Naphthalene as the sole source of carbon and energy was investigated by Annweiler *et al.* (2000). Apart from the typical Naphthalene metabolites known from mesophiles, intermediates such as 2-carboxycinnamic acid, and phthalic and benzoic acid were identified for the pathway of this bacterium. These compounds indicate that Naphthalene degradation by the thermophilic *B. thermoleovorans* differs from the known pathways found for mesophilic bacteria.

Pseudomonas cepacia was found to simultaneously degrade a mixture of three-, four-, five- and seven-benzene ring PAHs when inoculated at high cell densities and it was observed that the degradation of five- and seven-benzene ring PAHs improved when low molecular weight PAHs were present (Juhasz *et al.* 1996).

Pseudomonas stutzeri and *Vibrio mimicus* are two bacteria found to degrade BTEX (Benzene, Toluene, Ethylbenzene, Xylene) compounds (Babaarslan *et al.* 2003) and *Hydrocarboniphaga effusa* gen. nov., sp. nov., a novel member of the γ-*Proteobacteria* was found to be active in alkane and aromatic hydrocarbon degradation (Palleroni *et al.* 2004).

MacNaughton *et al.* (1999) investigated the microbial community development in an experiment simulating a coastal oil spill. They compared the communities for four different treatments (no oil control, oil alone, oil plus nutrients, and oil plus nutrients plus an indigenous inoculum). Using phospholipid fatty acid (PLFA) analysis and polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) they found a banding pattern in the no oil control that completely disappeared in the oiled samples, indicating that after introducing the oil the dominant bacterial community structure changed substantially. They did not find significant differences between the nutrient-amended and indigenous inoculum-treated plots, but both differed from the oil only plots. Sequence analysis of prominent DGGE bands showed the presence of α-*Proteobacteria*, which were not detected in un-oiled soil, and members of the *Flexibacter-Cytophaga-Bacteroides* phylum.

A *Stenotrophomonas maltophilia* strain, which was able to utilise Pyrene as a sole carbon and energy source and also degraded other high molecular weight PAHs containing up to seven benzene rings, was isolated by Boonchan *et al.* (2000b) from PAH-contaminated soil.

Hyphomicrobium, *Xanthomonas*, and *Bacillus* species were found by Li *et al.* (Li *et al.* 2000) to be most active in degrading petroleum hydrocarbons in a soil contaminated with hydrocarbons with concentrations up to 200 g kg^{-1}.

Characterising microbial communities from soil and groundwater of oil-contaminated sites Becker and Dott (1995) found, that the percentage of hydrocarbon-degrading micro-organisms in those communities was influenced by the diversity and amount of carbon supply. Additionally, they showed that even though the physiological potentials of the individual bacteria complemented each other and determined the distinctive profile characteristic of the microbial community, the individual members could differ in their metabolic abilities and in their taxonomic status.

Although the taxonomic status of isolates seemed to be highly dependent on the physicochemical factors of a site, the taxonomic status of the bacteria did not determine their activities with strains of the same species showing different degradation abilities for hydrocarbon substrates.

2.5.2. Genetically engineered organisms degrading PAHs

Another potential strategy for using organisms for the remediation of contaminated soil is to genetically manipulate a suitable organism. Since bacterial genes encoding catabolic enzymes for recalcitrant compounds started to be cloned in the late 1970s and early 1980s organisms have been genetically engineered (Cases and de Lorenzo 2005).

The first engineered and patented organism able to degrade a mix of recalcitrant compounds (e.g. camphor, octane, salicylate, and Naphthalene) was a *Pseudomonas* strain engineered by Gunsalus and Chakrabarty in

1981. This was followed by work on the degradation of petroleum components (Harvey *et al.* 1990) and chloro-aromatic compounds (Haugland *et al.* 1990). Since then genetic engineering has produced numerous strains able to degrade pollutants in the laboratory (Cases and de Lorenzo 2005).

Another area of study is the generation of transgenic plants able to express biodegradation or detoxification genes recruited from bacteria (Rugh *et al.* 1998; Hannink *et al.* 2001). Using plants has got the advantage, that via the roots the catalyst responsible for the degradation of recalcitrant materials can be introduced well below the soil surface; additionally horizontal gene transfer is less probable (Cases and de Lorenzo 2005).

2.5.3. Fungi degrading PAHs

A diverse array of fungi possesses the ability to non-specifically degrade a wide range of PAHs. Those fungi include members of the genera *Agrocybe, Bjerkandera, Coriolopsis, Crinipellis, Flammulina, Kuehneromyces, Laetiporus, Marasmiellus, Naematoloma, Phanerochaete, Pleurotus, Ramaria, Rhizoctonia, Rhodotorula, Trametes, Trichosporon.* Those genera belong to the phylum Basidiomycota which includes symbionts (mutualists, commensalists and parasites) and saprobes decaying dead organic matter (e.g. leaves and wood) and play an important role in the C cycle (Swann and Hibbett 2003).

Other fungi found to degrade PAHs are members of the genera *Aspergillus, Candida, Chrysosporium, Fusarium, Neurospora, Penicillium, Saccharomyces, and Trichoderma* which belong to the phylum Ascomycota. The Ascomycota, the sister group of the Basidiomycota, constitute the largest class of fungi characterised and includes plant pathogens, saprobes, and decomposers (Alexopoulos *et al.* 1996).

Members of the genera *Cunninghamella, Mortierella, Mucor,* and *Syncephalastrum* belonging to the phylum Zygomycota make up the third group of fungi capable of degrading PAHs. Even though most of the

taxonomic orders within the phylum are strongly supported as monophyletic, relationships among them are poorly resolved (James and O'Donnell 2004).

White rot fungi are ligninolytic fungi and include members of the phyla Ascomycota and Basidiomycota (Deacon 2005). Some of the common substructures of lignin, (e.g. catechol diethers, alkylarenes and biphenyls) resemble the chemical structure of many persistent compounds contaminating the environment (Yateem *et al.* 1998). Ligninolytic fungi and white rot fungi in particular are known to degrade PAHs and to detoxify PAH-polluted soils and sediments.

The major breakthrough on the potential of fungi for use in PAH bioremediation was in 1985 when Bumpus *et al.* reported that the white-rot Basidiomycete *Phaenerochaete chrysosporium* partially degraded Benzo[a]pyrene to carbon dioxide (CO_2). Following this study, it was shown that *P. chrysosporium* can metabolise a wide variety of PAHs under ligninolytic and non-ligninolytic conditions all the way to CO_2 (Haemmerli *et al.* 1986; Sanglard *et al.* 1986; Bumpus 1989).

Later studies showed the ability of other Basidiomycetes such as *Crinipellis stipitaria* (Lambert *et al.* 1994; Lange *et al.* 1994) and white-rot fungi such as *Bjerkandera sp.* (Field *et al.* 1992; Sack and Günther 1993), *Pleurotus ostreatus* (Sack and Günther 1993; Bezalel *et al.* 1996; Cerniglia 1997) , and *Trametes versicolor* (synonym: *Coriolus versicolor*) (Sack and Günther 1993; Collins and Dobson 1996) in metabolising PAHs such as Phenanthrene, Anthracene, Pyrene, Benzo[a]pyrene, Fluorene, and Fluoranthene. Those studies led to the assumption that *Bjerkandera* sp., *Pleurotus ostreatus*, and *Trametes versicolor* may be more promising than *P. chrysosporium* in their ability to completely mineralise PAHs (Field *et al.* 1992; Sack and Günther 1993; Vyas *et al.* 1994).

While discussing the research in Dave Gibsons' laboratory at the microbiology department of the University of Texas in Austin, Cerniglia (1997) reviewed the importance of non-ligninolytic and ligninolytic fungi in the bioremediation of those persistent organic pollutants.

In 1998, Yateem *et al.* investigated the influence of the strain type, inocula concentration and the addition of N on the degradation of hydrocarbons in oil-contaminated soil. They examined the effect of different concentrations of *P. chrysosporium* in remediating soil contaminated with weathered crude oil, under N limited and rich conditions and compared the oil biodegradation activity of three white rot fungi (*Phanerochaete chrysosporium*, *Trametes versicolor*, and *Pleurotus ostreatus*).

In their experiments they found that the reduction in TPH concentration in *T. versicolor*-treated microcosms was 78%, which is almost equal to the degradation rates of *P. chrysosporium* (77%) under N-limiting conditions while they proved that *T. versicolor* was capable of producing peroxidases enzymes under N-rich conditions.

In 2001, Canet *et al.* also determined the potential of the white-rot fungi *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes versicolor*, and a PAH-degrading fungi strain isolated from woodland on the Wye College Estate, S.E. England, by incubating the fungi and combinations of two or more of these fungi in microcosms containing wheat straw and non-sterilised coal-tar contaminated soil.

They found that highest PAH-degradation rates were achieved when no fungi were added to the soil. Their results suggested that without the added fungi native micro-organisms colonised the straw added as organic substrate and degraded PAH co-metabolically, whereas with fungi added, the colonisation of the straw by the natural microflora was inhibited by the fungi previously inoculated to the straw.

P. ostreatus differs from *P. chrysosporium* in its lignin degradation mechanism since it does not have lignin peroxidase activity. *P. ostreatus* has a pathway similar to those found in non-ligninolytic fungi and an oxidative pathway where it cleaves the aromatic ring and mineralises the PAH, as ligninolytic fungi do (Cerniglia 1997).

The potential of *P. ostreatus* to degrade PAHs was indicated by studies by Sack and Günther (1993) who, while screening for species able to degrade PAHs, showed that *P. ostreatus* is quite efficient in the degradation of Phenanthrene and Fluorene but less efficient with Fluoranthene, while Pyrene was not degraded to any significant extent.

Similarly, Vyas *et al.* (1994) showed that *P. ostreatus* was able to degrade Anthracene. In another study on *P. ostreatus,* Bezalel *et al.* (1996) showed that the fungus was able to mineralise 7% of [^{14}C]catechol, 3% of [^{14}C]-Phenanthrene, 0.4% of [^{14}C]-Pyrene, and 0.2% of [^{14}C]-Benzo[a]pyrene to $^{14}CO_2$ by day 11 of incubation. It also mineralised 0.2% of [^{14}C]-Fluorene within 15 days and 0.6% [^{14}C]-Anthracene in 35 days, but did not mineralise Fluoranthene. The authors observed laccase and manganese-inhibited peroxidase, enzymes considered to be part of the ligninolytic system during the experiments. However, although activity of both enzymes was observed they could not find a distinct correlation to PAH degradation.

Hatakka (1994) found that *P. ostreatus* showed slower degradation rates of oil hydrocarbons when compared to other fungi. This may be due to the selective degradation ability of this organism which classified it as one of the moderate lignin degraders.

Márquez-Rocha *et al.* (2000) found that the white-rot fungus, *P. ostreatus*, was able to metabolise four soil adsorbed PAHs of which three, Pyrene, Anthracene, and Phenanthrene, were mineralised and BaP just oxidised.

The effect of inoculation of *P ostreatus*, temperature, and adding fertiliser and bark as pre-treatment methods on PAH degradation in aged creosote contaminated soil was investigated by Eggen and Sveum (1999). Optimal degradation rates were achieved when bark was added and the soil was incubated at 22°C before inoculation with the white rot fungus. At low temperatures (8°C), fungal inoculation had the best effect when no fertiliser was added, whereas pre-treatment with fertiliser stimulated microbial activity at low temperature and enhanced PAH degradation even without addition of fungi.

The non-ligninolytic fungi *Cunninghamella elegans* has also been found to degrade PAHs (Cerniglia 1992). Since conjugation products are considered to be less toxic than the parent PAH, it has been suggested that *C. elegans* can be used to efficiently remediate PAH-contaminated soil as its metabolic pathway and conjugated metabolites are indicative of a detoxification pathway (Cerniglia *et al.* 1985; Cerniglia 1997).

Looking for PAH degrading fungi, Pickard *et al.* (1999) hypothesised that fungi capable of metabolising PCBs might also be able to degrade PAHs. In their experiments they tested the ability of three *Bjerkandera adusta* strains, three *P. ostreatus* strains, a *P. chrysosporium*, a *T. versicolor*, a *Coriolopsis gallica* and a *Ganoderma applanatum* strain to degrade Anthracene, Pyrene, or Phenanthrene while growing on a bran flakes medium. Their results showed significant variations but the trends showed that about half of the strains tested were able to metabolise Anthracene.

2.5.4. Comparison of micro-organisms that degrade PAHs

Genetically engineered organisms were thought to be an ideal way to biodegrade contaminants in soil, but while genetically modified organisms seem to have a great potential under controlled conditions, it is still a big challenge to deliver predictable organisms that are able to perform under natural conditions.

When trying to use those genetically engineered organisms in the field, several problems occurred such as the realisation that organisms like *Pseudomonas* and *Rhodococcus*, which are widely favoured as hosts of genes because of their fast growth, are far less significant under natural conditions (Cases and de Lorenzo 2005).

Engineered organisms which are released in an environment with complex microbial communities also face resistance to colonisation effects and a niche specificity which is not optimal and might make them vulnerable to predators (Cases and de Lorenzo 2005).

Furthermore, work on *E. coli* and *Pseudomonas* revealed that the heterogeneous texture of natural niches selects distinct populations that quickly diverge genetically from the initial inoculum (Rainey and Travisano 1998).

In addition it has been shown that a high amount of inoculum will be needed for bioaugmentation to be a viable bioremediation technology (Jobson *et al.* 1974; MacNaughton *et al.* 1999). Consequently, the use of genetically engineered organisms represents an uneconomical treatment strategy.

When looking within the groups of fungi and bacteria for the optimal organisms to degrade hydrocarbons both organismal strategies have advantages and disadvantages.

One advantage fungi have over bacteria is, that the fungal mycelium can grow into the soil and distribute itself through the solid matrix to degrade the PAHs (Cerniglia 1997) whereas other micro-organisms such as bacteria are mainly found in surface biofilms which might not allow them to reach the contaminants (Cases and de Lorenzo 2005).

Another advantage fungi, and white-rot fungi in particular, have is that their extracellular enzymes directly attack the PAHs and are able to degrade high molecular weight PAHs (≥four rings) whereas PAH degrading bacteria only have intracellular enzymes and are often limited to the degradation of low molecular weight PAHs (Grotenhuis *et al.* 1999).

However, some bacteria have also been found to degrade high molecular weight PAHs such as four-ring PAHs like Fluoranthene, a nonalternant PAH with a five-membered ring, which has been found to be metabolised by a variety of bacteria including *Alcaligenes denitrificans* and *Pseudomonas* (*Sphingomonas*) *paucimobilis* (Mueller *et al.* 1990; Weissenfels *et al.* 1990a; Kanaly and Harayama 2000). Pyrene, a pericondensed 4-ring PAH, was found to be metabolised by e.g. *Mycobacterium* (Heitkamp *et al.* 1988b; Cerniglia and Heitkamp 1990) and Chrysene was metabolised by a *Rhodococcus* sp. strain which was found to be capable of utilising Chrysene as well as Pyrene as sole sources of carbon and energy (Walter *et al.* 1991).

With respect to the bacterial degradation of PAHs containing more than four rings, several studies have focused on the biodegradation of the five-ring BaP molecule because of its potential hazard to human health. Although it has been shown that biodegradation of BaP by pure and mixed cultures of bacteria occurs, all biotransformations by bacteria take place under co-metabolic conditions and never with BaP as a sole source of carbon and energy.

For example *Beijerinckia* sp. Strain B8/36, a mutant created by treating *Beijerinckia* sp. with N-methyl-N'-nitro-N-nitrosoguanidine, degraded BaP when grown on succinate plus biphenyl (Gibson *et al.* 1975) and one strain was shown to degrade BaP when grown on succinate plus salicylate (Barnsley 1975).

However, bacteria can use other hydrocarbons as a sole source of C and energy and were found to mineralise PAHs more rapidly than fungi (which do not utilise PAHs as the sole source of C and energy). A medium for fungi must therefore be supplemented with an additional C source (Cerniglia 1997) and high N concentrations can also stimulate the growth of other soil micro-organisms which can create a synergistic environment for the degradation of hazardous chemicals, together with the fungi (Illman 1993).

Cerniglia (1997) hypothesised that the production of hydroxylated intermediates by fungi will be important for the bioremediation of PAH-polluted sites if the initial ring oxidation reaction is the rate-limiting step for the bacterial degradation of PAHs, since it could accelerate the mineralisation of these compounds. Additionally, fungi produce metabolites with higher water solubility and enhanced chemical reactivity. This could enhance the mineralisation of these compounds by indigenous soil bacteria.

Boonchan *et al.* (2000) later investigated the biodegradation of high-molecular-weight PAHs in liquid media and soil by bacteria and the fungus *Penicillium janthinellum*. In agreement with Cerniglias hypothesis, they found significantly improved degradation rates and reduction in the mutagenicity of organic soil extracts, when co-inoculating PAH-contaminated soil with a fungal-bacterial co-culture.

Mineralisation of PAHs and other persistent pollutants is influenced by a range of biotic and abiotic factors. Not only have the pollutants to be available, but also the composting conditions must be optimal for degrader organisms. Those PAH-degrading organisms are either able to grow on PAHs by using them as their C source or organisms that are degrading PAHs co-metabolically or by unspecified oxidation metabolism using exoenzymes.

Examining the fate of 5 radio-labelled PAHs in five soils, Carmichael and Pfaender (1997) found that neither the characteristics of the soils (such as PAH concentration and particle size), nor of the PAHs (such as solubility) were usually correlated with the numbers of PAH-degrading micro-organisms within each soil. The absence of any significant correlation of community size or degrader numbers with bioavailability characteristics suggest that the degradation of some PAHs may be largely a co-metabolic process where the degraders are growing on C sources other than PAHs wherefore their numbers are not necessarily related to PAH availability.

2.6. ANALYSIS OF MICROBIAL CONSORTIA

Microbial communities can be analysed at the functional, phenotypic and genetic level. Advances in molecular microbiological tools for direct extraction of total community DNA and RNA from environmental samples and their analysis by methods like temperature or denaturing gradient gel electrophoresis (TGGE or DGGE) and terminal-restriction fragment length polymorphism (T-RFLP) have made studies of microbial communities much easier while it has been a time-consuming task to analyse entire microbial communities before. However, DNA and RNA based techniques have problems in reproducibility of the nucleic acid extraction and selectivity of the polymerase chain reaction (PCR) step (Loick 2004). Special problems arise when an unknown microbial community is to be analysed. Due to the nature of the genetic methods the total diversity of micro-organisms will be revealed and depending on the diversity of the microbial community identification of important groups can be time consuming and effects of environmental conditions might be overlooked in cases where communities do not respond with a quick change in their structure.

An alternative method to investigate microbial community responses to environmental conditions is by determination of phospholipid fatty acid (PLFA) profiles. Fatty acids are the key component of cellular membranes of all living cells and phospholipid fatty acids (PLFA) do not appear as storage lipids (figure 2.3). PLFAs consist of a single molecule of glycerol where two of the OH groups are bound to the two fatty acid chains and one to a phosphate group (Figure 2.4).

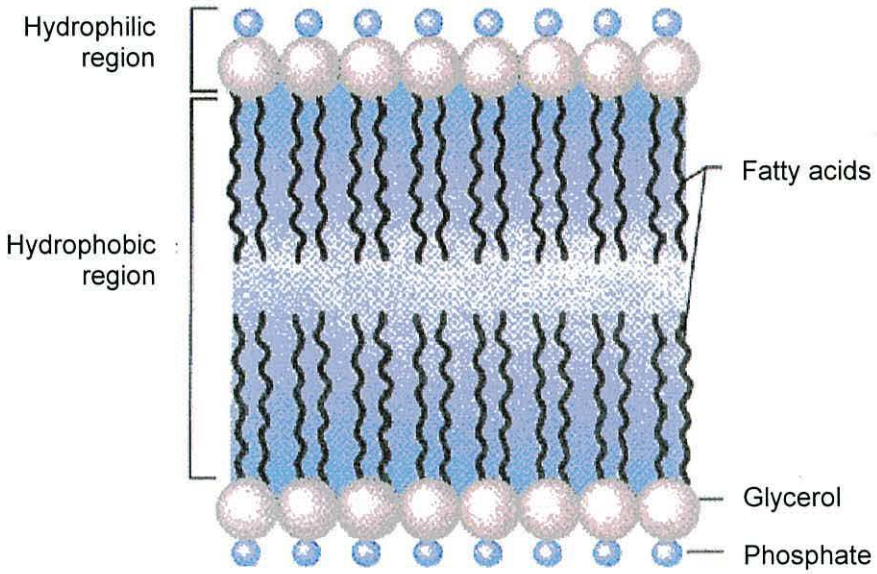

Figure 2.3: arrangements of phospholipids in membranes of living cells.

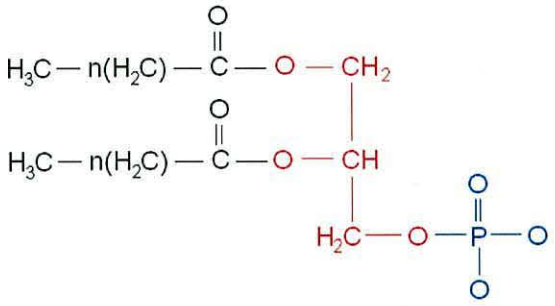

Figure 2.4: generalised structure of a PLFA with glycerol backbone (red), fatty acid chains (black), and phosphate (blue)

Phospholipids are subjected to a quick turn-over rate and are quickly degraded by phospholipases after cell death. They can be extracted qualitatively as well as quantitatively. Phospholipids are not only suitable

bio-markers for the quantification of living microbial biomass in sediments (White *et al.* 1979), composts (Hellmann *et al.* 1997), and soils (Zelles *et al.* 1995). Because of their taxonomically important phospholipid fatty acid which is attached to the glycerine frame, they can be used to determine microbial diversity in ecosystems (Tunlid and White 1992; Zelles *et al.* 1992; Zelles 1999).

Classification is achieved by separating PLFAs in functional groups (chain-structure, degree of saturation, and OH-substitution). Phylogenetic classification of micro-organisms in accordance with their "natural" relationships supports the assumption that fatty acid synthesis is similar in taxonomically related groups. Therefore PLFA profiles from non culturable micro-organisms can be related to their culturable relatives.

PLFA profiles have been proven useful to get fingerprints of complex microbial communities and to compare populations, however, for interpretation those biomarkers are drawn upon that have been determined from pure cultures. The problem arising is to prove that a single PLFA is solely present within a single genus or species. Branched fatty acids which are biomarkers for Gram-positive bacteria are also present in some anaerobic Gram-negative sulphate reducing bacteria and cyclopropyl fatty acids which are generally common in Gram-negative bacteria are also present in some anaerobic strains of Gram-positive bacteria (Kaur *et al.* 2005). For these reasons it is advisable to use several PLFAs combined to functional groups for biological interpretation and to take the environmental conditions (e.g. aerobic or anaerobic) into consideration.

Beside structural diversity, it is also possible to use PLFA biomarkers to describe the physiological status of microbial communities. Most environmental stresses affect micro-organisms in the way that they increase the fluidity of the cell membrane of the micro-organism. One adaptation mechanism to counteract and compensate for such stresses and effects is to alter the PLFA composition by isomerisation of *cis*- to *trans*-unsaturated PLFAs or from iso- to anteiso- branched PLFAs (Kieft *et al.* 1994; White 1994; Kaur *et al.* 2005).

An increase in those ratios in response to environmental stress conditions such as temperature, organic compound toxicity (e.g. toluene), starvation, osmotic stress, and pH has been shown (Guckert *et al.* 1986; Frostegard *et al.* 1993b; Heipieper *et al.* 1996; Gattinger 2000; Bååth and Anderson 2003).

2.7. CONCLUSIONS

The removal of PAHs by biodegradation has been investigated in several studies under a range of conditions and with different kinds of contaminated materials. Those changing conditions and different preconditions make it difficult to compare individual studies and determine optimal conditions (i.e. in some cases it works, in other cases it fails).

This is significantly hampering the adoption of the technology within industry. For example, it was shown that nutrient amendment and changes in pH and temperature had no effect on PAH degradation in a study by Thomas *et al.* (1989), whereas other studies found an increase in PAH degradation rates when nutrients were added, pH levels adjusted or temperatures changed (Wang and Bartha 1990; Wang *et al.* 1990; Antizar-Ladislao *et al.* 2005a, 2005b, 2006).

Investigation of the magnitude of PAH losses by volatilisation also give seemingly contradicting results with some studies showing that volatilisation represents a negligible PAH loss pathway (Kirchmann and Ewnetu 1998; Guerin 2000) whereas others show that volatilisation plays an important role in contaminant removal (Bossert and Bartha 1984; Cousins and Jones 1998).

Similarly when inoculating contaminated soil with organisms some studies have found no differences between inoculated and non-inoculated systems (Weissenfels *et al.* 1990a; McFarland *et al.* 1992; McFarland and Qiu 1995; Canet *et al.* 2001; Ahtiainen *et al.* 2002) whereas other studies found a positive effect on PAH degradation (ŠaŠek *et al.* 2003b).

The behaviour of PAHs in soil is guided by several processes. After introduction of the PAH to the soil, the contaminant can diffuse into pores or between particles, adsorb to particle surfaces, become entrapped within humic complexes or remain in the water-soluble fraction. A removal of PAHs from the soil occurs through leaching, volatilisation, or biodegradation by micro-organisms such as bacteria and fungi. Sorption, diffusion, and entrapment on the other hand might reduce the extractable PAH amounts, however, the components linger in the soil and their future behaviour remains uncertain. Figure 2.5 gives an overview of the processes governing PAH behaviour in soil.

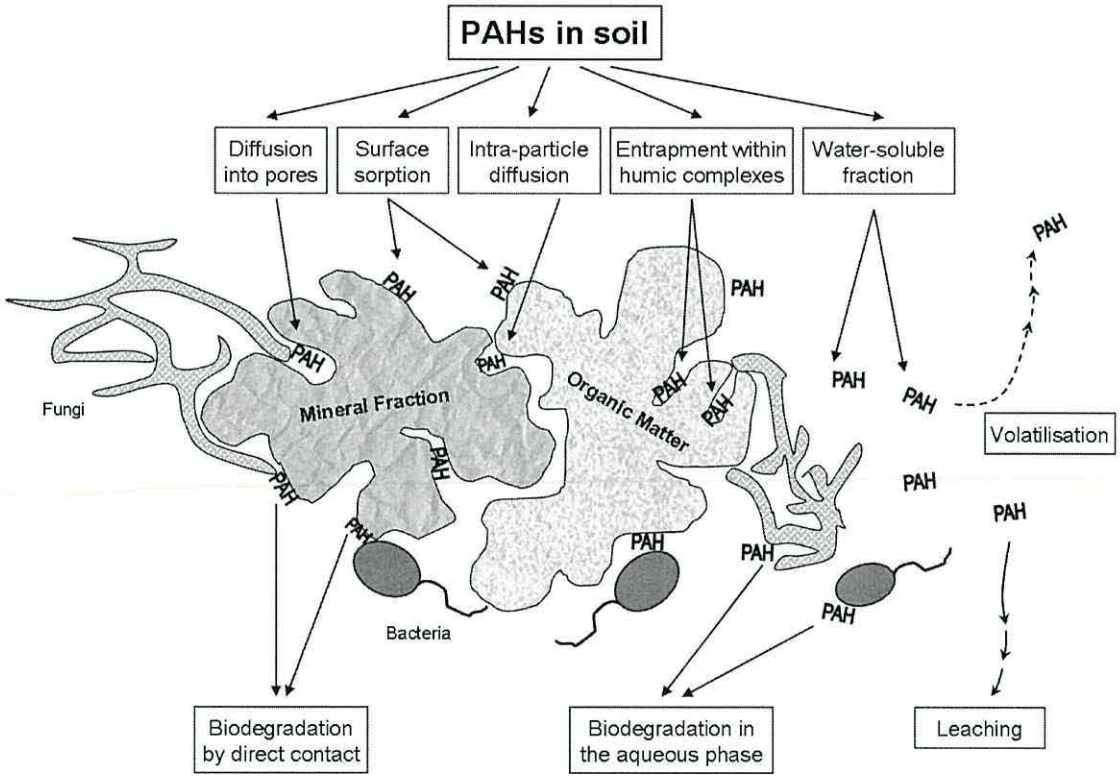

Figure 2.5: Summary of the processes governing PAH behaviour in soil. For explanation see text.

As degradation and mineralisation of PAHs depends on many interacting factors (Figure 2.6) it is important to describe the process completely and as detailed as possible. Emphasis should be laid on developing a standardised method to study the effects of different conditions by

assessing the given conditions and characteristics of the contaminated material, including co-contaminations and indigenous microbial communities. This should then lead to developing methods to systematically treat contamination.

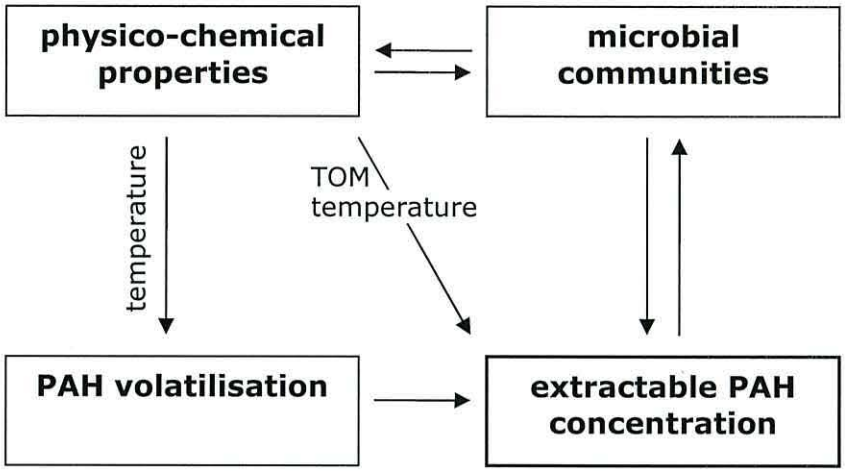

Figure 2.6: Schematic showing the main factors influencing a co-composting process of PAH contaminated soil. TOM = total organic matter

Efficient and cost effective bioremediation should include either complete mineralisation of the PAHs or at least biotransformation to less hazardous compounds. The use of genetically engineered organisms is hindered by many factors such as their poor competitive ability in soil and the costs of producing such organisms particularly on a large scale.

Typically, in comparison to bacteria, fungal rates of PAH degradation are slow and inefficient; however, the ecological role of fungi could be significant as they have the ability to hydroxylate a wide variety of PAHs, and these polar intermediates can be mineralised by soil bacteria or detoxified to innocuous compounds (In der Wiesche *et al.* 1996).

As most studies have investigated the use of bacteria OR fungi and only a few have looked at the combined use of organisms of both kingdoms, future studies should focus on harnessing the advantages of both bacteria and fungi by using or creating a synergistic environment for the degradation of hydrocarbons.

Complementary studies will be needed to obtain a better understanding on the interactions between bacteria and fungi and their contributions to PAH degradation. This will result in a better process-based understanding of the competitive and symbiotic relations of PAH degrading organisms and lead to the development of a PAH biodegradation method combining the positive effects of both microbiological kingdoms.

Whichever method is chosen or investigated, all factors of PAH degradation should be considered, especially the potential of the process to distribute PAHs to other places through leaching or volatilisation.

Chapter 3

Materials and Methods

Unless otherwise stated all chemicals used were of GC-grade purchased from VWR International Ltd, UK or Sigma-Aldrich Company Ltd, UK.

3.1. CONTAMINATED SOIL AND ADDITIVES

The PAH contaminated soil used in this study was excavated from a former refinery site. The site sits on Thames alluvium basically comprising silty clays deposited in estuarine conditions. The clays become slightly siltier at a few metres depth. A thin peaty band (ca. 30 cm thick) is evident across some of the site at between 1 and 4 m below ground level which is believed to imply a marine transition. The Thames Alluvium continues to 15 to 18 m below ground level where sands and gravels are encountered. Chalk is found below the sands and gravels.

The contaminated soil was excavated down to a depth of 6 m and placed in an area to bioremediate; i.e. the soil was formed into windrows, elongated stockpiles measuring about 100 m long, 5 m wide at the base and up to 1.5 m tall, with a triangular cross section. The soil was then turned once to twice a week to introduce oxygen to encourage bioremediation. The windrows were left open to the elements. Irrigation was implemented to increase the moisture content which became very low during the summer months.

The soil was kept in windrow piles for 2 months in which the total PAH concentration was reduced by about 70% leaving a still highly contaminated soil with total PAH concentrations ranging from 1800 to 2000 mg kg^{-1}. While the smaller PAHs have been degraded to a large extent during the windrow pile treatment, the larger and more difficult to access PAHs are still present in the soil, often at higher proportions than at the start.

It has been found that with increasing molecular size the electrochemical stability as well as the hydrophobicity of PAHs increases which contributes to an associated increase in their persistence (Cerniglia 1992), explaining a higher degradation of smaller sized PAHs during the initial windrow treatment. Additionally, it has been shown that bound residue formation was more extensive for hydrophobic pollutants such as the heavier PAHs (McFarland and Qiu 1995). Therefore grouping of single PAHs in low, medium, and high molecular weight (LMW, MMW, and HMW respectively) PAHs categories is a good and frequently-used method to describe contamination (see below).

3.2. EXPERIMENTAL SETUP

Stones were removed from the soil which was then air dried, passed through a 7 mm sieve, and homogenised. The soil was then mixed with sawdust as a bulking agent and additional carbon source, and different additives (see below and in Chapters 4 to 6).

The soil had a silty clay texture and clay content of 35 to 40%; further characteristics of the soil and the sawdust are given in Table 3.1, characteristics of the different additives are given in the corresponding Chapters (Chapters 4 to 6).

For the manure experiment, the different types of manure were chosen by their nutrient and organic matter content, as well as the type of animal and its diet and the different microbial community profile resulting from this (see Chapter 4).

As white rot fungi are expected to be the most effective fungi to bioremediate complex organic contaminants in soil, the two white rot fungi *Pleurotus ostreatus* (Oyster mushroom) and *Trametes versicolor* (Turkey-tail mushroom) as well as a combination of both were selected for the fungal experiment (see Chapter 5).

The third experiment aimed to investigate the combined effects of manure and fungal inoculum addition (see Chapter 6). The type of manure (chicken manure) and fungal additive (*P. ostreatus*) were chosen according to the results from the previous two experiments.

Table 3.1. Physico-chemical characteristics and microbial community profiles of the soil and sawdust used for the experiments. Values represent mean ±S.D. where applicable; n=3

	Soil	**Sawdust**
Physico-chemical characteristics		
Dry matter (%)	78.5	83.2
pH	8.18 ± 0.03	4.31 ± 0.02
Total C (% of dry matter)	13.5 ± 0.7	46.7 ± 1.8
Total N (% of dry matter)	0.36 ± 0.02	0.49 ± 0.07
C/N ratio	37.3 ± 3.3	95.7 ± 11.8
Electrical conductivity ($\mu S/cm^{-1}$)	214 ± 27	40 ± 4
Microbial community profile (PLFA)		
Total bacterial PLFA (nmol/g dry material)	7.66	2.63
Total fungal PLFA (nmol/g dry material)	0.15	1.49
Bacteria (% of total PLFAs)	41.77	25.68
Actinobacteria (% of total PLFAs)	9.91	2.83
Ratio of fungal-to-bacterial PLFAs	0.02	0.57
Ratio of Gram-negative-to-Gram-positive bacterial PLFAs	0.74	0.15
Ratio of iso-to-anteiso monounsaturated PLFAs	0.44	0.52

The moisture content of the soil mixtures were adjusted to 40 ± 4% on a dry weight basis. The mixtures were then separated into triplicates of 4.5 litres which were filled into 5 litre glass vessel with a surface area of 227 cm^2. The glass vessel had a grid at the bottom where a pipe ended to aerate the mixture (Figures 3.1 and 3.2).

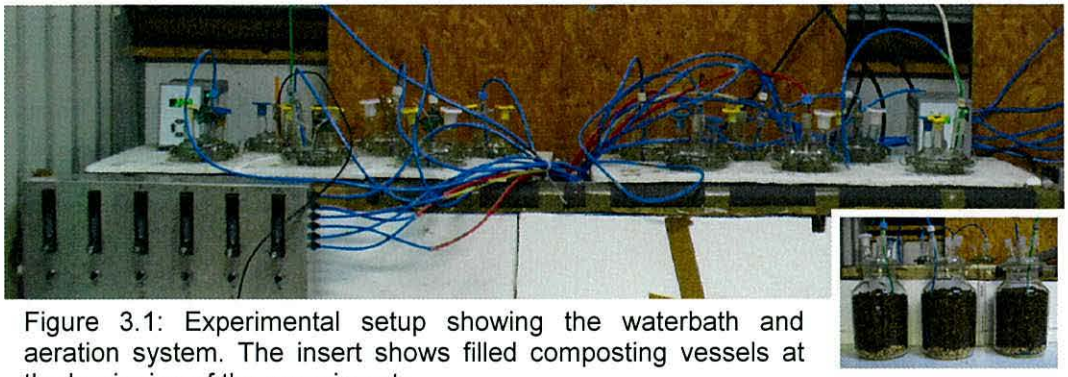

Figure 3.1: Experimental setup showing the waterbath and aeration system. The insert shows filled composting vessels at the beginning of the experiment.

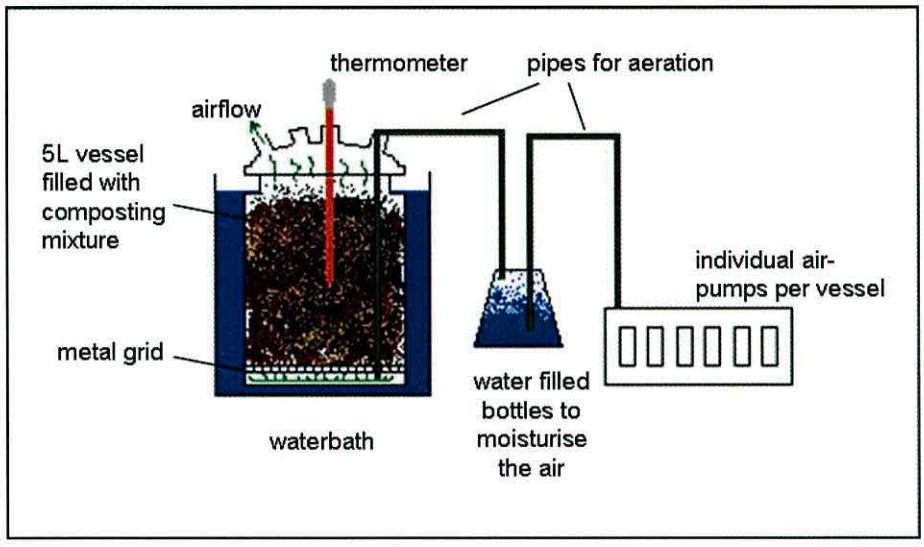

Figure 3.2.: Schematic representation of the experimental setup for one pilot-scale vessel. All experimental vessels were placed in the same waterbath but had individual aeration systems.

To investigate the effect of different temperatures on the degradation process, the glass vessels were placed into a water-bath. The temperature of the water-bath was changed weekly to reflect different temperatures that would occur during a composting process (see Chapters 4 to 6 for temperature profiles). To sustain aerobic conditions and maintain the moisture content of the soil-mixture, the soil-mixtures were forcefully aerated (200 ml vessel^{-1} min^{-1}) with air pre-humidified by being previously bubbled through water of the same temperature as the water-bath.

3.3. SAMPLING AND PRE-TREATMENT OF SAMPLES

To ensure a homogeneous mixture and a homogeneous sample, the soil-mixtures were thoroughly mixed prior to the removal of 200-250 g from each vessel at each sampling point at the end of each temperature step. Due to the nature of the mixture (high clay content) the sample taken needed to be dried to ensure the analysed sample was representative. Additionally moisture was found to inhibit PAH extraction (Berset *et al.* 1999; Belkessam *et al.* 2005) and the authors also tested different drying techniques and found that Naphthalene was lost to some extent during freeze-drying and no quantitative results could be obtained for this PAH. Similarly, Belkessam *et al.* (2005) reported that freeze-drying of clay rich soil induced volatilisation of light compounds. However, this study aimed to investigate PAH removal by micro-organisms; to ensure no biological degradation of the contaminants occurred after sampling, the samples were frozen to suppress microbial activity. Additionally, the soil used in this study had already lost larger amounts of the lighter PAH components during the pre-treatment and during this study emphasis was laid on the removal of larger components where the pre-treatment was less successful. The samples were therefore frozen and freeze-dried using an Edwards Super Modulyo freeze-dryer (BOC Ltd. Crawley, Sussex, UK) and then stored at -20°C until further analysis. Nonetheless, losses of lighter aromatic components during freeze-drying have to be considered.

3.4. ANALYSIS

3.4.1. Physico-chemical analysis

Physico-chemical characteristics influence the growth and survival conditions of micro-organisms as well as the behaviour of the contaminants (e.g. solubility and availability). Measurements of those characteristics can also be used to monitor the progress of the composting process.

The moisture content (H_2O %) was determined by using the difference in weight of the sample before and after freeze-drying by the following formula:

$$H_2O\ \% = w_d / w_f * 100 \qquad (E.3.1)$$

where w_d is the weight of the freeze-dried sample; w_f is the weight of the fresh sample.

To determine the total organic matter (TOM) and ash content of the samples around 3 g of the freeze-dried sample were weighed into crucibles and placed into a muffle furnace at 550 °C for 16 hours. The ash content (ash %) was calculated by the following formula:

$$\text{ash}\ \% = (w_t / w_0) * 100 \qquad (E.3.2)$$

where w_0 is the weight of the initial sample; w_t is the weight of the sample after ignition. TOM is the matter lost during ignition and is calculated as:

$$\text{TOM}\ \% = 100\ \% - \text{ash}\ \% \qquad (E.3.3)$$

The pH and conductivity were determined on aliquots of 1 g freeze-dried sample in 10 ml deionised water using a Jenway pH meter 3320 and a HI 8733 conductivity meter (Hanna Instruments, Italy), respectively.

The total C and N content were determined simultaneously by analysing the freeze-dried, ground, samples using an automated Dumas procedure on a Carlo Erba NA 1500 analyser (Erba Science UK).

CO_2 and NH_3 emissions were measured from the headspace of the 5-litre-vessels using Gas detection tubes (RAE Systems, San Jose, CA, USA) following the manufacturers instructions.

3.4.2. PAH extraction from soil

After each temperature step a homogenised, freeze-dried sample of between 7 and 9 g was used for PAH extraction. PAHs were extracted with acetone and petroleum ether followed by the removal of acetone by washing the extract with water as prescribed in ISO 18287 (International Standard 2006). D-Perylene was used as internal standard and results were corrected by referring to the amount of ash, to take account of the weight loss of the compost mixture during the composting process.

The ISO 18287 method "Soil quality – Determination of polycyclic aromatic hydrocarbons (PAH) – Gas chromatographic method with mass spectrometric detection (GC-MS)" was followed with slight modifications. In summary, 85 to 150 µg internal standard (Perylene-d12 dissolved in cyclohexane) was added to 7-9 g of the freeze-dried soil mixture. To extract PAHs, 25 ml acetone was added to the sample which was then shaken at 200 movements min^{-1} for 15 min. The sample was then centrifuged for a few seconds to allow the smaller soil particles to settle. The supernatant was then decanted into a separating funnel. The soil sample was again extracted with acetone, shaken, centrifuged, and the supernatant added to the first extract. 50 ml petroleum ether was added to the extracts and shaken. To remove the acetone and other polar components the extract was shaken twice with 200 ml distilled water and the acetone-water phase was removed. The organic layer was then dried over anhydrous sodium sulphate and the dried extract was transferred to a clean test vial. Following this, 100 µl isooctane was added as a keeper and the extracts were concentrated to 10-20 ml under a gentle stream of nitrogen. The extracts were sufficiently clean to be directly analysed by GC-MS (see below).

3.4.3. Extraction of metabolic breakdown products from PAH degradation

To investigate possible accumulation of PAH breakdown products after each temperature step a homogenised, freeze-dried sample of 2 g was used. A modified Bligh and Dyer extraction followed by a trimethylchlorosilane (TMCS) derivatisation and GC-MS analysis was used to extract and detect PAH breakdown products.

In the modified Bligh and Dyer extraction 1.6 ml phosphate buffer (8.7g K_2HPO_4 in 1 l ultra-pure H_2O, adjusted to pH 7.4 with HCl, and stored over chloroform), 2 ml chloroform, and 4 ml methanol were added to the sample. The sample was then vortexed and sonicated for 2 min and then left for 2 hrs with an occasional vortex. The sample was then centrifuged at 3000 rpm for 10 min before the liquid phases were decanted into a separating funnel. The whole procedure was repeated and the second extract added to the first extract. 4 ml ultra-pure H_2O and 4 ml chloroform were added to the combined extracts, shaken and left over night. The bottom layer of the separated phases was collected in a clean scintillation vial and evaporated to dryness under a stream of nitrogen using a hotplate (< 37°C). The dried extracts were then stored at -20°C until further processing.

The following derivatisation step relied on reaction of keto groups with methoxyamine to the oxime derivative and then subsequently trimethyl silylation of the analytes by *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine, which acts as a catalyst to increase the efficiency of end product formation (Allison 2003; Thermo Fisher Scientific Inc. 2008).

At the beginning of the derivatisation step methoxyamine was dissolved in pyridine to give a final concentration of 20 mg ml^{-1}. Next, 1 ml of the solution was added to the dried extracts and the samples were subsequently incubated at 90°C for 15 min. After incubation 300 µl freshly opened BSTFA (containing 1 % Trimethylchlorosilane (TMCS)) was added to the sample solution and the mixture was again incubated at 90°C for 15 min. While BSTFA is an effective trimethylsilyl donor, TMCS aids in

derivatising amides, many secondary amines and hindered hydroxyls that are not derivatised by BSTFA alone. After incubation 10 µl alpha-cholestane hydrochloride was added as an internal standard to the extracts which were then evaporated to dryness under nitrogen using a hot plate (< 37°C). The dried extracts were stored at -20°C until GC-MS analysis. Samples were transferred to GC-MS (see below) by re-dissolving the extract in 200 µl hexane.

Data were analysed statistically using correlation as well as multivariate analyses. Even though breakdown-products such as 9,10-anthraquinone and different carboxylic acids (e.g. 1,2-benzenedicarboxylic acid) were found, no accumulation of metabolites could be detected, neither were significant differences found between treatments or sampling points. With this extraction method it was tried to make extraction conditions relatively broad to include a wide range of metabolites. However, this also made those components that were present in smaller amounts difficult to detect in the large amounts of other components in the extract. Similar effects have been found by McElroy (1989) stating the difficulty to detect PAH metabolites in field samples. It can therefore not be guaranteed that undetected changes in some metabolite concentrations did not happen. As statistical analysis did not reveal any changes in metabolite concentrations results are not presented.

3.4.3.1. Analysis of extracted PAH data

In this study the removal of 16 PAHs classified as priority pollutants by the U.S. Environmental Protection Agency (EPA), namely Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benz[a]anthracene, Chrysene, Benz[b]fluoranthene, Benz[k]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-c,d]pyrene, Dibenz[a,h]anthracene, and Benzo[g,h,i]perylene, was to be investigated (see also Chapter 2 part 2.2.2). Benz[k]fluoranthene concentrations, however, were below the detection limit at all times. Therefore the removal of the remaining listed 15 PAHs was investigated.

Added organic matter is decomposed during the experiment. This results in loss of weight due to CO_2 and other gaseous emissions. This weight loss of material will cause a distortion of results when not considered during analysis (i.e. when expressing amounts on a per weight basis). To take the weight loss of the soil/sawdust/additive (manure and/or fungi) mix into account the measured PAHs were corrected by referring to the amount of ash which is considered the most chemically stable parameter during the degradation process (Amir *et al.* 2005).

The PAH contamination was very heterogeneously distributed in the soil matrix and even though best efforts were made to thoroughly mix the soil, a homogeneous distribution of the contaminants could not be guaranteed when handling larger amounts. To account for initial differences in concentrations between vessels, PAH concentrations were compared by referring to the changes in the percentage remaining in the vessels relative to the start as follows:

$$\text{percentage remaining} = (C_t/C_0) \times 100 \qquad \text{(E.3.4)}$$

where C_t is the concentration of the PAH at sampling time t and C_0 is the concentration of the PAH at the beginning of the experiment.

As one of the main factors controlling biodegradation, microbial activity brings about changes in temperature which are one of the main characteristics of a composting process. Those temperature changes subsequently result in a change in the microbial community structure. Temperature also has an influence on PAH availability making PAHs more available with increasing temperatures (Pignatello and Xing 1996). This can result in a temporal increase in their amounts at higher temperatures followed by a decrease when the now available compounds can be degraded, or re-bound to the soil.

During the experiments, different temperatures were applied during each time period. Possible changes in degradation rates during single temperature steps can give an insight into the effects of the implied temperature on the PAH degradation behaviour. Degradation rates (degR) were calculated as:

$$degR = 1- (C_t/C_{t-1}) \quad (E.3.5)$$

with C_t being the concentration of the measured PAH at the end of the temperature(time) period and C_{t-1} being the concentration of the PAH at the beginning of the same period. Positive degR values indicate a decrease in PAH concentration, while negative values indicate an increase.

The three different methods by which PAH results have been looked at are referred to as follows:

- "percentage change": PAH amounts at each sampling point in % relative to that measured at the start of the experiment – this takes initial differences in PAH concentrations into account

- "rates of degradation": Factor by which the extractable PAH concentration decreased during the given sampling (temperature) period – this reflects possible influences of temperature changes.

- "concentration": PAH amount (in µg) per g ash – this reveals possible effects on the actual concentrations of PAHs as well as effects of PAH concentrations on PAH removal

The chemical properties of PAHs depend in part upon their molecular size as well as molecule topology, where an increase in size and angularity generally results in an associated increase in hydrophobicity and electrochemical stability which contributes to their persistence (Cerniglia 1992; Kanaly and Harayama 2000). Additionally, it has been shown that bound residue formation was more extensive for the more hydrophobic pollutants, such as the heavier PAHs (McFarland and Qiu 1995). For a more general analysis of changes in PAH contamination, PAHs have been grouped into three groups according to their molecular weight:

- low molecular weight (LMW) PAHs (2- and 3- ring PAHs: Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene).
- medium molecular weight (MMW) PAHs (4-ring PAHs: Fluoranthene, Pyrene, Benz[a]anthracene, Chrysene).
- high molecular weight (HMW) PAHs (5- and 6-ring PAHs: Benz[b]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-c,d]pyrene, Dibenz[a,h]anthracene, Benzo[g,h,i]perylene).

3.4.4. PAH volatilisation

At each sampling point, volatilisation of PAHs was measured before the soil was homogenised by pulling 500 ml of air from the headspace through a TENAX thermo desorption tube (Markes Int.) and subsequent GC-MS analysis (see below).

3.4.5. Microbial community analysis

A modified one phase extraction was used for phospholipid fatty acid (PLFA) extraction (Steinberger *et al.* 1999). After each temperature step a homogenised, freeze-dried sample of between 1 and 1.5 g was used.

In a modified Bligh and Dyer extraction, 1.6 ml phosphate buffer (8.7g K_2HPO_4 in 1 L ultra-pure H_2O, adjusted to pH 7.4 with HCl, and stored over chloroform), 2 ml chloroform, and 4 ml methanol were added to the sample. The sample was then vortexed and sonicated for 2 min and then left for 2 hrs with an occasional vortex. The sample was then centrifuged at 3000 rpm for 10 min before the liquid phases were decanted into a separating funnel. The whole procedure was repeated and the second extract added to the first extract. 4 ml ultra-pure H_2O and 4 ml chloroform were added to the combined extracts, shaken and left over night. The bottom layer of the separated phases was collected in a clean scintillation vial and evaporated to dryness under a stream of nitrogen using a hotplate (< 37°C). The dried extracts were then stored at -20°C until further processing.

In a second step, the material was fractionated into neutral lipids, glycolipids and phospho-(polar)-lipids on a silica-bonded phase column (Supelclean™ LC-Si SPE, Sigma-Aldrich) by consecutive elution with chloroform and acetone. In detail, the dried lipid extract was re-dissolved in 2.5 ml chloroform and added on top of the silica-bonded phase column. To ensure that the whole dry lipid was transferred onto the column another 2.5 ml of chloroform were added to the lipid-extract-tube and transferred onto the column. To elute the glycolipids the column was washed three-times with 2.5 ml acetone. The phospholipids were then washed from the column by adding 5 ml methanol which was collected in a clean test-tube and evaporated to dryness under a stream of nitrogen using a hotplate (< 37°C). The dried extracts were stored at -20°C until further processing.

The dried phospholipids were methylated by adding 1 ml toluene:dry-methanol (1:1) and 1 ml methanolic KOH (280 mg KOH in 25 ml dry-

methanol) to the phospholipid extract, vortexing and placing in an oven at 37°C for 30 min. After cooling to room temperature, 2 ml hexane:chloroform (4:1 v/v) and 2 ml ultra-pure H_2O were added. 1 M acetic acid was added to adjust the pH of the lower aqueous phase to pH 6-7. The extract was vortexed for 30 s and then centrifuged at 3000 rpm for 5 min. The top layer was transferred to a clean centrifuge tube and another 2 ml hexane:chloroform (4:1 v/v) were added to the former tube. After vortexing again for 30 s followed by centrifugation at 3000 rpm for 5 min the top layer was combined with the first and after addition of 15 µg nonadecanoic acid methyl ester (dissolved in hexane) the extract was evaporated to dryness under nitrogen using a hotplate (< 37°C). The dried extracts were stored at -20°C until GC-MS analysis. Samples were transferred to GC-MS (see below) by redissolving the extract in 250 µl hexane.

3.4.5.1. Groupings of PLFAs

Individual PLFA markers were used to quantify the total and relative abundance of specific cell types through the different composting treatments and stages. PLFAs were designated using the nomenclature described in Frostegard *et al.* (1993). PLFAs are designated by the number of C-atoms followed by the degree of unsaturation, expressed by the number after the colon. For unsaturated PLFAs, the degree for unsaturation is followed by wx, where x indicates the position of the double bond nearest to the aliphatic end w. The prefixes i, a, and cy refer to iso, anteiso, and cyclopropyl branching respectively. A number followed by Me indicates the position of a methyl group and c at the end indicate a *cis* structure at the unsaturated position.

Total bacteria were identified by the PLFAs: 15:0, 17:0, i14:0, i15:0, a15:0, i17:0, a17:0, 10Me16:0, 10Me17:0, 10Me18:0, cy17:0, cy19:0 (Bååth *et al.* 1992; Zelles 1996; Cooper *et al.* 2002; Fierer *et al.* 2003; Grayston *et al.* 2004) and fungi with the PLFA 18:2w6c (Frostegard *et al.* 1993; Zelles

1996). The sum of bacteria and fungi indicating PLFA was used to identify microbial PLFA.

Bacterial PLFAs were further grouped into PLFAs indicative for gram-positive bacteria (i14:0, i15:0, a15:0, i17:0, a17:0, 10Me16:0, 10Me17:0, 10Me18:0)(Fierer *et al.* 2003; Grayston *et al.* 2004), for Actinobacteria (10Me16:0, 10Me17:0, 10Me18:0)(Cooper *et al.* 2002; Fierer *et al.* 2003), and gram-negative bacteria (cy17:0, cy19:0)(Fierer *et al.* 2003; Grayston *et al.* 2004).

Bacteria are known to alter their membrane lipids in response to stress (White 1994; Kieft *et al.* 1997) and increases in the ratio of iso- to anteiso branched PLFAs typically show increases in stress levels (Kaneda 1991; Kieft *et al.* 1994; McKinley *et al.* 2005). The ratio of iso- to anteiso branched PLFAs was calculated as the sum of i15:0 and i17:0 divided by the sum of a15:0 and a17:0.

3.4.6. GC-MS analysis

GC-MS analysis was performed with a Agilent Technologies gas chromatograph 6890N Network GC System equipped with a mass detector Agilent Technologies 5973 Network Mass Selective Detector and a data analysis station MSD ChemStation D.01.02.16. Details of GC-MS settings are given in Table 3.2.

Columns were purchased from Agilent Technologies UK Limited (Stockport, Cheshire). The electronic pressure control system was set to adjust the pressure according to the heat resistance of the column oven and the mass spectrometer was auto-tuned every day for maximum sensitivity.

For the analysis of volatilised PAH from the soil mixtures a Unity thermal desorber (Markes Int. Ltd.) was joined up with the GC-MS system. PAH compounds were pre-concentrated from a 500 ml air sample from the headspace of the reaction vessel by adsorption onto a Tenax TA, carbotrap

B, and molecular sieve adsorbents in series in a silico-sulphure inert tube. The sample was then further concentrated onto a second adsorption surface containing a Peltier cold trap device in a thermal desorption instrument (Unity from Markes Int. Ltd., Cardiff, Wales) before being thermally desorbed into the GC-MS system for separation, identification and quantification.

The Unity thermal desorption unit was used to desorb PAH headspace samples from the adsorbents and was initially set at room temperature and heated to 325°C for 5 min. The secondary cold trap was set at 5°C and heated at 16°C s^{-1} until a temperature of 325°C was obtained and held for 3 min. The latter stage was used to introduce the sample onto the GC column. The mass spectrometer was auto-tuned every day for maximum sensitivity and scanned from 35 to 250 mass units every 0.2 s to give responses in the ng range. Chromatographic retention time from the column and mass spectral matching were used to confirm the identity of the volatiles. Quantification was performed by desorbing a standard PAH mixture (PAH Calibration Mix, Supelco/Sigma-Aldrich, UK) from the same adsorbent.

Table 3.2: GC-MS settings for the different PAH and PLFA analyses.

Analysis	PAH extracts from soil	PAH from gas samples	PLFA extracts from soil	Metabolite extraction from soil
Column type	30 m HP-5 MS type (5%-Phenyl-methyl-polysiloxane; 0.25 mm internal diameter, 0.25 µm film thickness)	30 m HP-5 MS type (cross linked 5%-Phenyl-methylpolysiloxane; 0.25 mm internal diameter, 0.25 µm film thickness)	30 m DB-225 type (methylpolysiloxane; 0.25 mm internal diameter, 0.25 µm film thickness)	25 m HP-1 type (methylpolysiloxane; 0.20 mm internal diameter, 0.11 µm film thickness)
Carrier gas and flow rate	high grade helium (99.9999%) at 1 ml m^{-1}	high grade helium (99.9999%) at 1 ml m^{-1}	high grade helium (99.9999%) at 1 ml m^{-1}	high grade helium (99.9999%) at 0.6 ml m^{-1}
Split ratio	5.0 with a flow of 6 ml min^{-1}	splitless	5.0 with a flow of 6 ml min^{-1}	100 with a flow of 60 ml min^{-1}
Electronic pressure control	1 ml min^{-1}	1 ml min^{-1}	1 ml min^{-1}	0.6 ml min^{-1}
Injector temperature	280°C	280°C	280°C	280°C
Injector purge flow	1 ml min^{-1}	1 ml min^{-1}	1 ml min^{-1}	1 ml min^{-1}
GC-MS oven temp.-profile	80°C for 1 min; increase at a rate of 15°C min^{-1} up to 320°C; hold for 10 min	80°C for 1 min; increase at a rate of 15°C min^{-1} up to 320°C; hold for 10 min	80°C for 1 min; increase at a rate of 15°C min^{-1} up to 180°C; increase at a rate of 3°C min^{-1} up to 215°C; hold for 1 min	80°C for 1 min; increase at a rate of 20°C min^{-1} up to 150°C; increase at a rate of 5°C min^{-1} up to 250°C; hold for 1 min
GC-MS interface temp.	280°C	280°C	280°C	280°C

3.4.7. Statistical analysis

Statistical Analysis was performed using SAS 9.1 with Enterprise Guide 3.0 and Genstat© 10th Edition (Lawes Agricultural Trust: Rothamsted Experimental Station). The effects of the independent variables, namely sampling temperature (time) and treatment on PAH concentrations in soil and gas samples, degradation rates, PLFAs and physico-chemical characteristics were analysed using one- and two-way Analysis of Variance (ANOVA). To investigate differences between treatments within one temperature period and differences between temperature periods within one treatment, one-way ANOVAs with data grouped by treatment or temperature (time)-period were performed. A two-way (split plot) ANOVA was performed to investigate possible interactions of temperature (time) and treatment and to assess the effects of the additives used. If required, dependent variables were normalised prior to analysis using $\log_{10}$-, square root-, or reciprocal transformation. Spearman's rank correlation was carried out to look at possible relationships between results of different analyses.

Diagnostic Biplot analysis on the basis of principal component analysis was performed using Genstat© 10th Edition to investigate associations of treatments and PLFA profiles. Principal component analysis was used to create a new set of variables based on linear functions of the original variables, whilst retaining as much information about the original variation as possible. A biplot graphing procedure based on matrix outputs from principal component analysis was used to visualise the results.

CHAPTER 4

Comparison of the effects of different types of manure at different temperatures on the bioremediation of PAH contaminated soil

4.1. ABSTRACT

This Chapter investigates the potential of co-composting animal waste and aged PAH-contaminated soil as a mechanism to stimulate PAH decontamination. The influence of animal waste addition (horse, cattle or chicken manure), incubation temperature, and composting time on the loss of PAHs biotically (biodegradation) or abiotically (volatilisation) from soil were determined using gas chromatography-mass spectrometry (GC-MS). Simultaneous changes in microbial community structure were examined using phospholipid fatty acid (PLFA) profiling. Results revealed that PAH availability increased with temperature and that PAH loss through volatilisation was minimal in comparison to other removal processes. PLFA analysis revealed influences of manure type and temperature on microbial community development during composting and suggested that PAHs were degraded co-metabolically with degradation rates dependent upon PAH molecular weight and structure. No accumulation of PAH metabolites was detected. Whilst the addition of cattle manure was most effective at stimulating the degradation of low and medium molecular weight (LMW and MMW) PAHs (degradation of 75 and 62% respectively), the addition of chicken manure was the only treatment to induce a loss of high molecular weight (HMW) PAHs (17%). It was concluded that co-composting PAH contaminated soil with chicken manure can provide an easy-to-implement and economic strategy for improving soil quality and removing additional HMW PAHs from an aged contaminated soil where simple oxygen introduction did not show any further success.

4.2. INTRODUCTION

Co-composting contaminated soil requires an additive that supplies organic matter which can be degraded by micro-organisms initiating the composting process. To perform co-composting processes at a large scale those additives should be readily-available.

Manures are materials that are readily available, rich in organic matter and nutrients, and are an abundant natural source of micro-organisms. Competition with soil organisms has been found to be a major factor in the reduction of the bacterial populations introduced to soils (Acea *et al.* 1988; Soda *et al.* 1998). Micro-organisms able to degrade the present feedstock are essential for a successful co-composting process and manure adds micro-organisms within their natural habitat. This decreases the risk of competition and vulnerability towards predators and therefore increases the viability of the introduced organisms. At the same time, the manure supplies nutrients to the indigenous microbial community which can improve their growth conditions.

Manure contains mineral nutrients (e.g. ammonium, phosphate, potassium, sodium, magnesium, calcium, zinc, and copper), organic compounds including endocrine disrupting substances and antibiotics, and complex breakdown products resulting from digestion of plant material and by-products of animal metabolism which all influence the microbial composition of the manure as well as the soil micro-organisms (Unc and Goss 2004).

Characteristics of manures depend on the type of animal and their diet. Digestion of plant material increases as the passage time through the animal body (from intake to excretion) increases. The passage time increases with the length of the digestive tract and most animals depend on the large intestine (colon) and its appendages for fermentation. In some animals, the capacity for fermentation is increased by the cecum - an appendage of the colon located near the junction of the small and large intestines.

The horse has a very large and well developed cecum, which in combination with a long passage time allows it to survive solely on forages. In poultry, cellulosic fibres are ground by the grizzard, a specialised stomach constructed of thick, muscular walls. Grinding increases the surface area and the subsequent rate of digestion of the fibres in a small cecum and the rectum. Nutrient-rich cecal pellets are re-ingested for a second passage. In ruminants such as cattle, sheep, and goat the rumen - a multi-chamber stomach, serves as the principal site for fermentation. This organ can retain fibre for continued attack while screening and passing digested materials to the intestine. Ruminants can digest most cellulosic materials. Therefore, cattle manure is much lower in fibre and protein while horse dung contains a considerable amount of undigested cell wall material and associated protein (MacLean *et al.* 1983; Loomis and Connor 1992).

Even though micro-organisms in the digestive tract of animals are digested themselves (Pounden *et al.* 1950) and survival conditions for enteric bacteria are considered unfavourable once they have left the animal organism, some can leave the animal with the manure and survive for extended periods (Fenlon *et al.* 2000). The survival-rate of bacteria, however, depends on the source, microbial species and manure application method, as well as the nutrient content of the manure (Unc and Goss 2004).

Previous studies have looked at the usage of manure as a co-composting agent (Kirchmann and Ewnetu 1998; Wellman *et al.* 2001; Wong *et al.* 2002; Atagana 2004). However, little research has been performed to directly compare effects of different manure types and due to different conditions in experimental setups, soil types, and co-contaminations it is very difficult to compare those effects between different studies. The manure types used in this study were chosen to cover a broad range of possible manure properties and characteristics. It was expected that the physico-chemical characteristics as well as the microbial composition of the manures chosen showed the highest possible differences between readily available manures.

The aim of this part of the thesis is:

> To identify the effect of manures with different characteristics on the removal of PAHs from aged contaminated soil.
> (Thesis aim 1)

and thereby hypothesising that:

> Successfulness of a co-composting process to remove PAHs from an aged contaminated soil depends on the manure-type.

4.3. MATERIALS AND METHODS

4.3.1. Additives

Manure from three types of animals with different digestive systems resulting in manures with contrasting organic matter and nutrient content, as well as different microbial community profiles, were selected for the trials, namely horse-, cattle- and chicken-derived manure (Table 4.1).

4.3.2. Treatments

The aged PAH contaminated soil (see Chapter 3 "Materials and Methods") was mixed with sawdust as a bulking agent and manure was added to give a soil:sawdust:manure ratio of 3:1:2 (v/v/v) for the manure treatments. A mixture of aged PAH contaminated soil with sawdust in a ratio 1:1 (v/v) was used as the temperature-control treatment (TC).

After manure addition, the moisture content of the treatment mixtures was adjusted to 40 ± 4% on a dry weight basis by the addition of tap-water. The characteristics of the soil and the sawdust as well as the methods used are described in Chapter 3 "Materials and Methods".

The temperature profile was changed weekly as follows: days 0-4 20°C (room temperature), days 4-11 35°C, days 11-18 50°C, days 18-25 65°C, followed by a period at a lower temperature from day 25-32 at 35°C representing the heating and cooling/maturation phase of a composting process.

The treatments were as follows:

- horse manure treatment : representing co-composting with manure from a herbivore
- cattle manure treatment : representing co-composting with manure from a ruminant
- chicken manure treatment : representing co-composting with manure from poultry
- TC : temperature treated control treatment

Table 4.1. Physico-chemical characteristics and microbial community profiles of the manure types used for the experiment (± S.D.) if applicable.

	Horse manure	Cattle manure	Chicken manure
physico-chemical characteristics			
Dry matter (%)	20.6	16.6	54.1
pH	6.10 ± 0.11	8.30 ± 0.08	7.20 ± 0.07
Total C (% of dry matter)	44.3 ± 0.3	41.5 ± 0.7	44.9 ± 2.8
Total N (% of dry matter)	2.12 ± 0.10	3.04 ± 0.18	3.50 ± 0.17
C:N ratio	20.9 ± 0.9	13.7 ± 0.6	17.5 ± 1.0
Electrical conductivity (µS cm^{-1})	606 ± 17	1343 ± 92	874 ± 49
Microbial community profile (PLFA)			
Total bacterial PLFA (nmol g^{-1} dry sample)	8874	604	195
Total bacterial PLFA added to the mixture (nmol g^{-1} soil)	440.3	42.5	20.5
Total fungal PLFA (nmol g^{-1} dry sample)	433.8	25.7	31.2
Total fungal PLFA added to the mixture (nmol g^{-1} soil)	21.5	1.81	3.28
Bacteria (% of total PLFAs)	40.8	40.8	43.1
Actinobacteria (% of total PLFAs)	1.24	6.24	6.84
Ratio of fungal-to-bacterial PLFAs	0.05	0.04	0.16
Ratio of Gram-negative-to-Gram-positive bacterial PLFAs	0.04	0.18	0.19
Ratio of iso-to-anteiso monounsaturated PLFAs	0.83	4.25	0.45

4.4. RESULTS AND DISCUSSION

4.4.1. Physico-chemical analysis

4.4.1.1. Total organic matter content

The TOM content was significantly higher in the chicken manure treatment until day 4 ($p < 0.0001$). Even though TOM contents were higher with the chicken manure treatment no significant differences could be detected between the treatments after the initial phase of the experiment (Figure 4.1). Surprisingly the TOM content in the TC treatment was higher than in the horse and cattle manure treatments. However, differences were not significant and are probably attributable to the addition of larger amounts of sawdust to the contaminated soil for the TC treatment. The decline of TOM in the treatments results from decomposition of organic matter and is an indication for the progression of the composting process. Differences in changes in TOM were similar in all treatments.

4.4.1.2. pH

The pH values range from 7.99 (chicken manure treatment, day 32) to 8.68 (TC treatment, day 25) (Figure 4.1). Values are generally increasing for the horse manure and TC treatment. With the cattle- and chicken manure treatment the pH increased until day 11 followed by a decrease until the end of the experiment which is significant in the chicken manure treatment ($p < 0.0005$). Compared to the other treatments the chicken manure treatment had a significantly lower pH on days 0 (20°C), 25 (65°C), and 32 (35°C) ($p < 0.0001$; $p < 0.005$; $p < 0.001$ respectively).

In a composting process a decrease in pH is usually detected when organic acids are formed during the initial stages of the composting process (Williams 1996). This behaviour was only detected for the horse manure treatment. The increase with the other treatments could be from high metabolic activities possibly resulting in the production of intermediate metabolites in the compost systems. The subsequent decreases may be attributed to manure and hydrocarbon degradation which may have resulted in the release of acidic intermediate and final products that can lower the pH (Atagana 2008). However, no accumulation of intermediate or final breakdown products was found.

4.4.1.3. Total N

The percentage of total N in the compost mixtures ranged from 0.46% (TC) to 1.12% (chicken manure treatment) (Figure 4.1). The manure amended treatments had higher N contents than the TC treatment with the chicken manure treatment showing the highest percentage of total N over the whole course of the experiment.

The changes in total N contents were similar for the cattle-, chicken manure and TC treatments where highest percentages were measured at day 25 and lower percentages at day 11. The differences between total N concentrations between days 11 and 25 were significant in the cattle manure ($p < 0.05$) and TC treatment ($p < 0.01$). The horse manure treatment showed a slightly different temporal change in total N contents with highest amounts measured at day 18 and decreasing during the highest temperature step (65°C from days 18 to 25).

4.4.1.4. Total C

The total C content of the treatments did not show any significant differences between the sampling points within each treatment, nor between the treatments at each sampling point (Figure 4.1).

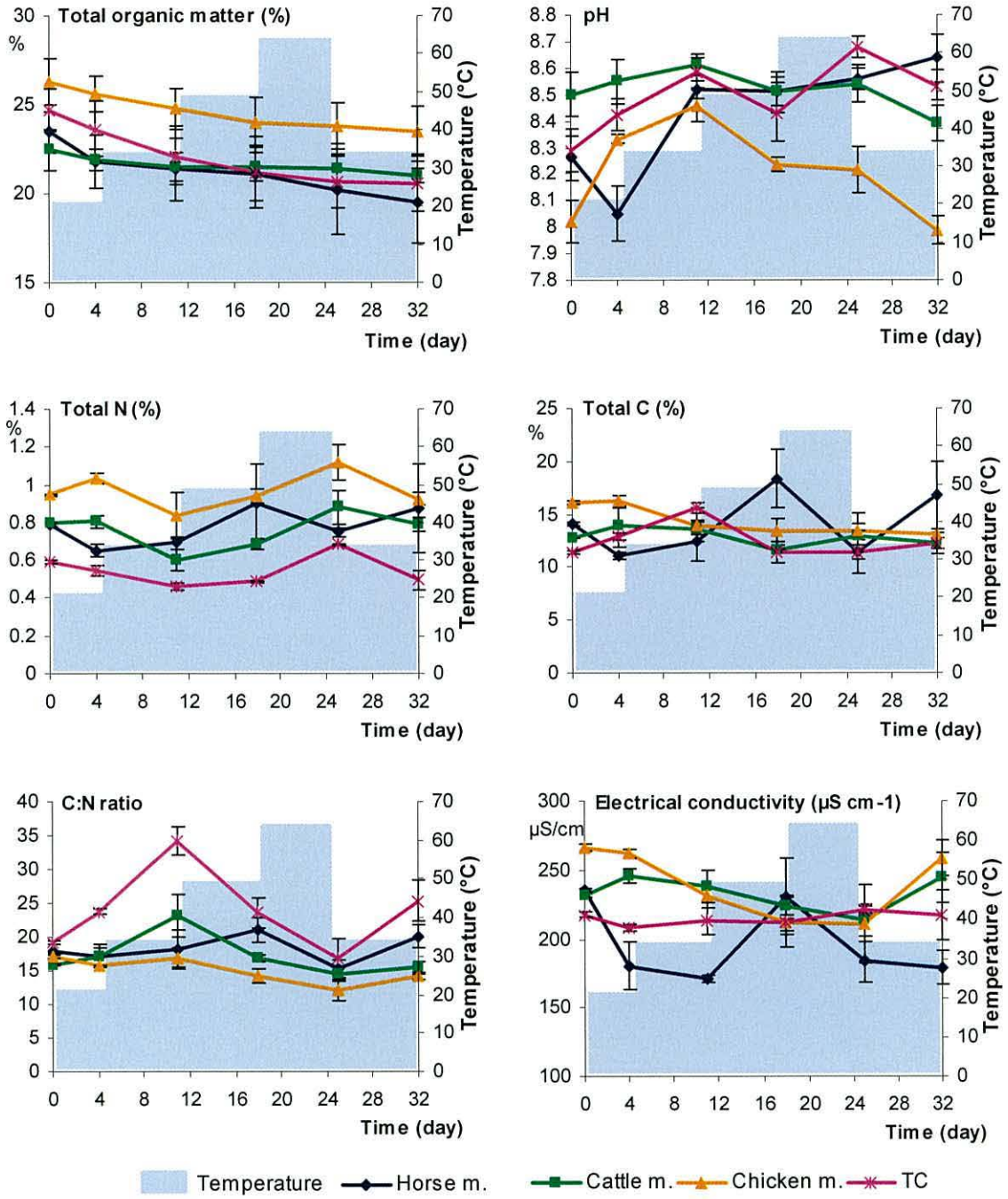

Figure 4.1: Mean values of different physico-chemical analyses with the different treatments during the different stages of the 32 day experiment. Treatments are labelled as Horse manure = blue, Cattle manure = green, Chicken manure = yellow, TC = pink. The light blue background represents the applied temperature profile. Error bars represent mean ±S.E.; n=3

4.4.1.5. Carbon to Nitrogen Ratio

The C:N ratio was highest for the TC treatment during the whole course of the experiment (Figure 4.1). This was expected as no manure was added. The C:N ratios were lowest for the chicken manure treatment except at day 0 where the cattle manure treatment had the lowest C:N ratio. With no significant changes in the total C content the C:N ratio was mainly influenced by the changes in total N.

The cattle-, chicken manure and TC treatment show a similar behaviour over the experiment with rates increasing until day 11 and decreasing to significantly lower rates by day 25 ($p < 0.05$). Rates do not differ significantly between the different sampling points for the horse manure treatment.

C:N ratios between 25:1 and 35:1 have been recommended for effective compost bioremediation (Anderson 1991; Kubota and Nakasaki 1991). Except for the TC treatment at day 11 ratios were well below those recommended ratios. However, similar ratios were found by Atagana (2008) during composting of hydrocarbon contaminated soil with sewage sludge. Even though in his study ratios were slightly higher in the beginning (23:1), they had decreased to 15:1 after 6 months. Previous studies have indicated that in composts with low C:N ratios (11:1) the microbial biomass and respiration are higher and cause faster degradation of cellulose and hemicellulose in comparison to composts with high initial C:N ratios (54:1) (Eiland *et al.* 2001).

4.4.1.6. Electrical conductivity

Measurements of electrical conductivity (EC) (Figure 4.8) showed significantly higher ($p < 0.05$) amounts of soluble ions in the cattle and chicken manure treatments than in the other treatments until day 11. Amounts of soluble ions decreased in the cattle- and chicken manure

treatments during the heating phase followed by an increase during the last temperature period (35°C days 25 to 32). No differences in EC values were found in the TC treatment. The horse manure treatment on the other hand showed generally decreasing EC values with only an increase during the 50°C temperature period (day 11 to 18). Due to a large variability between the replicate vessels differences in soluble ion concentrations between sampling points were not significantly different within the same treatments.

4.4.1.7. Ammonia emissions

As found for total N, ammonia emissions were significantly higher for the chicken manure treatment during the whole experiment ($p < 0.05$) (Figure 4.2). Chicken manure contains a higher proportion of inorganic nitrogen (Cogger 2005), which may explain the over 10-fold higher NH_3 emissions. The other treatments all had extremely low rates of NH_3 emissions and between them showed no significant treatment effect.

NH_3 emissions show an influence of temperature with significantly higher emissions at day 25 (65°C) for all but the TC treatment ($p < 0.01$) where no NH_3 emissions were detectable at any time.

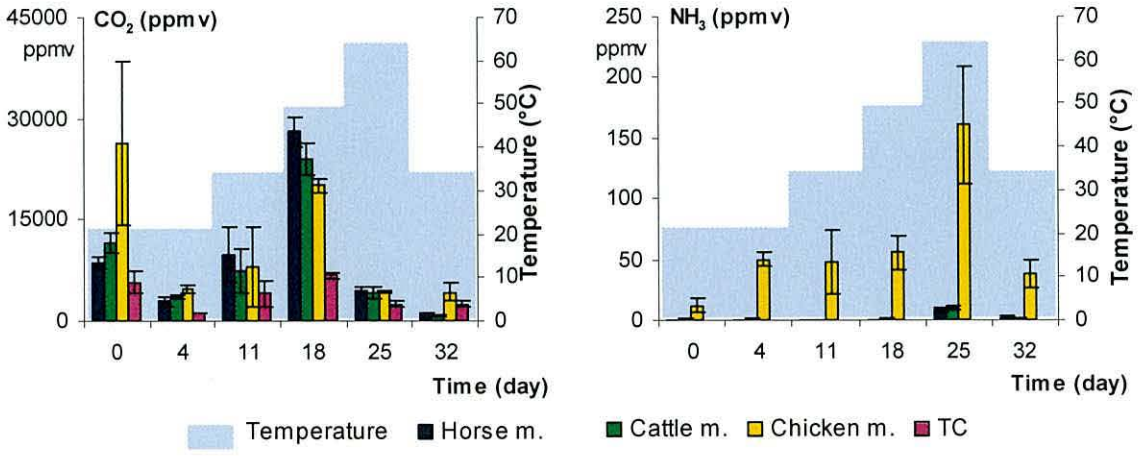

Figure 4.2: Mean values of CO_2 and NH_3 analyses with the different treatments during the different stages of the 32 day experiment. Treatments are labelled as Horse manure = blue, Cattle manure = green, Chicken manure = yellow, TC = pink. The light blue background represents the applied temperature profile. Error bars represent mean ±S.E.; n=3

4.4.1.8. Carbon dioxide emissions

CO_2 emissions decreased within the first 4 days of the experiment for all treatments (Figure 4.2). This initial decrease is followed by a 5- to 9-fold increase until day 18 (50°C) where CO_2 emissions were highest. By day 25, CO_2 levels are similar to those measured at day 4. With amounts ranging from about 1200 to 6700 ppm volume CO_2, emissions showed the least variation for the TC treatment. Except for day 32 the TC treatment also showed the lowest CO_2 emissions. CO_2 evolution has been used to estimate microbial activity (Alef 1995; Atagana 2008). Up to 4 times higher CO_2 concentrations were measured for the manure treatments indicating a higher microbial activity, however, no correlation of CO_2 emissions with microbial PLFA analysis results could be found.

4.4.2. Microbial community changes

4.4.2.1. Total microbial and bacterial PLFA and differences in the PLFA profiles of the manures added

Total amounts of microbial PLFAs recovered from the soil showed the same behaviour as amounts of bacterial PLFAs (Figure 4.3). Total amounts of microbial as well as bacterial PLFAs were significantly different between all treatments up to day 11 ($p < 0.001$). After this initial phase, significant differences were no longer detected between the different manure treatments; however, the amounts of extracted PLFAs within those treatments were about twice as high as for the TC treatment.

The bacterial load added with the horse manure was 10 times higher than with the cattle manure and 20 times higher than with the chicken manure. Accordingly, the amounts of the different microbial groups were highest with the horse manure and lowest within the chicken manure treatment.

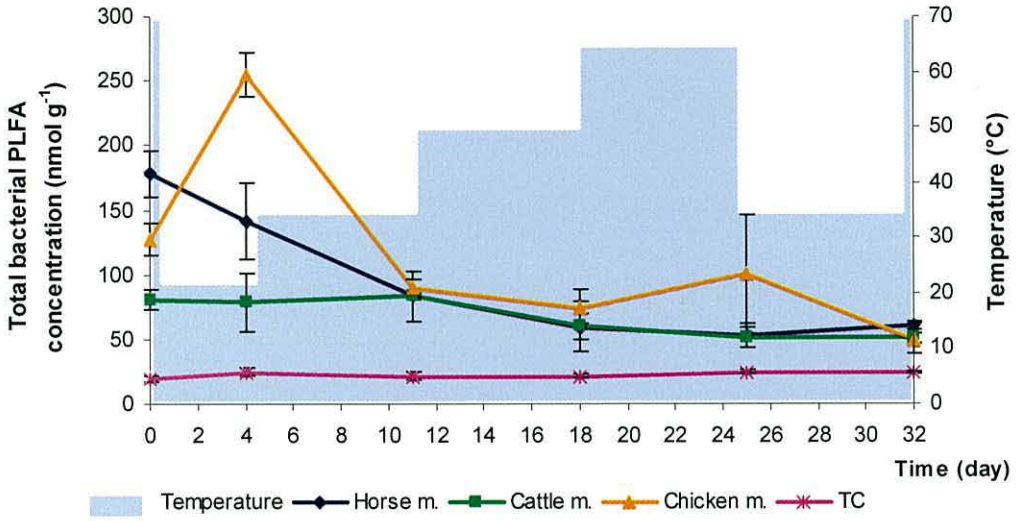

Figure 4.3: Mean concentrations of total bacterial PLFAs in nmol per g dry sample detected in the different treatments over the different stages of the 32 day experimental period. Error bars represent mean ±S.E.; n=3

The ratios of different microbial groups differed within the used manure types. All microbial communities of the pure manures were dominated by Gram-positive bacteria. While no difference in the ratio of Gram-negative-to-Gram-positive bacterial PLFAs was found between the cattle- and chicken manure, the ratio within the horse manure was much lower and the ratio of fungal-to-bacterial PLFA was highest with the chicken manure while the horse manure and cattle manure showed similar ratios (Table 4.1).

Those differences between the manure types were reflected in the initial treatment mixtures. Even though the microbial load was significantly different in the beginning of the experiment, the PAH degradation was not correlated to those numbers. The TC treatment had a significantly lower microbial load than the manure treatments over the whole course of the experiment ($p < 0.005$).

4.4.2.1.1. Gram-negative and Gram-positive bacteria indicating PLFAs

The ratio of Gram-negative-to-Gram-positive PLFAs was significantly higher for the TC treatment over the whole course of the experiment ($p < 0.01$), whereas no significant differences were detected between the manure amended treatments (Figure 4.4). It has been found that Gram-negative bacteria usually dominate the system in hydrocarbon contaminated environments (MacNaughton *et al.* 1999), explaining the higher Gram-negative-to-Gram-positive bacterial PLFA ratio in the aged PAH contaminated soil (Table 3.1). While manure addition lowers this ratio with the manure amended treatments by introducing higher amounts of Gram-positive bacteria, the ratio stays higher with treatment TC.

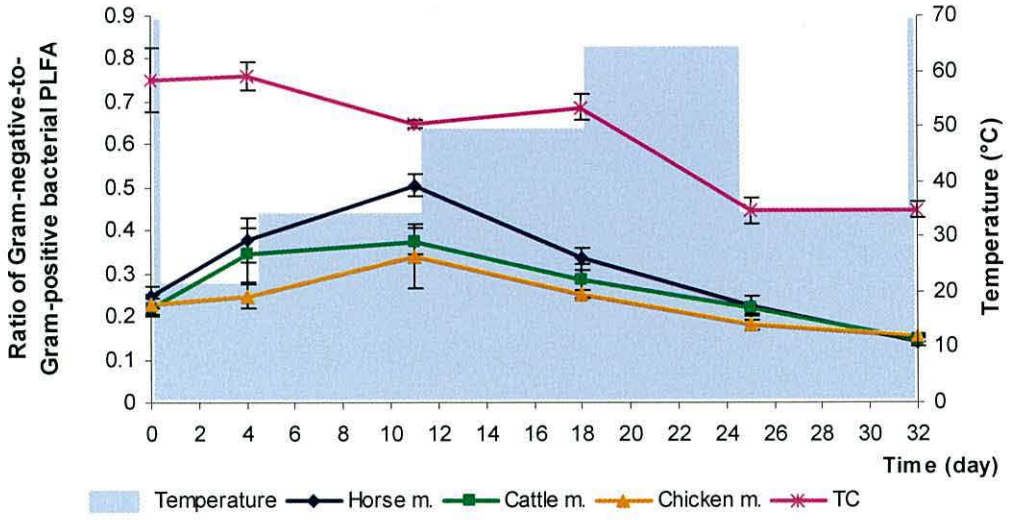

Figure 4.4: Mean values of the ratio of Gram-negative-to-Gram-positive bacteria indicating PLFAs detected in the different treatments during the different stages of the 32 day experimental period. Error bars represent mean ±S.E.; n=3

The Gram-negative-to-Gram-positive bacteria indicating PLFA ratio decreased significantly in the TC treatment ($p < 0.0001$). With the manure treatments it increased until day 11 followed by a decrease until the end of the experiment ($p < 0.05$).

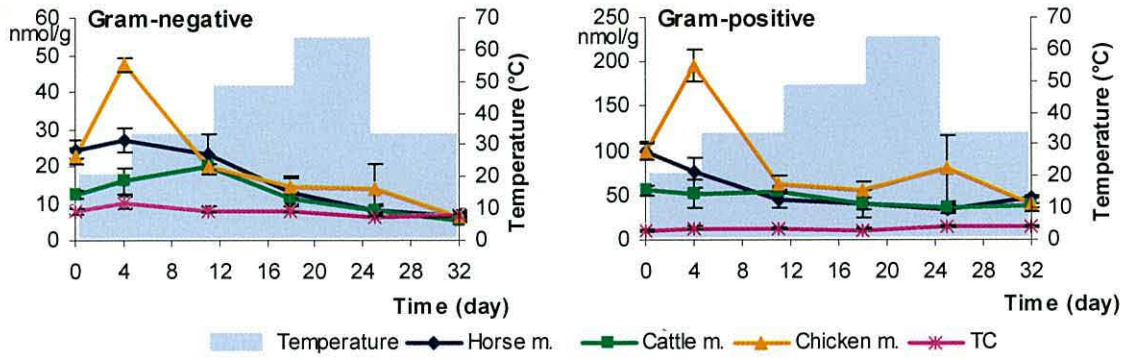

Figure 4.5: Mean values of the concentration of Gram-negative and Gram-positive bacteria indicating PLFAs in nmol per g dry sample detected in the different treatments during the different stages of the 32 day experimental period. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

It has been found that amounts of Gram-negative bacteria increase with temperatures increasing during composting of straw material (Klamer and Bååth 1998). The increasing Gram-negative-to-Gram-positive ratios in the manure treatments is probably due to a combined effect of the increasing temperature and the contaminant favouring the growth of Gram-negative bacteria. The decreases in the ratio during the rest of the experiment was caused by a decrease in Gram-negative bacteria, while the abundance of Gram-positive bacteria did not change significantly with any treatment except for an increase with the chicken manure treatment during the first experimental week followed by a subsequent decrease (Figure 4.5).

4.4.2.1.2. Actinobacteria indicating PLFA

The percentage of PLFA attributable to Actinobacteria decreased significantly during the course of the experiment for the TC treatment ($p < 0.0001$) (Figure 4.6a). However, the ratio of Actinobacteria indicating PLFA to total bacterial PLFA remained significantly higher in the TC treatment up to day 25. For the manure treatments, ratios of Actinobacteria-to-bacterial PLFA increased significantly over time reaching similar ratios to the ones found with the TC treatment by the end of the experiment.

With an exception for total amounts of Actinobacteria indicating PLFAs measured at day 4 with the chicken manure treatment those amounts did not differ significantly between the treatments and no significant changes were detected during the course of the experiment (Figure 4.6b).

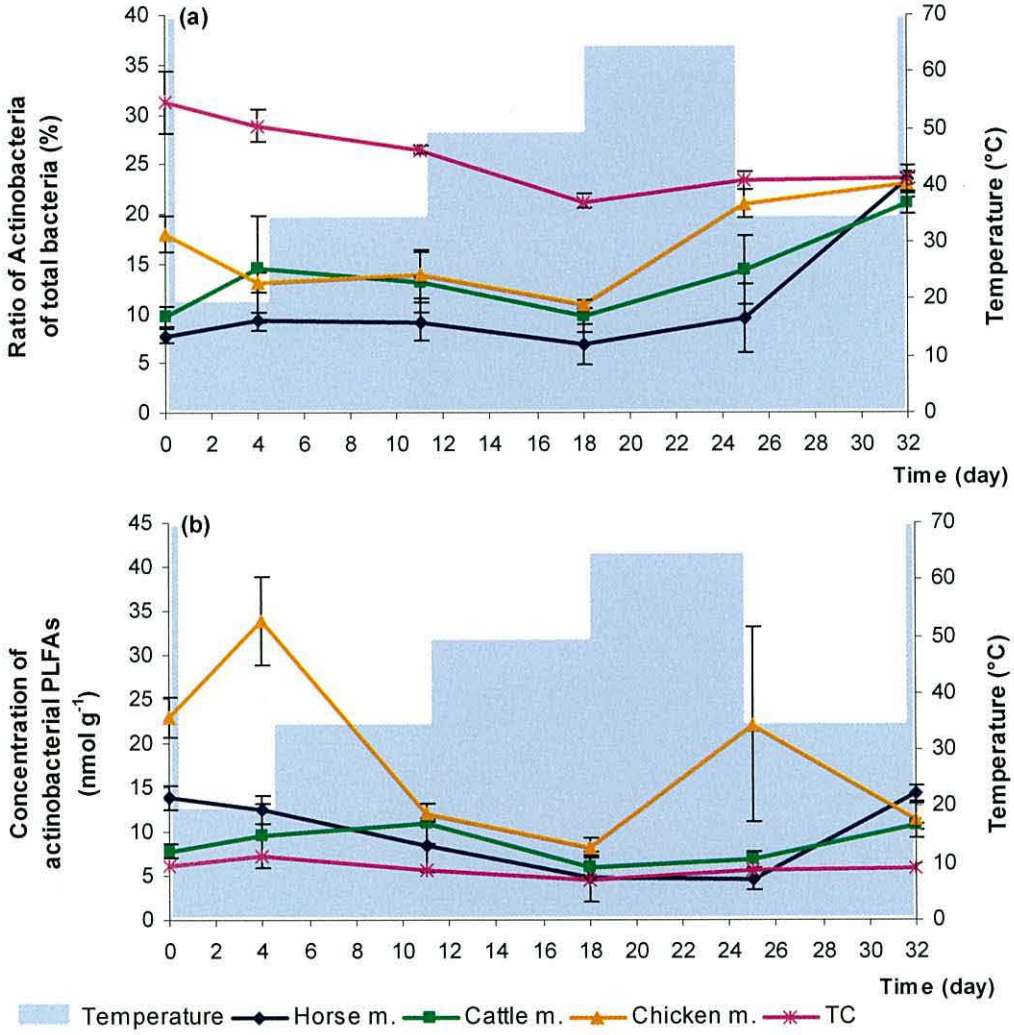

Figure 4.6a/b: Mean ratio of Actinobacteria indicating PLFAs in % of bacterial PLFAs (a) and concentration of Actinobacteria indicating PLFA in nmol per g dry sample detected in the different treatments over the 32 day experimental period. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

Actinobacteria are of special interest in bioremediation processes. The class of Actinobacteria includes genera such as Mycobacterium, Nocardia, and Streptomyces which have been found to degrade PAHs (Sutherland *et al.* 1995; Mrozik *et al.* 2003). Some members of the Actinobacteria form

branching filaments which, like mycelia of fungi, enables them to get in contact with contaminants that would otherwise be inaccessible. However, the profile of Actinobacteria indicating PLFAs did not correlate with PAH degradation but showed a similar behaviour as detected for normal composting of straw material with amounts being low at all times only showing a slight increase towards the end of the composting process (Klamer and Bååth 1998).

4.4.2.2. Fungi indicating PLFA

Within all treatments, the ratio of fungal-to-bacterial PLFAs in soil decreased significantly towards the end of the experiment ($p < 0.05$) (Figure 4.7a). Ratios of fungal-to-bacterial PLFAs were higher for the chicken and horse manure treatments than for the TC and the cattle manure treatments at the start of the experiment. Although at the start ratios of fungal-to-bacterial PLFAs were different between the manure treatments, they became increasingly similar to each other during the course of the experiment, reaching similar values to the TC treatment by the end of the experiment.

A significantly higher amount of fungal PLFAs were detected in the chicken and horse manure treatment and numbers increased in the chicken manure treatment before decreasing as in the other treatments (Figure 4.7b). Certain fungi such as *Phanerochaete chrysosporium, Pleurotus ostreatus* and *Trametes versicolor* (Canet *et al.* 2001; ŠaŠek *et al.* 2003) have been shown to be effective in degrading PAHs. However, in the present study fungi indicating PLFAs did not correlate with PAH degradation. In a study by Klamer and Bååth (1998) investigating the development of PLFA profiles during composting of straw material the authors found an initial increase of fungal PLFAs which then decreased once the temperatures reached 50°C. A similar behaviour was detected in the present study.

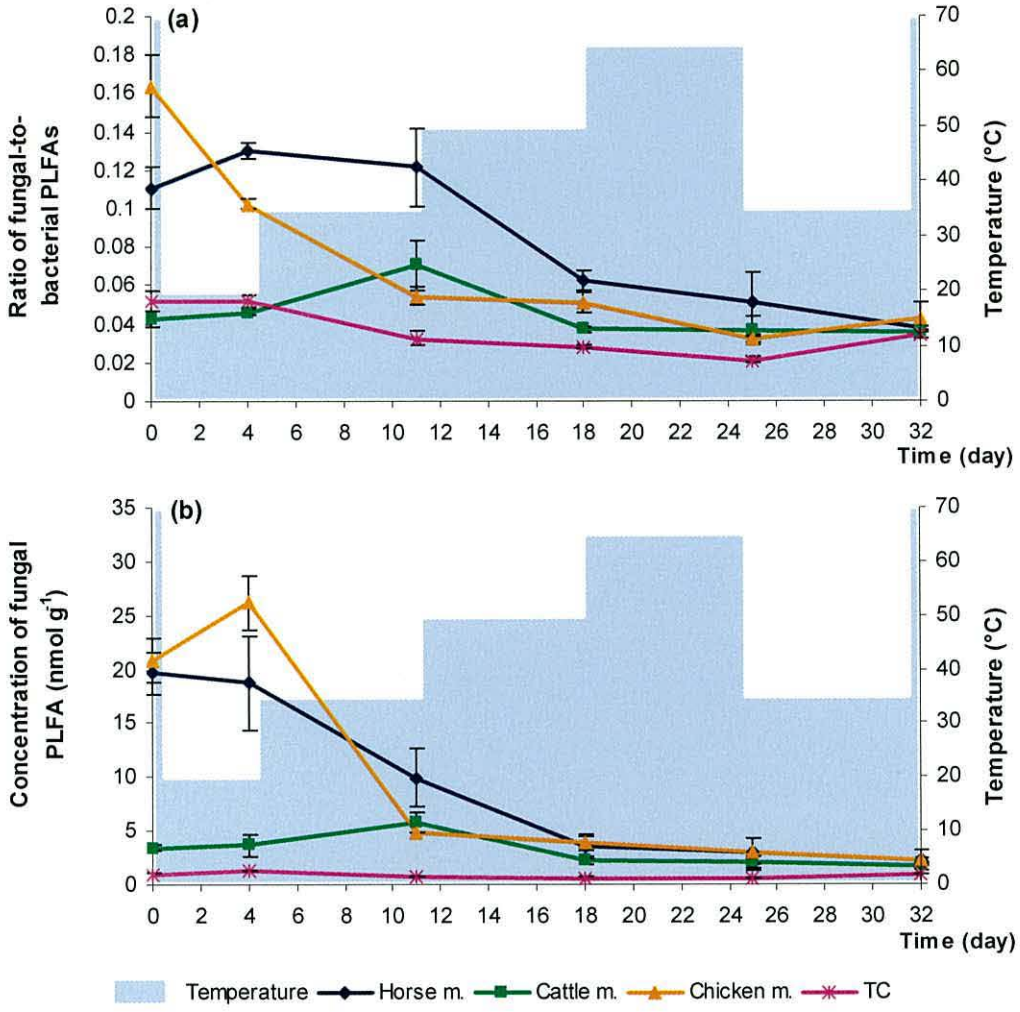

Figure 4.7a/b: Mean ratio of fungal-to-bacterial PLFAs (a) and concentration of fungal PLFA in nmol per g dry sample detected in the different treatments over the 32 day experimental period. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

4.4.2.3. Stress indicating PLFAs

The ratios of iso-to-anteiso branched PLFAs were similar between the treatments in the beginning as well as at the end of the experimental period with only the horse manure treatment having higher values. The ratio of iso-to-anteiso branched PLFAs increased for all treatments but at different time-points during the experiment (Figure 4.8).

The TC treatment did not show an increase in stress levels as indicated by the ratio of iso-to-anteiso branched PLFAs until the temperatures were changed to 65°C, whereas with the manure treatments this ratio increased constantly until the end of the 65°C temperature step. With the manure amended treatments soil as well as manure derived micro-organisms had to adapt to the new conditions introduced by mixing manure and soil. For the TC treatment soil organisms only had to adapt to a change in aeration and moisture and the changing temperatures.

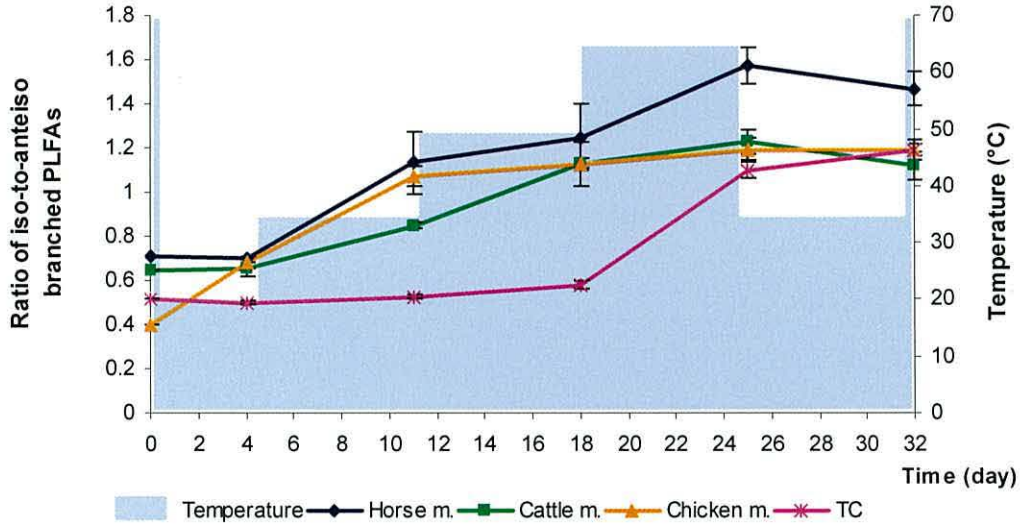

Figure 4.8: Mean ratio of iso-to-anteiso branched PLFAs detected in the different treatments over the 32 day experimental period. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

Results showed that with increasing temperatures stress indicating PLFAs increased. It has been found that factors such as temperature, pH, osmotic stress, and starvation increase those stress indicating PLFA ratios (Kaur *et al.* 2005). Even though correlation analysis (n=58, DF=56) only revealed a modest to strong positive correlation ($0.5<r<0.8$) of the ratios with time and temperature an influence of the changing physico-chemical conditions as well as the presence of the contaminants is probable. This might, however, not be detected as a combined influence of different factors and/or the more prominent influence of time and temperature might obscure a clear correlation.

Supporting this is the TC treatment where ratios only increase when temperatures increase above 50°C. The earlier increase with the manure amended treatments indicates that physico-chemical changes in the environment have another important effect on the microbial community.

4.4.2.4. Influence of additives on the PLFA profile development

As indicated by the analysis of different groups of micro-organisms as identified by PLFA extraction, profiles were different at the beginning of the experiment, becoming more similar over the experimental period.

Diagnostic Biplot analysis revealed associations of treatments and PLFAs (Figures 4.9). At the beginning of the experiment the horse manure treatment (vessels 1a-c) was highly associated with the bacteria indicating PLFA 15:0 and 17:0 and the Gram-positive bacteria indicating PLFA i14:0. The chicken manure treatment (vessels 3a-c) was mainly associated with the Actinobacteria indicating PLFAs 10Me17:0 and 10Me18:0 as well as with the Gram-positive bacteria indicating PLFA a17:0 and the Gram-negative bacteria indicating PLFA cy19:0. The TC (vessels Ca-c) and cattle manure (vessels 2a-c) treatments on the other hand where not positively associated with any PLFA but negatively with the remaining PLFAs (i:15:0, a15:0, i17:0, 10Me16:0; cy17:0, 18:2w6c).

During the following temperature steps differences between treatments become less pronounced and "outliers" (e.g. vessels 1a (horse manure) and 3a (chicken manure) at day 7) appear with individual PLFAs becoming associated with single replicate vessels. Those "outliers" emphasise the difficulty in predicting microbial community development and its dependency on a range of factors guiding those developments. However, the chicken manure treatment still shows an association with the Actinobacteria indicating PLFAs (10Me16:0, 10Me17:0, 10Me18:0) and while the cattle and horse manure treatment become more similar they are mainly associated with the bacteria indicating PLFA 15:0.

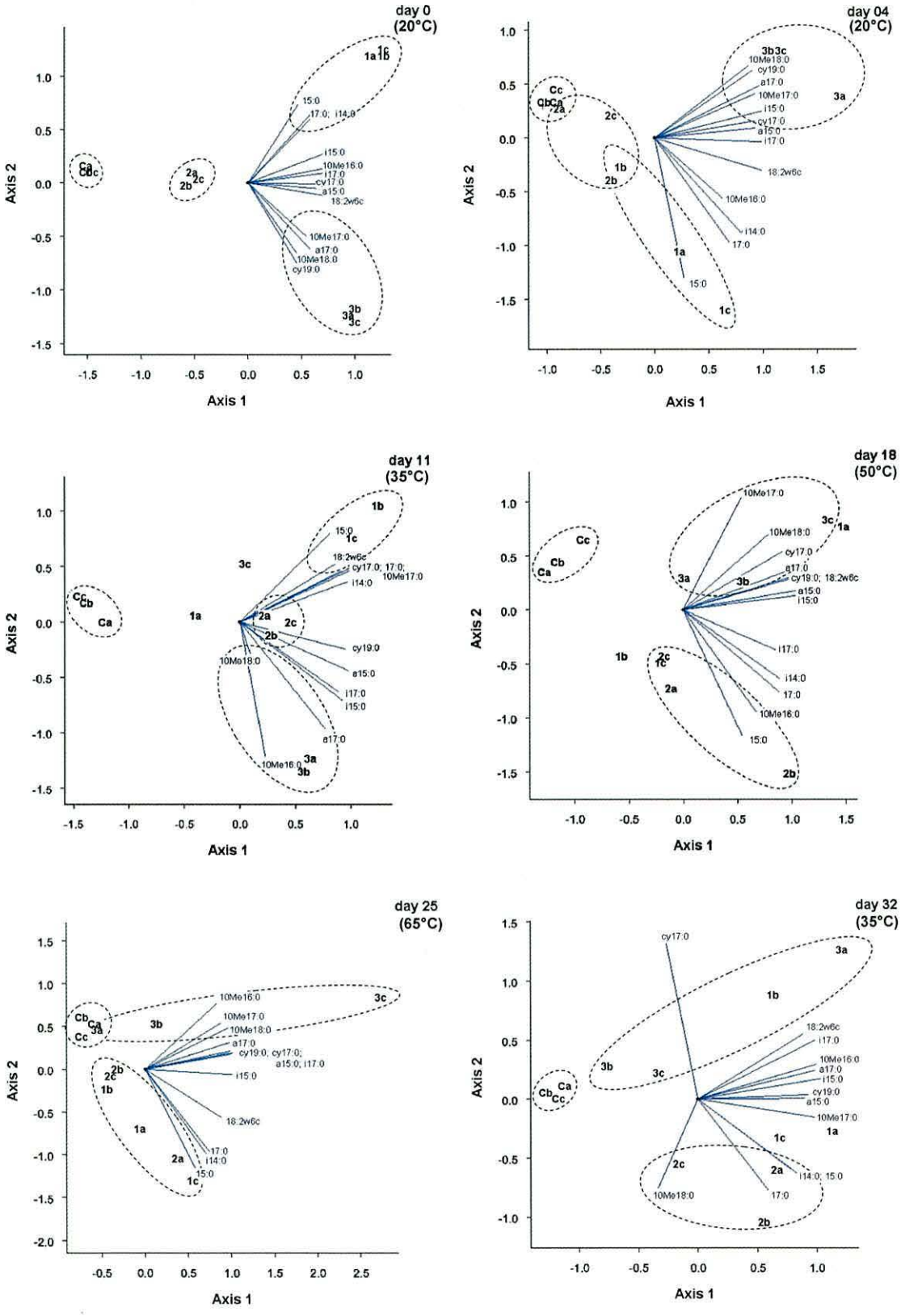

Figure 4.9: Diagnostic Biplots showing associations of PLFAs with the different treatments at each sampling point. The treatments are indicated as follows: 1 = horse manure, 2 = cattle manure, 3 = chicken manure, C = TC, a-c indicate replicate vessels.

Treatments were most similar at the end of the heating phase (day 25). During the following temperature step at 35°C treatments become more different again. The horse manure treatment seems to become more closely associated with the Actinobacteria indicating PLFA 10Me17:0 (vessel 1a) and the bacteria and Gram-positive bacteria indicating PLFAs 15:0 and i14:0 (vessel 1c), respectively while the cattle manure treatment becomes more associated with the bacteria indicating PLFAs 15:0 and 17:0, and the Gram-positive bacteria and Actinobacteria indicating PLFAs i14:0 and 10Me18:0, respectively. The chicken manure treatment on the other hand is not highly associated with any PLFA, but closer linked with the Gram-negative bacteria indicating PLFA cy17:0 and the fungi indicating PLFA 18:2w6c. The TC treatment is the only treatment that did not show any substantial changes in the PLFA profiles and replicate vessels did not develop in different ways.

PLFA profile development and changes in associations of treatments with individual PLFAs show the influence of the manure on the composting process as well as the temperature influence. It also shows that the cattle and horse manure are more closely related to each other than to the chicken manure, which was expected due to the diet of the animals, which was assumed to influence manure composition to a larger extent than the digestive system of the animal the manure derived from, both in terms of physico-chemical, as well as microbial properties.

4.4.3. Changes of extractable PAH amounts

Taking the weight loss of the soil/sawdust/manure mix into account the data measured of the extracted PAHs were corrected by referring to the amount of ash which is considered the most chemically stable parameter during the degradation process (Amir *et al.* 2005).

Due to the inhomogeneous distribution of the contaminants in the aged PAH contaminated soil, as well as in order to investigate temperature effects on PAH removal, results have been looked at in three different ways ("percentage change", "rates of degradation" and "concentration") as described fully in Chapter 3 (Materials and methods).

4.4.3.1. Changes in percentages of PAH concentrations remaining in the treatment mixtures over the 32 day experiment

4.4.3.1.1. Effects of treatments on extractable USEPA PAHs

Total USEPA PAH (as identified by the USEPA and listed in Table 4.2) concentrations at the end of the experimental period (day 32) were not significantly different to concentrations measured at the beginning for any of the treatments (Figure 4.10).

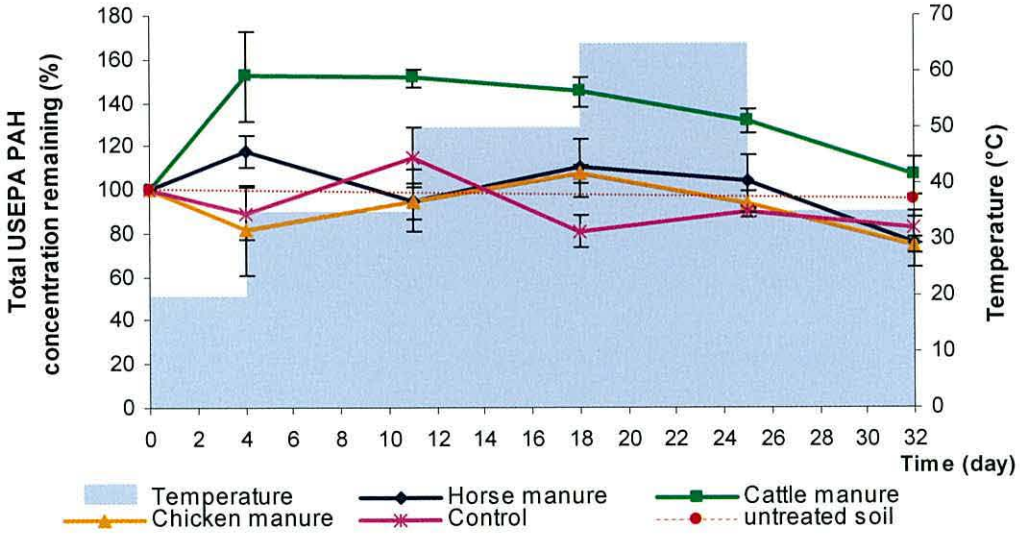

Figure 4.10: Mean percentage of total PAHs remaining (%) in the different treatments at the end of different temperature steps of the 32 d experiment. The red dotted line connects the percentage of the remaining total PAHs detected with the untreated soil after 32 days with the 100% in the beginning of the experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

The percentage of measured PAHs remaining in the soil at the end of the 32 d composting period are shown in Table 4.2. With exceptions for Naphthalene and Benz[a]anthracene percentages of LMW and MMW PAHs (up to MW 228) remaining were significantly lower at the end of the experiment for the TC, horse and cattle manure treatments relative to those present at the start ($p < 0.05$). With the chicken manure treatment only Pyrene (MW 202) concentrations have not been reduced.

Table 4.2. Percentage of PAHs remaining at the end of the experiment.

	Horse manure %±S.E.	**Cattle manure** % ±S.E.	**Chicken manure** %±S.E.	**TC** %±S.E.
LMW PAHs				
Naphthalene	103.3±81.5^{a}	106.0 ±47.1^{a}	34.5 ±12.9^{a*}	91.1±21.5^{a}
Acenaphthylene	59.7±9.6^{a*}	53.5 ±5.6^{a*}	78.4 ±7.1^{a*}	75.7±8.5^{a*}
Acenaphthene	56.3±8.9^{a*}	47.2 ±9.1^{a*}	53.8 ±17.4^{a*}	81.0±13.7^{a}
Fluorene	39.4±3.2^{ab*}	26.2 ±4.5^{b*}	55.0 ±10.0^{ab*}	64.5±10.6^{a*}
Phenanthrene	31.6±2.4^{ab*}	12.8 ±1.7^{b*}	47.2 ±9.9^{ab*}	37.1±4.0^{a*}
Anthracene	49.5±0.7^{ab*}	35.7 ±5.2^{b*}	68.6 ±8.4^{ab*}	54.0±6.6^{a*}
MMW PAHs				
Fluoranthene	36.0±7.1^{b*}	27.2 ±2.7^{b*}	66.4 ±1.7^{a*}	35.5±3.6^{b*}
Pyrene	52.2±12.4^{b*}	44.9 ±3.5^{b*}	103.7 ±4.3^{a}	56.4±4.4^{b*}
Benz[a]anthracene	107.9±16.0^{a}	102.7 ±7.4^{a}	74.1 ±1.7^{a*}	117.4±9.3^{a}
Chrysene	41.1±22.4^{a*}	19.8 ±1.2^{a*}	29.8 ±20.7^{a*}	15.4±0.8^{a*}
HMW PAHs				
Benzo[b]fluoranthene	122.9±3.3^{a}	106.6 ±6.5^{a}	67.9 ±1.9^{b*}	118.0±8.2^{a}
Benzo[a]pyrene	133.5±16.1^{a}	126.2 ±7.6^{a*}	65.8 ±1.4^{b*}	109.0±6.0^{a}
Indeno[1,2,3-c,d]pyrene	334.8±5.2^{a*}	279.3 ±23.0^{ab*}	189.0 ±10.0^{c*}	251.1±23.2^{bc*}
Dibenz[a,h]anthracene	189.8±12.0^{a*}	164.2 ±15.3^{ab*}	117.2 ±10.9^{b}	118.8±8.85^{b}
Benzo[g,h,i]perylene	503.5±14.6^{b*}	178.6 ±12.4^{c*}	4830.4 ±355.8^{a*}	4209.3±439.3^{a*}
Grouped PAHs				
Total LMW PAHs	41.4±1.8^{ab*}	25.2 ±3.5^{b*}	58.9 ±7.3^{a*}	48.7±5.9^{a*}
Total MMW PAHs	47.2±8.4^{b*}	38.3 ±3.2^{b*}	78.0 ±3.1^{a*}	47.9±4.0^{b*}
Total HMW PAHs	153.8±7.4^{a*}	130.4 ±8.6^{a*}	83.1 ±2.8^{b*}	136.5±9.4^{a*}
Total USEPA PAHs	75.5±11.4^{a*}	106.3 ±8.8^{a}	74.6 ±3.5^{a*}	82.4±7.2^{a*}

Values are means of the percentage of extractable PAHs remaining in % of the amount found in the soil at the beginning of the experiment ±S.E., values with the same letter are not significantly different between the treatments and * indicates a significant difference to the concentrations found at the beginning of the experiment at the $p < 0.05$ level as determined using a Tukey post-hoc test. n=3

PAH concentrations in the untreated soil were not significantly different by the end of the 32 day experimental period. Final remaining percentages were between 91 and 100% for all measured PAHs except Benz[a]anthracene and Chrysene for which only 84 and 81% of the initial concentrations could be measured, respectively. However, due to a larger variance for the concentrations of these PAHs final concentrations were not significantly different to initial ones.

The smaller PAHs showed a decrease or no net change in their concentrations during the experiment, while the concentration of most HMW PAHs (Figures 4.11a and b) increased significantly during the first temperature step in the horse manure, cattle manure, and TC treatments.

After the initial increase, concentrations of Benzo[b]fluoranthene, Benzo[a]pyrene, and Dibenz[a,h]anthracene subsequently decreased with the horse manure treatment to a level that was not significantly different to that initially present at the start of the experiment. For the cattle manure treatment only Benzo[a]pyrene decreased to a non-significantly different level, while the Benzo[b]fluoranthene concentration did not change significantly over the whole experimental period.

With the TC treatment, Benzo[a]pyrene and Dibenz[a,h]anthracene concentrations did not change significantly during the experiment. The other HMW PAHs increased significantly during the initial phase. While concentrations of Indeno[1,2,3-c,d]pyrene and Benzo[g,h,i]perylene remained higher only Benzo[b]fluoranthene concentrations were found to decrease to non significantly different concentrations by the end of the experiment.

The chicken manure treatment behaved slightly differently from the other treatments as no initial increases in PAH concentrations were observed, however, concentrations of Benzo[b]fluoranthene and Benzo[a]pyrene were significantly reduced by the end of the 32 day composting period ($p < 0.05$).

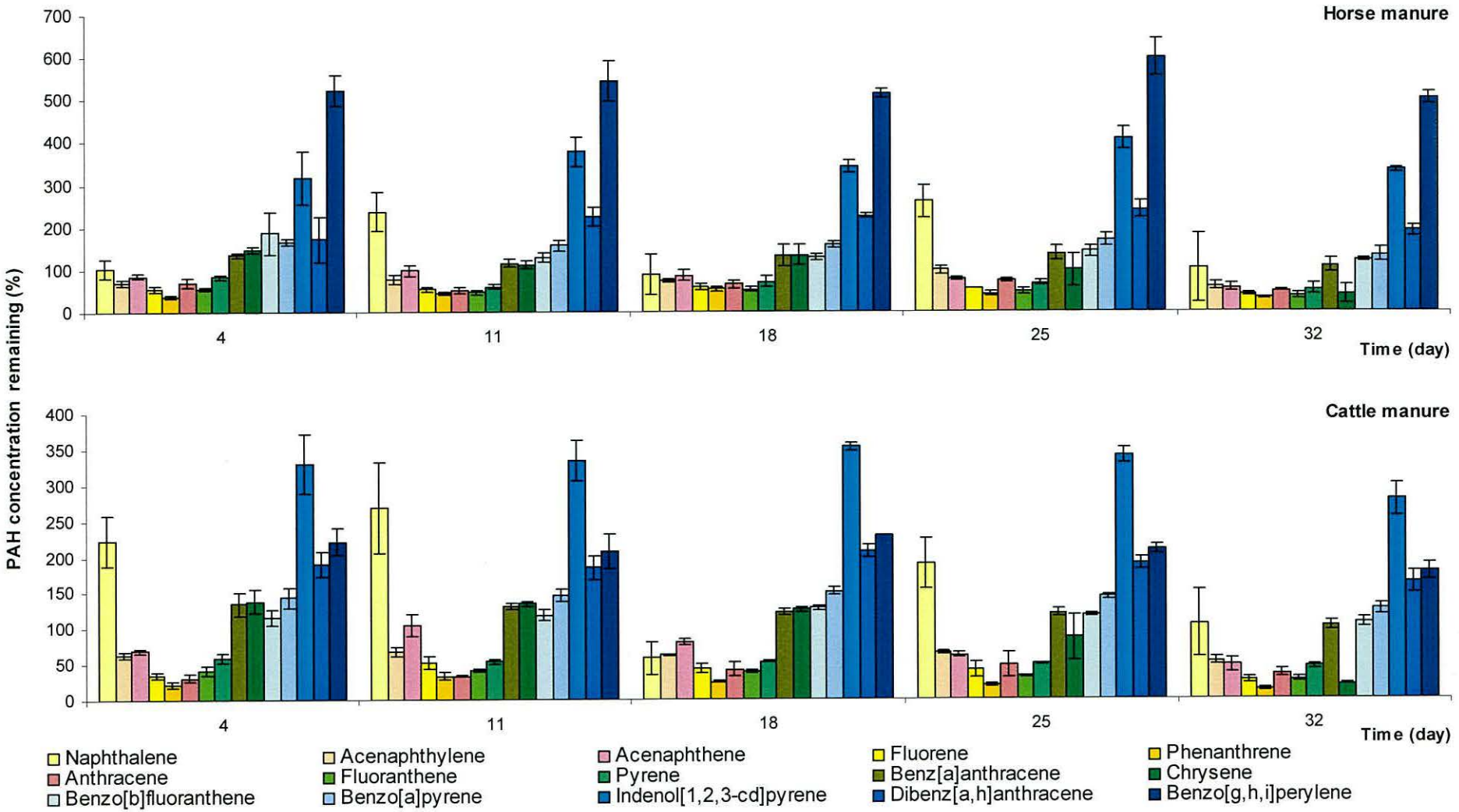

Figure 4.11a: Mean concentrations of individual USEPA PAHs remaining (%) in the Horse and Cattle manure treatments at the end of different time periods steps during the 32 day experiment in % from concentrations measured at the beginning of the experiment. Error bars represent ±S.E.; n=3

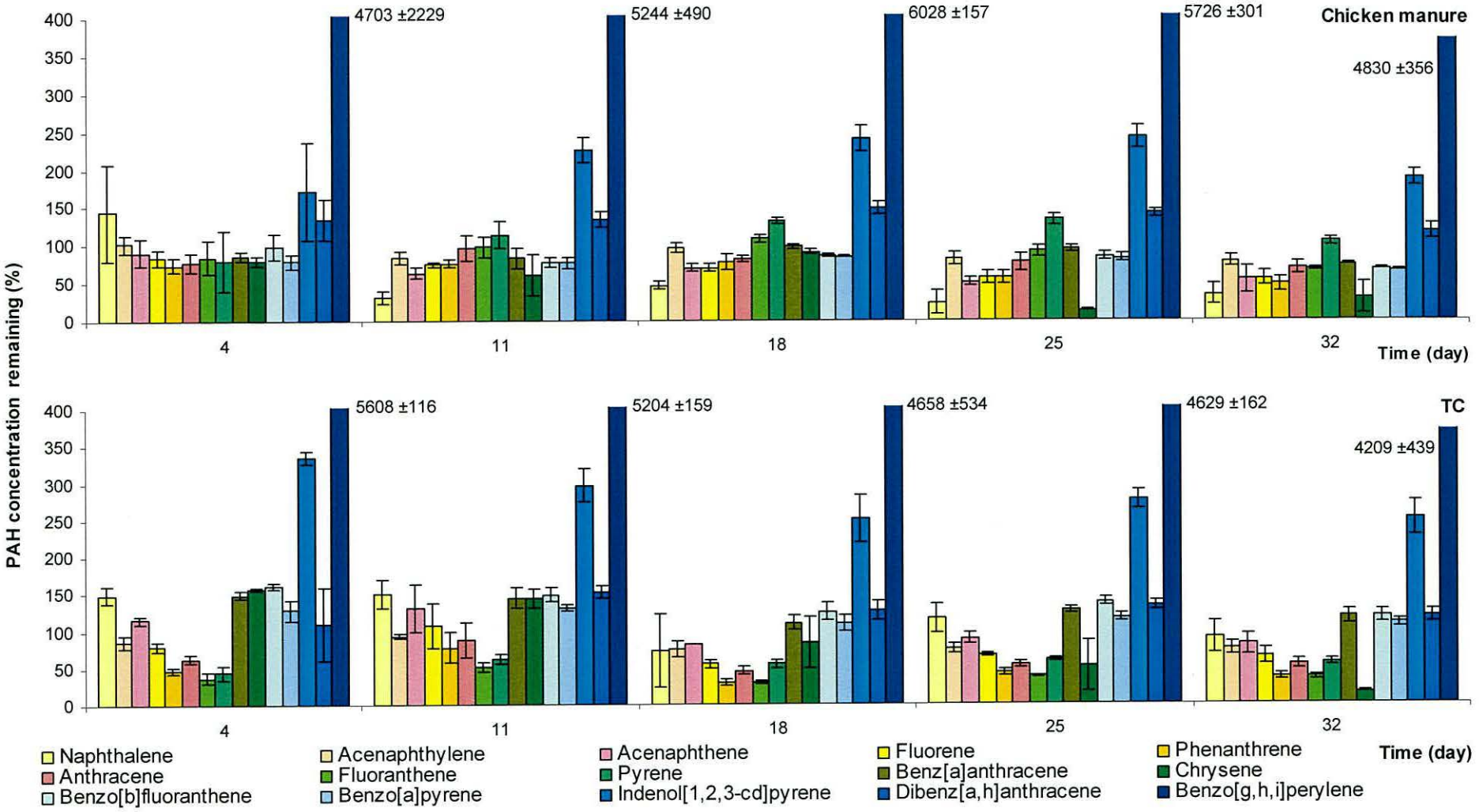

Figure 4.11b: Mean concentrations of individual USEPA PAHs remaining (%) in the Chicken manure and TC treatments at the end of different time periods steps during the 32 day experiment in % from concentrations measured at the beginning of the experiment. Error bars represent ±S.E.; n=3

The lower MW PAHs generally seemed to show a larger reduction in their percentages remaining in the soil than the higher MW ones. Similar findings have been reported from other studies investigating the use of different additives to enhance remediation of hydrocarbons from contaminated soil. The investigated processes were found to be successful in degrading smaller PAHs while the larger PAHs remain problematic compounds due to their stronger hydrophobic characteristics and stability, especially when dealing with an aged contaminated soil (Aprill *et al.* 1990; Morgan and Watkinson 1990; Weissenfels *et al.* 1990; Weissenfels *et al.* 1990; Mueller *et al.* 1991; Potter *et al.* 1999; Wong *et al.* 2002; Antizar-Ladislao *et al.* 2005).

Supporting the findings in those studies, it has also been found that with increasing molecular size the electrochemical stability as well as the hydrophobicity of PAHs increases which contributes to an associated increase in their persistence (Cerniglia 1992). This explains a higher degradation of smaller PAHs during the initial 2-month windrow pre-treatment as well as during the imposed 32 day treatment. Additionally, it has been shown that bound residue formation was more extensive for the more hydrophobic pollutants, such as the heavier PAHs (McFarland and Qiu 1995).

For the aforementioned reasons, grouping of single PAHs into LMW, MMW, and HMW PAHs is a good and often used way to summarise contamination. However, results of this study reveal some differences of individual PAHs within those groups which can distort results. This appeared to be especially the case for high MW PAHs such as Benzo[g,h,i]perylene, which seemed to have been present in the soil in very low concentrations (~0.3 µg g^{-1} ash) but whose extractable amounts increased over 40-fold. Therefore, results for grouped PAHs have to be interpreted with care.

4.4.3.1.2. Treatment effects of PAHs group into low, middle, and high molecular weight categories

Temporal changes in the extractable concentrations of grouped PAHs present in the soil over the course of the experiment are presented below. With the untreated soil (red dotted line) amounts of grouped PAHs only change slightly remaining at 95.5% for total measured USEPA PAHs, and 93.6%, 95.8%, and 97.6% for grouped LMW, MMW, and HMW PAHs respectively.

Effects of treatments on LMW PAHs

The grouped extractable LMW PAHs (Figure 4.12) demonstrated the least changes in their concentration during the experiment. However, percentages remaining were significantly lower at the end of the experimental period for all treatments ($p < 0.05$).

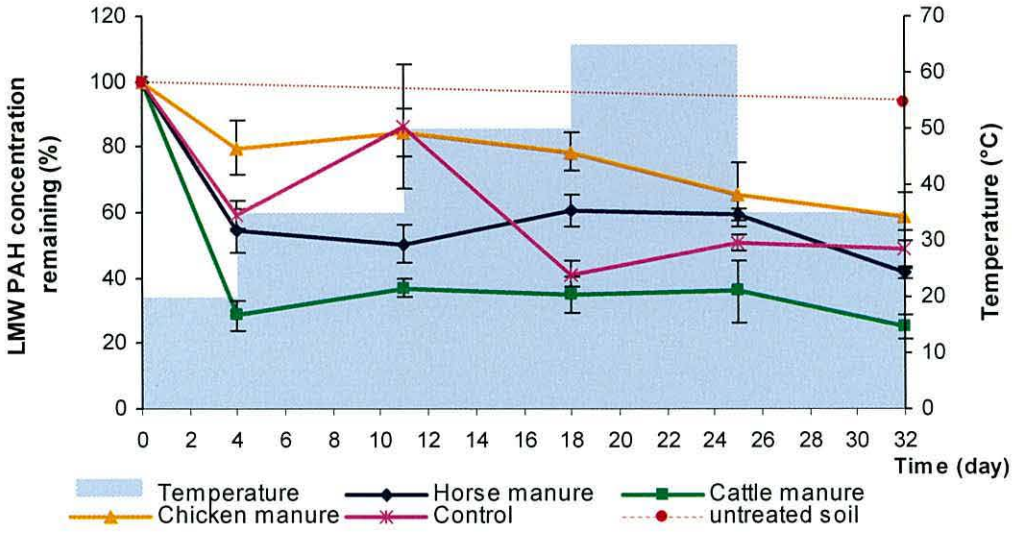

Figure 4.12: Mean concentration of LMW PAHs in the different treatments at the end of different temperature steps of the 32 day experiment. The red dotted line connects the percentage of the remaining grouped LMW PAHs detected with the untreated soil after 32 days with the 100% in the beginning of the experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

The reduction in extractable LMW PAHs mainly occurred during the first temperature step and after this initial reduction no further significant changes were detected with any of the treatments over the remaining 28 d. At the end of the 32 day experiment with the cattle manure addition LMW PAHs had decreased to a significantly lower remaining percentage compared to the chicken manure or no manure (TC) treatments ($p < 0.01$).

Effects of treatments on MMW PAHs

With the horse-, cattle manure, and TC treatments the concentrations of MMW PAHs (Figure 4.13) declined significantly ($p < 0.005$) over time with PAH amounts in the treatment mixture being lower at day 32 compared to day 0. As with the LMW PAHs, most of the MMW PAHs were lost in the first few days of incubation. In contrast, the concentrations of MMW PAHs in the chicken manure treatment showed slightly different temporal dynamics to the other treatments with an increase in extractable PAHs until day 18 followed by a decrease resulting in 78% of the initial PAHs remaining in the soil-compost mixture after 32 d.

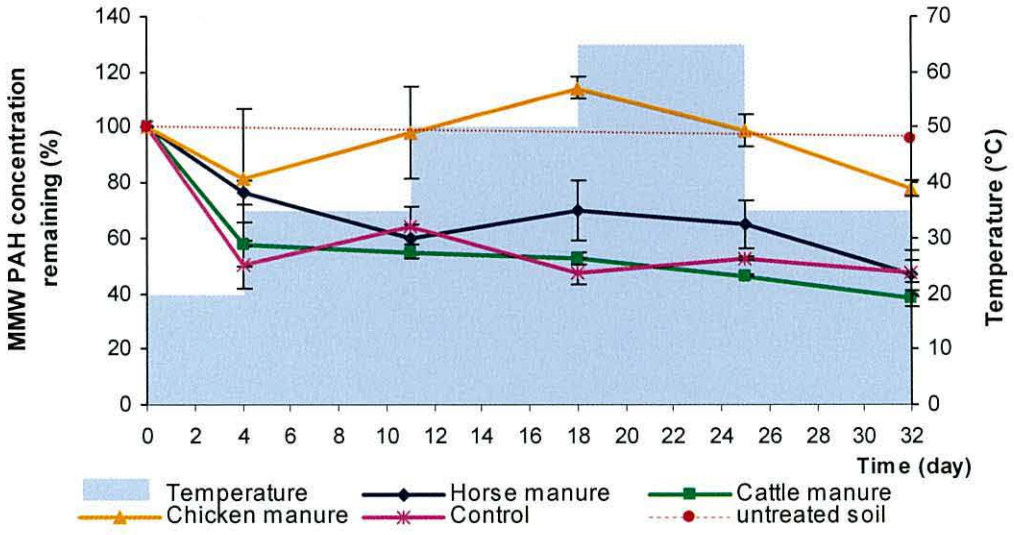

Figure 4.13: Mean concentration of MMW PAHs in the different treatments at the end of different temperature steps of the 32 day experiment. The red dotted line connects the percentage of the remaining grouped LMW PAHs detected with the untreated soil after 32 days with the 100% in the beginning of the experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

Effects of treatments on HMW PAHs

Grouped HMW PAH concentrations (Figure 4.14) increased significantly during the first 4 days for all but the chicken manure treatment. After this initial increase, percentages remaining in the treatment mixtures stayed significantly higher than at day 0 until the end of the experiment where concentrations started to drop to a level where they were not significantly different from the initial concentrations.

Percentages of HMW PAH concentrations remaining in the treatments were significantly lower with the chicken manure treatment than with the other treatments ($p < 0.01$) which were not significantly different to each other. The increase in PAH concentrations, especially for the HMW PAHs in the beginning of the experiment, could have been caused by the added organic matter of the manure and/or sawdust. Dissolved organic matter tends to interact with free ions and hydrophobic pollutants increasing their solubility and hence favouring their transport (Smith *et al.* 1991) and its addition to contaminated soil can cause an increase in contaminant concentration by occupying sites the contaminant was bound to previously and therefore making it solvent-extractable (Kästner and Mahro 1996).

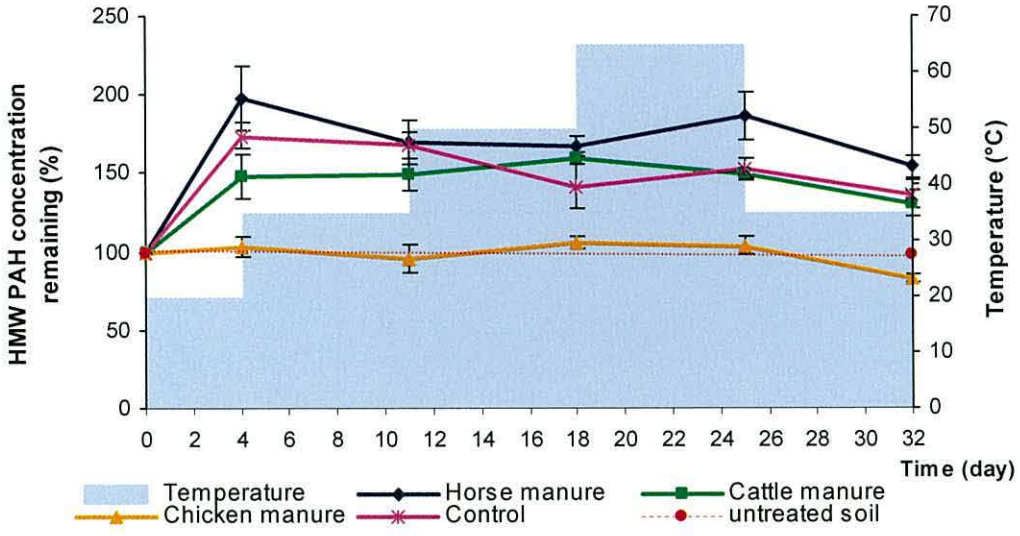

Figure 4.14: Mean concentration of HMW PAHs in the different treatments at the end of different temperature steps of the 32 day experiment. The red dotted line connects the percentage of the remaining grouped LMW PAHs detected with the untreated soil after 32 days with the 100% in the beginning of the experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

Additionally, co-composting contaminated soil with nutrient rich materials such as manure can lead to micro-organisms attacking the more readily degradable organic material first while the ligno-cellulosic structures are less susceptible to microbial decomposition and can therefore have a high potential for PAH adsorption, resulting in a counter-decrease in extractable PAH amounts. Later, when these lignin-cellulosic structures (e.g. straw material) are degraded their disappearance can contribute to PAH release (Amir *et al.* 2005). This can explain the increase in extractable PAH amounts during the experiment.

The biggest difference between the treatments for the changes in HMW PAH concentrations were that the chicken manure treatment showed the best results in enhancing the degradation of HMW PAHs. Initially HMW PAH concentrations increased significantly with the other treatments.

The TC treatment showed a similar increase in percentages of HMW concentrations to the horse- and cattle manure treatments. Assuming that the processes that release those PAHs within those treatments also take place in the chicken manure treatment, suggests that the lack of increase in HMW PAH concentrations with the chicken manure treatment might be due to a simultaneous degradation of released HMW PAHs. It also suggests that the chicken manure has a significantly higher potential to remove HMW PAHs counteracting the release of the PAHs. This is possibly attributed to the micro-organisms in the chicken manure; however, the higher TOM content might have caused PAH removal by binding as well.

4.4.3.2. Influence of incubation temperature on changes in PAH concentrations

Analysis of differences in degradation rates between the implied temperature steps revealed that highest decreases in concentration mainly occurred during the lower temperature steps at the beginning and the end of the experiment, as found by other authors (Beaudin *et al.* 1999; Antizar-Ladislao *et al.* 2005).

Higher degradation rates during the early stages of the process were mainly found for LMW and MMW PAHs (Figure 4.15), as for the HMW PAHs a significant increase in PAHs was measured during the first temperature step (see below).

With the cattle manure treatment, degradation rates were highest during the first temperature step for LMW PAHs and Fluoranthene. With the horse manure treatment, degradation rates were significantly higher during the first temperature step for Phenanthrene and during the last temperature step for Acenaphthylene. The chicken manure treatment only had a significantly higher degradation rate for Chrysene during the 65°C temperature step (day 18 to 25). For the TC treatment highest degradation rates were detected during the 50°C step (day 11 to 18) for Phenanthrene and Benz[a]anthracene and during the first temperature step for Fluoranthene.

LMW PAHs had significantly higher degradation rates during the first temperature step for the horse- and cattle manure treatment ($p < 0.005$), while significant differences between degradation rates of the summed MMW PAHs only occurred with the TC treatment ($p < 0.005$) where the highest degradation rate was found during the first temperature step (Figure 4.15).

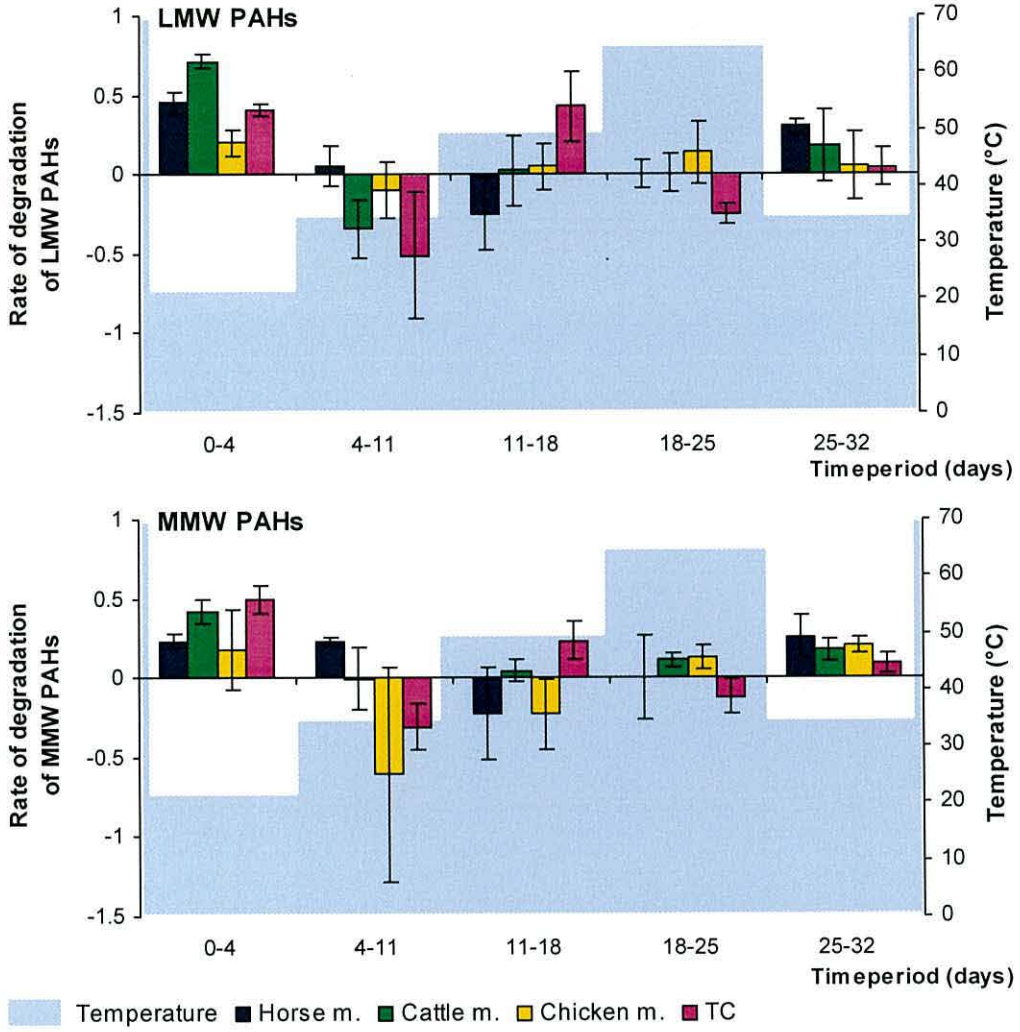

Figure 4.15: Mean rate of degradation of grouped LMW and MMW PAHs during the different temperature steps of the 32 day experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

For the HMW PAHs (Figure 4.16) the degradation rate was significantly different during the first temperature step due to the increase in the amounts of extractable PAHs. Statistical analysis revealed that if there were significant differences between degradation rates, those only occurred for the first temperature step and degradation rates were not significantly different between the subsequent temperature steps. Even though not statistically significant, higher degradation rates of total HMW PAHs were detected during the last temperature step for all treatments.

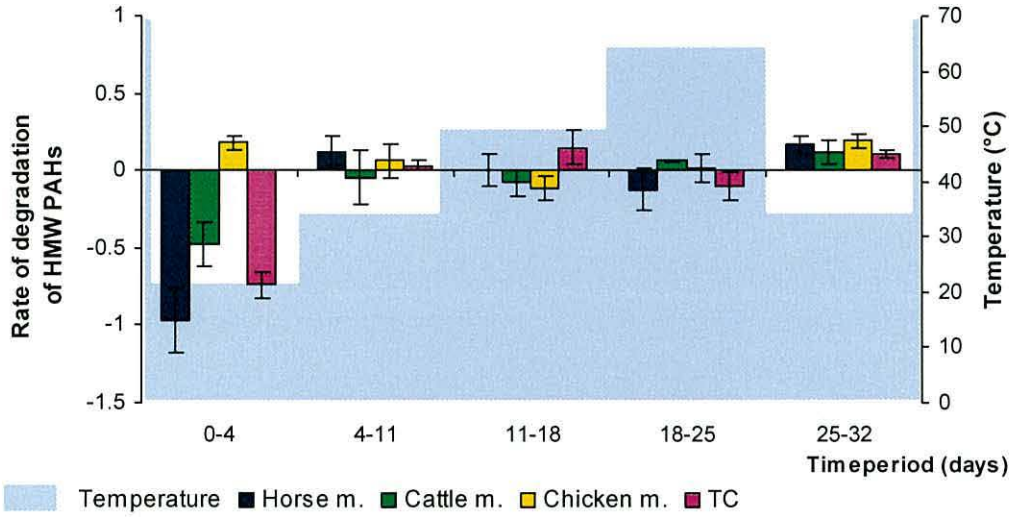

Figure 4.16: Mean rate of degradation of grouped HMW PAHs during the different temperature steps of the 32 day experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

Comparing treatments, degradation rates were only significantly different between the treatments during the first temperature step. During this step degradation rates for the LMW PAHs were significantly higher with the cattle manure treatment ($p < 0.005$) whereas degradation rates for the higher molecular weight PAHs Benz[a]anthracene, Chrysene, and Benzo[a]pyrene as well as the total HMW PAHs were significantly higher with the chicken manure treatment ($p < 0.005$). Degradation rates for total MMW PAHs did not show significant differences between treatments.

Degradation rates during single temperature steps give an insight into the effects of the implied temperature on the PAH degradation behaviour. Temperature has been shown to have an influence on PAH availability making PAHs more available with increasing temperatures (Pignatello and Xing 1996). No significant increases in PAH concentrations could be detected with increasing temperatures. However, a temporal increase in their amounts at higher temperatures might have been followed by a decrease when the now available compounds can be degraded, or re-bound to the soil.

4.4.3.3. Effects of extractable PAH concentrations

Differences in PAH concentrations were greater between the treatments at the beginning of the experiment than at the end (Table 4.3).

Table 4.3. Mean concentrations of individual and grouped PAHs (µg g^{-1}) at the beginning (day 0) and end (day 32) of the experiment. Values are concentrations in µg per g ash ±S.D.; n=3

		Horse manure	Cattle manure	Chicken manure	TC
	day	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.
LMW PAHs					
Naphthalene	0	0.6 ±0.1	0.8 ±0.1	2.6 ±0.4	1.4 ±0.2
	32	*0.6 ±0.8*	*0.8 ±0.6*	*1.3 ±0.7*	*0.9 ±0.6*
Acenaphthylene	0	2.3 ±0.2	2.9 ±0.3	2.0 ±0.2	2.2 ±0.2
	32	*1.4 ±0.3*	*1.5 ±0.2*	*1.7 ±0.5*	*1.6 ±0.2*
Acenaphthene	0	3.1 ±0.3	3.3 ±0.3	3.3 ±0.3	2.8 ±0.3
	32	*1.7 ±0.4*	*1.5 ±0.4*	*2.3 ±0.9*	*1.8 ±1.0*
Fluorene	0	9.6 ±0.4	12.4 ±0.5	6.3 ±0.1	7.5 ±0.2
	32	*3.8 ±0.4*	*3.2 ±1.0*	*4.8 ±1.4*	*3.5 ±1.0*
Phenanthrene	0	78.1 ±7.5	131.7 ±12.6	38.9 ±3.7	74.4 ±7.5
	32	*24.7 ±4.5*	*16.6 ±2.3*	*27.8 ±7.5*	*18.1 ±5.5*
Anthracene	0	79.0 ±10.0	128.6 ±16.7	55.9 ±6.6	88.5 ±12.6
	32	*39.1 ±4.7*	*45.2 ±7.5*	*48.7 ±17.8*	*39.0 ±12.8*
MMW PAHs					
Fluoranthene	0	426.4 ±42.0	541.6 ±58.7	236.5 ±24.1	591.9 ±110.8
	32	*150.1 ±42.2*	*145.6 ±14.2*	*213.6 ±74.2*	*156.6 ±8.9*
Pyrene	0	439.1 ±66.4	555.3 ±93.2	288.8 ±42.1	615.6 ±80.2
	32	*221.5 ±78.0*	*246.6 ±33.7*	*350.1 ±88.8*	*300.8 ±58.4*
Benz[a]anthracene	0	54.6 ±10.5	57.7 ±15.3	93.9 ±4.6	63.6 ±13.0
	32	*57.2 ±9.6*	*58.0 ±9.0*	*75.6 ±24.9*	*69.7 ±6.2*
Chrysene	0	56.6 ±6.1	56.3 ±6.7	101.7 ±10.3	63.8 ±6.4
	32	*23.1 ±21.3*	*11.2 ±2.1*	*9.8 ±1.3*	*29.9 ±35.6*
HMW PAHs					
Benz[b]fluoranthrene	0	50.2 ±8.5	55.3 ±10.2	79.8 ±13.1	50.7 ±6.5
	32	*61.6 ±9.4*	*58.4 ±8.1*	*60.1 ±13.2*	*54.4 ±11.0*
Benzo[a]pyrene	0	32.4 ±3.2	36.1 ±3.4	63.2 ±6.3	45.4 ±4.7
	32	*42.8 ±6.8*	*45.3 ±3.1*	*49.8 ±9.6*	*41.7 ±5.6*
Indeno[1,2,3-cd]pyrene	0	6.3 ±1.1	7.1 ±0.8	9.2 ±0.9	8.3 ±1.5
	32	*20.9 ±3.4*	*19.6 ±1.4*	*21.3 ±7.3*	*17.4 ±2.5*
Dibenz[a,h]anthracene	0	3.4 ±1.0	4.1 ±1.3	5.2 ±1.1	5.8 ±1.2
	32	*6.3 ±1.4*	*6.5 ±1.4*	*6.9 ±2.2*	*6.3 ±2.3*
Benzo[ghi]perylene	0	3.3 ±0.6	8.7 ±2.5	0.3 ±0.0	0.4 ±0.1
	32	*16.8 ±4.1*	*15.3 ±3.3*	*16.3 ±6.1*	*14.2 ±3.3*

Differences in PAH concentrations in µg per g ash were detected between the vessels. Possible interactions of absolute PAH amounts with physico-chemical properties, microbial community changes, PAH volatilisation, or PAH degradation were analysed using Spearman's rank correlation. Values of correlation coefficients (n=58, DF=56) were below 0.4 representing only a weak correlation which might not have been caused by the PAH but might be due to third factors influencing both variables in the same way.

The possibility that concentrations detected at day 32 might represent a threshold value below which no further degradation can occur (at least under the present circumstances) was investigated. The reaching of a threshold value within a treatment would result in PAH concentrations reaching a plateau. All treatments show reductions of extractable PAH concentrations during the last temperature step for all PAHs suggesting that this plateau was not reached.

4.4.3.4. PAH volatilisation

The amounts of PAHs lost in the vapour phase were related to the amount of contaminated soil in the vessel. Overall, the loss of PAHs from the soil due to volatilisation was relatively low and represented an insignificant loss pathway in comparison to the total amount remaining in the soil. However, a temperature dependency of PAH volatilisation was obvious with volatilisation rates increasing with higher temperatures. Additionally, volatilisation rates were higher for smaller PAHs (Figure 4.17). No HMW PAHs were detected within the gas phase.

Volatilisation rates were highest for the chicken manure treatment for all PAHs except for Fluoranthene and Pyrene, where highest rates were detected within the TC treatment. However, significant differences in volatilisation rates between the treatments were only detected until day 18 with no treatment effects seen thereafter.

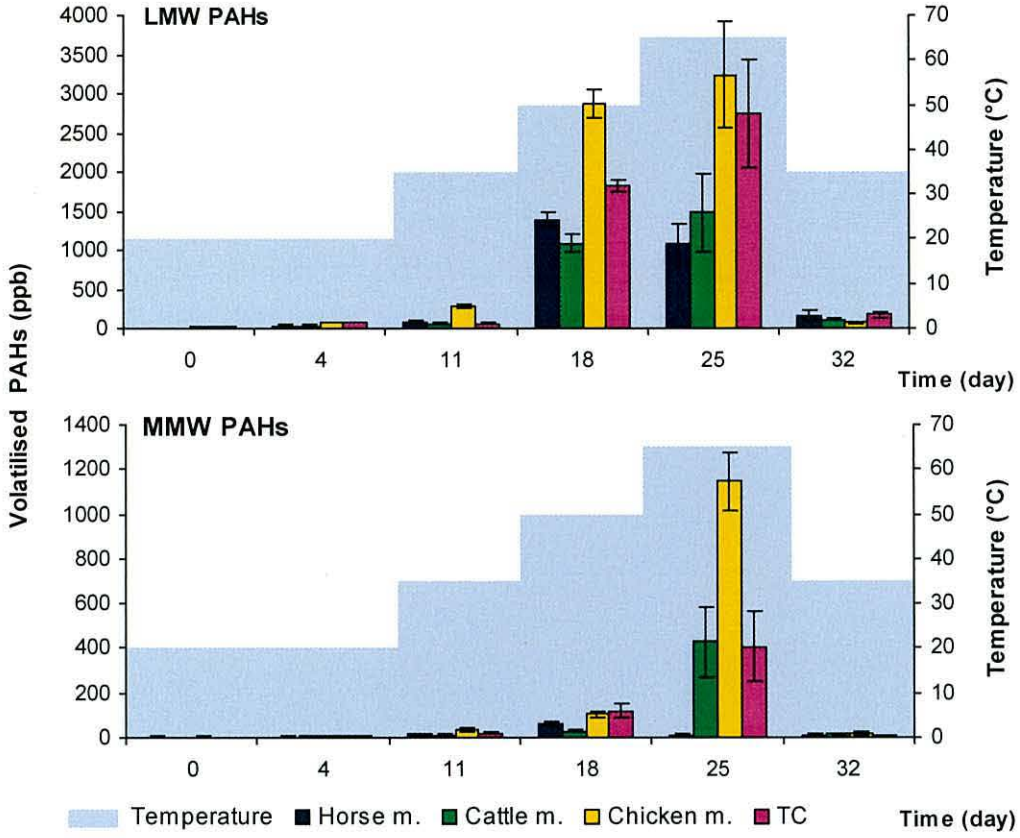

Figure 4.17: Amounts of volatilised grouped LMW and MMW PAHs with the different treatments during the different temperature steps of the 32 day experiment. The light blue background shows the applied temperature profile. Error bars represent mean ±S.E.; n=3

Even though PAH losses through volatilisation were negligible, results showed the influence of high temperatures on the release of PAHs, with higher volatilisation rates during the higher temperature steps suggesting an increased release of bound PAHs with increasing temperatures. They also support that larger PAHs are more difficult to release from bound sites than the LMW PAHs whose volatilisation was up to three times higher than the ones for the larger PAHs. Extractable amounts increased up to the end of the 50°C temperature step for the majority of PAHs. Even though the temperature is increasing most PAH concentrations show a decrease during the last 2 temperature steps. This decrease might be detected because most of the bound PAHs that can be released at the temperature range of 50° to 65°C have already been released during the 50°C temperature step. It has to be assumed that there are still PAHs being released during the 65°C temperature step, which will lead to an underestimation of PAH degradation during this phase.

4.4.4. Discussion of effects of different process characteristics on PAH removal

One of the most important factors governing the success of bioremediation strategies is bioavailability and desorption processes are widely accepted to be the rate-limiting factors in biodegradation of an aged contamination (Pignatello and Xing 1996; Carmichael and Pfaender 1997; Semple *et al.* 2001).

During the composting process decreases as well as increases in PAH amounts were detected. Decreases of extractable PAH amounts may be attributed to biodegradation due to microbial activity (co-metabolic degradation) as well as to sorption to the compost matrix. Increases in extractable PAH amounts can appear when PAHs are released from sorptive sites. PAHs can be released from those sites by increasing temperatures (Gilot *et al.* 1997) by other components taking their place (Kästner and Mahro 1996) or by substances acting as surfactants which can be indigenous to the additives used for co-composting (Wong *et al.* 2004).

4.4.4.1. Interactions of microbial community changes and PAH contamination

Initial significant differences within the microbial community were attributed to the different additives. The microbial community profile obtained by PLFA analysis is similar to profiles obtained by other studies investigating microbial community developments during a composting process (Hellmann *et al.* 1997; Klamer and Bååth 1998). For the manure treatments the gradual decrease in biomass, estimated as total measured PLFAs, during the process is consistent with results obtained by others. This suggests that the microbial community is influenced more by factors introduced by the composting process, such as feedstock and temperature, than by the presence of the contaminants in the soil. Conversely, signs of a change in microbial communities to those that have been found to be important for

PAH degradation were also detected. Although similar to findings in the study by Kästner and Mahro (1996), no correlation was found between the microbial community profile and its changes and the changes in PAH concentrations. A lack of correlation between microbial communities and PAH degradation suggest that the organisms present are probably not able to grow on the PAHs, but that PAHs are degraded by co-metabolic or unspecified oxidation metabolism which can lead to the accumulation of metabolites and might therefore pose a risk as metabolites might be more toxic than the parent compound (Kästner and Mahro 1996). However, no accumulation of metabolites could be detected during the experiment.

PLFA results have to be interpreted with care though, as the profile obtained by PLFA extraction does not show changes within the different microbial groups. As conversion factors from the amounts of PLFAs characteristic for different micro-organisms to actual biomass are lacking, one must also bear in mind that PLFA results and changes in community patterns do not give absolute amounts of biomass of different groups (Klamer and Bååth 1998).

Fungi have been found to successfully degrade contaminants including PAHs (Gramss *et al.* 1999; Husain 2008; Mancera-López *et al.* 2008). A larger amount of fungal biomass has been introduced with the chicken manure resulting in a higher ratio of fungal-to-bacterial PLFA (Figure 4.7). Even though the rate of fungal-to-bacterial PLFAs quickly declines, falling below the ratio within the horse manure treatment, this initial high amount of fungi might have been responsible for the degradation of HMW PAHs. Additionally a higher amount of total Actinobacteria indicating PLFAs was detected, which have also been found to have a higher potential for PAH degradation (Pizzul 2006).

Overall the similarity between the pattern of the manure and the TC treatment suggests that the indigenous soil microbial community might be more important for the degradation of PAHs than the introduced micro-organisms and might profit from the nutrient addition or the introduced ecological niches, when manure is added, as suggested by Kästner and Mahro (1996).

4.5. CONCLUSIONS

Effects of the different manure types on the co-composting process could be identified (Thesis aim 1) and differences in the success of the co-composting processes were found to be treatment dependent. Key findings include:

- Physico-chemical characteristics differed between the manure treatments with the chicken manure treatment being most different while the horse and cattle manure treatments showed greater similarities.
- Differences between the treatment responses were attributed to the differences between the manure types used.
- Physico-chemical properties generally develop in a similar way with all treatments indicating that the same processes are taking place independent of the amendments.
- Manure addition increased the amounts of micro-organisms and changed the microbial composition by altering ratios of microbial groups depending on the manure type.
- The chicken manure treatment was mainly associated with Actinobacteria
- PLFA profiles of the treatments became generally more similar over the course of the experiment indicating that the degradation process was leading to a similar end product independent of the manure type used.

Decreases as well as increases in extractable PAH concentrations were detected for all groups of PAHs and with all treatments.

- With possible degradation processes taking place, releases of PAHs can lead to an underestimation of degradation rates, when looking at PAH concentrations obtained by solvent extraction.

- Extractable concentrations of LMW and MMW PAHs were decreased in all treatments by the end of the experiment.

- Extractable HMW PAH amounts were decreased slightly in the chicken manure treatment while increases were detected with the other treatments by the end of the experiment.

- Assuming that the processes that released the HMW PAHs within all but the chicken manure treatments also take place in the chicken manure treatment, leads to the conclusion that the chicken manure has a significantly higher potential for HMW PAHs removal counteracting the release of the PAHs.

- The higher potential for HMW PAH removal with the chicken manure might be due to adsorption to the higher TOM content of the chicken manure.

- Cattle manure addition increased the extractable amounts of total USEPA PAHs during the first temperature step. However, after 32 days total USEPA PAH concentrations decreased to amounts similar to those found at day 0.

- All treatments but the cattle manure treatment showed a removal of total USEPA PAHs.

- Degradation rates were highest during the low temperature steps at the beginning (LMW and MMW PAHs) and end of the experiment (all PAHs).

- The loss of PAHs through volatilisation was insignificant; however, a temperature dependency was detectable.

- Treatments showed potential for further PAH removal following the 32 day experimental period.

Even though the chicken manure amended treatment showed advantages in PAH removal, overall the similar behaviour of the TC treatment indicated that the factors influencing the changes in PAH concentrations were not inherent to the added manure, but rather the changes in temperature and the forced aeration with moisturised air.

For a large scale remediation strategy practical considerations have to be made. Even though the TC treatment shows similar results to the manure amended treatments, without manure addition a composting process, including an increase in temperatures, would not take place. Applying heat from the outside may prove too expensive to be a viable option for remediation. Additionally, manure addition can increase soil quality which might be another incentive to use a co-composting process. In this case, results of this study recommend the use of chicken manure before horse and cattle manure.

CHAPTER 5

Comparing the effects of different fungal inocula at different temperatures to bioremediate PAH contaminated soil

5.1. ABSTRACT

This Chapter investigates the potential of white-rot fungi in the form of fungi-inoculated sawdust to bioremediate aged PAH contaminated soil. Fungi have different degradation pathways to bacteria and have been found to be able to degrade even the higher molecular weight PAHs as well as those that cannot be physically reached by bacteria. The influence of the addition of sawdust inoculated with *Pleurotus ostreatus*, with *Trametes versicolor*, and a mix of both, incubation temperature and composting time on the loss of PAHs biotically (biodegradation) or abiotically (volatilisation) from soil were determined using gas chromatography-mass spectrometry. Simultaneous changes in microbial community structure were examined using phospholipid fatty acid (PLFA) profiling. Results revealed that PAH availability increased with temperature but that PAH loss through volatilisation was minimal in comparison to other removal processes. No correlation was found between PAH degradation and PLFA patterns. While no significant changes in LMW PAH concentrations were found MMW and HMW PAH concentrations decreased with all treatments and to a significantly larger extent with *P. ostreatus* addition with only 9% (MMW) and 17% (HMW) of the PAHs remaining in the soil. However, an influence of the level of contamination which was higher with the *P. ostreatus* amended vessels has to be considered. It was concluded that addition of *P. ostreatus* can be used as a successful strategy to remove additional HMW PAHs from an aged contaminated soil where simple oxygen introduction did not show any further success.

5.2. INTRODUCTION

Bioremediation of PAH contaminated soil relies on organisms degrading the contaminant. Those organisms mainly belong to the kingdom of bacteria or fungi. While bacteria have been found to degrade PAHs faster, certain fungi were found to be able to reach the contaminants more easily and to be able to degrade higher molecular weight PAHs (Cerniglia 1997).

White-rot fungi are wood decaying fungi able to degrade lignocellulose. PAHs have a similar structure to lignin wherefore white-rot fungi also have the potential to oxidise PAHs via non-specific extracellular enzymes (Grotenhuis *et al.* 1999). Since extracellular peroxidases are able to oxidise complex aromatic compounds, white-rot fungi are expected to be the most efficient fungi capable of degrading poorly available PAHs (Field *et al.* 1992).

Previous studies have looked at the ability of fungi to degrade PAHs in soil and positive effects as well as no effects on PAH degradation have been reported (Canet *et al.* 2001; Hestbjerg *et al.* 2003; ŠaŠek *et al.* 2003). However, due to different conditions in experimental design, soil types, and co-contaminants as well as inoculation-methods and different types of fungal inocula used it is very difficult to draw conclusions on the general suitability of different fungi for bioremediation of contaminated sites.

Even though white-rot fungi have been shown to have the highest potential to biodegrade PAHs, when introduced into soil they are confronted with an environment that tends to contain less nutrients than are normally present in wood and furthermore, those nutrients that are present in the soil tend to be in different forms/complexes to those generally found in wood. The soil is also a much more spatially heterogeneous environment (Baldrian 2008). Different soil types vary greatly in their physico-chemical properties, organic matter content, inorganic nutrient contents, texture, and microbial biomass. Nevertheless, the growth of wood-inhabiting ligninolytic fungi is limited due to the low amount of available carbon and nitrogen in most soils (Boyle 1995).

To provide the introduced fungi with more optimal starting conditions, one method is to introduce the fungi within a medium that gives them good growth conditions and from where they can grow into the soil matrix. With a suitable inoculum or substrate added to the soil most wood-inhabiting ligninolytic fungi are able to colonise sterile soil (Baldrian 2008).

The main factor affecting the success of wood-inhabiting ligninolytic fungi colonising soil is, however, the presence of indigenous soil organisms (Rayner and Boddy 1988). While different fungi vary in their ability to colonise soil (Novotný *et al.* 1999), it has also been found that, in general, the establishment of a fungus in the soil is faster and more successful, the larger the inoculum biomass (Baldrian 2008). This is especially important when the fungus is introduced to non-sterile soil where species can differ significantly in their ability to colonise the soil (Lang *et al.* 1997; Tornberg *et al.* 2003).

Interactions between wood-inhabiting ligninolytic fungi and soil organisms are mostly combative. *Pleurotus* spp., *Phanerochaete* spp., and *Trametes versicolor* have been classified as strong competitors and therefore as having a good ability to colonise non-sterile soil (Martens and Zadražil 1998; Baldrian 2008). However, colonisation ability has been found to be variable even within a species and partly depends on the soil type and fungal strain (Tornberg *et al.* 2003).

When aiming to develop a bioremediation strategy for large amounts of contaminated soil the material to be added to the soil will preferably be readily-available, inexpensive, and easy to handle/use. Most naturally found and readily-available additives are bacteria dominated. To investigate the behaviour and influence of white-rot fungi on the degradation of PAHs under different temperatures, sawdust inoculated with one of the white-rot fungi fungi *P. ostreatus* and *T. versicolor* was used as an additive suitable to be used in a co-composting process.

The aim of this part of the thesis is:

> To examine the effects of white-rot fungi on PAH removal from aged contaminated soil.
> (Thesis aim 2)

It is hypothesised that:

> White-rot fungi enhance the degradation of PAHs in soil during a co-composting process.

5.3. MATERIALS AND METHODS

5.3.1. Additives

The two white-rot fungi *Pleurotus ostreatus* (Oyster-mushroom) and *Trametes versicolor* (Turkey-tail-mushroom) were selected for the trials (Figure 5.1). Sawdust inoculated with either *P. ostreatus* or *T. versicolor* (Table 5.1) was delivered by Fruiting Bodies (Llangadog, Wales, UK).

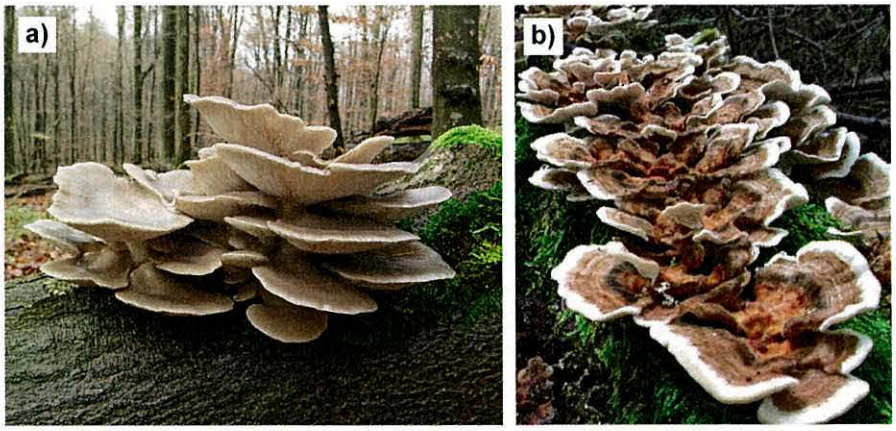

Figure 5.1: White-rot fungi *Pleurotus ostreatus* (a) and *Trametes versicolor* (b) growing on decaying wood.

Table 5.1: physico-chemical characteristics and microbial community profiles of the sawdust inoculated with the two different fungi used for the experiment (± S.D.) if applicable.

	Pleurotus ostreatus	*Trametes versicolor*
physico-chemical characteristics		
Dry matter (%)	27.6	19.3
pH	4.41 ± 0.03	3.96 ± 0.08
Total C (% of dry matter)	46.8 ± 0.6	47.1 ± 1.0
Total N (% of dry matter)	1.10 ± 0.30	0.80 ± 0.05
C:N ratio	44.5 ± 10.7	59.2 ± 4.5
Electrical conductivity (µS cm^{-1})	91 ± 6	145 ± 11
Microbial community profile (PLFA)		
Total bacterial PLFA (nmol g^{-1} dry sample)	50.9	108.3
Total bacterial PLFA added to the mixture (nmol g^{-1} soil)	6.18	9.19
Total fungal PLFA (nmol g^{-1} dry sample)	19.0	26.5
Total fungal PLFA added to the mixture (nmol g^{-1} soil)	2.31	2.24
Bacteria in % of total PLFAs	50.3	37.6
Actinobacteria in % of total PLFAs	1.53	2.57
Ratio of fungal to bacterial PLFAs	0.37	0.24
Ratio of Gram-negative to Gram-positive bacterial PLFAs	5.76	2.44
Ratio of iso-to-anteiso monounsaturated PLFAs	0.25	0.11

5.3.2. Treatments

The contaminated soil (see Chapter 3 "Materials and methods") was mixed with the inoculated sawdust by hand. For the fungi-amended treatments the soil was mixed with either *P. ostreatus* or *T. versicolor* inoculated sawdust in a ratio of 1:1 (v/v) or with both fungi in a ratio of 2:1:1 (v/v/v). A mixture of contaminated soil with sawdust in a ratio 1:1 (v/v) was used as the temperature control treatment. The treatments were as follows:

- P.ost : addition of *P. ostreatus* inoculated sawdust
- T.vers : addition of *T. versicolor* inoculated sawdust
- P&T-Mix : addition of *P. ostreatus* and *T. versicolor* inoculated sawdust
- TC : temperature treated control treatment

After sawdust addition, the moisture content of the soil mixtures was adjusted to 40 ± 4% on a dry weight basis by adding of tap-water. The characteristics of the soil and the sawdust as well as the methods used are described in Chapter 3 "Materials and Methods".

The temperature profile was changed weekly as follows: days 0-6, 20°C (room temperature); days 6-13, 25°C; days 13-20, 30°C; days 20-27, 35°C; days 27-34, 50°C; days 34-41, 65°C; followed by days 42-48 at 35°C representing the cooling/maturation phase of a composting process.

5.4. RESULTS AND DISCUSSION

5.4.1. Physico-chemical analysis

5.4.1.1. Total organic matter content

The TC treatment consistently had the lowest TOM in comparison to the other treatments ($p < 0.0001$). It also exhibited the lowest rate of TOM loss, decreasing from 20.8 to 19.8% over the 48 d incubation period (Figure 5.2). The fungi amended treatments had similar TOM contents initially. Treatments with only one added fungus (P.ost and T.vers) showed quicker and greater decreased in TOM than the TC or the P&T-Mix treatments. Greater losses in TOM indicate a higher decomposition during the simulated composting process.

5.4.1.2. pH

The pH values ranged from 7.69 (P.ost, day 41) to 8.70 (P&T-Mix, day 20). Values showed a similar behaviour for all treatments (Figure 5.2). After a decrease until day 13 the pH increased significantly ($p < 0.05$) during the next 2 weeks (until day 27) and then decreased over the next 14 days to

levels similar to those measured at day 13. Compared to the other treatments the pH with the P.ost treatment was significantly lower ($p < 0.005$) at days 0 and 6. These lower pH values might have been caused by the added fungus *P. ostreatus* which has been found to lower the pH (Lang *et al.* 1997).

5.4.1.3. Total N

The percentage of total N in the compost mixtures ranged from 0.07% (day 6, TC treatment) to 0.84% (day 48, P.ost treatment). No significant differences were detected between the different treatments (Figure 5.2). For all treatments total N contents decreased significantly from day 0 to day 6 followed by an increase until day 27 ($p < 0.01$). After a slight decrease concentrations increased again in a significant way from day 41 to 48, reaching concentrations similar to or slightly higher than the initial starting concentrations.

5.4.1.4. Total C

Total C content of the mixtures did not show any significant differences between the treatments (Figure 5.2). Similar to total N concentrations total C contents decreased during the first week. No major changes were detected during the following 5 weeks before total C concentrations increased again during the last experimental week.

5.4.1.5. Carbon-to-nitrogen ratio

The C:N ratio of the treatment mixtures was not significantly different between the treatments with an exception of day 20 where a significantly higher ratio ($p < 0.005$) was detected for the T.vers treatment compared to the P&T-Mix and the TC treatment (Figure 5.2). The temporal dynamics of substrate C:N ratio behaved similarly for all treatments. The ratios

increased significantly during the first 6 days. After this initial increase the ratios decreased to significantly lower ($p < 0.0001$) values by day 27 followed by an increase before values decreased again during the last week of the experiment.

5.4.1.6. Electrical conductivity

Electrical conductivity (EC) measurements showed a similar behaviour for all of the treatments (Figure 5.2). All the EC values were similar until day 34. During the 65°C temperature step (days 34 to 41) the EC declined significantly ($p < 0.01$) for all but the P&T-Mix treatment followed by a significant increase during the last temperature step (35°C, days 41 to 48).

5.4.1.7. Carbon dioxide emissions

The TC treatment had significantly higher CO_2 emissions than the other treatments at the start of the experiment (Figure 5.2). The CO_2 emissions increased nearly 4-fold for the P.ost treatment during the first 13 days, while decreases were detected for the other treatments. CO_2 emissions stayed low for the TC and the P&T-Mix treatment and decreased with the P.ost treatment. The P.ost and T.vers treatments show a similar CO_2-emission-pattern from day 20 onwards, with low levels at day 27, a peak at day 34 (50°C) and a slight increase from day 41 to 48. Due to a relatively high variability between the replicate vessels those differences where not statistically significant.

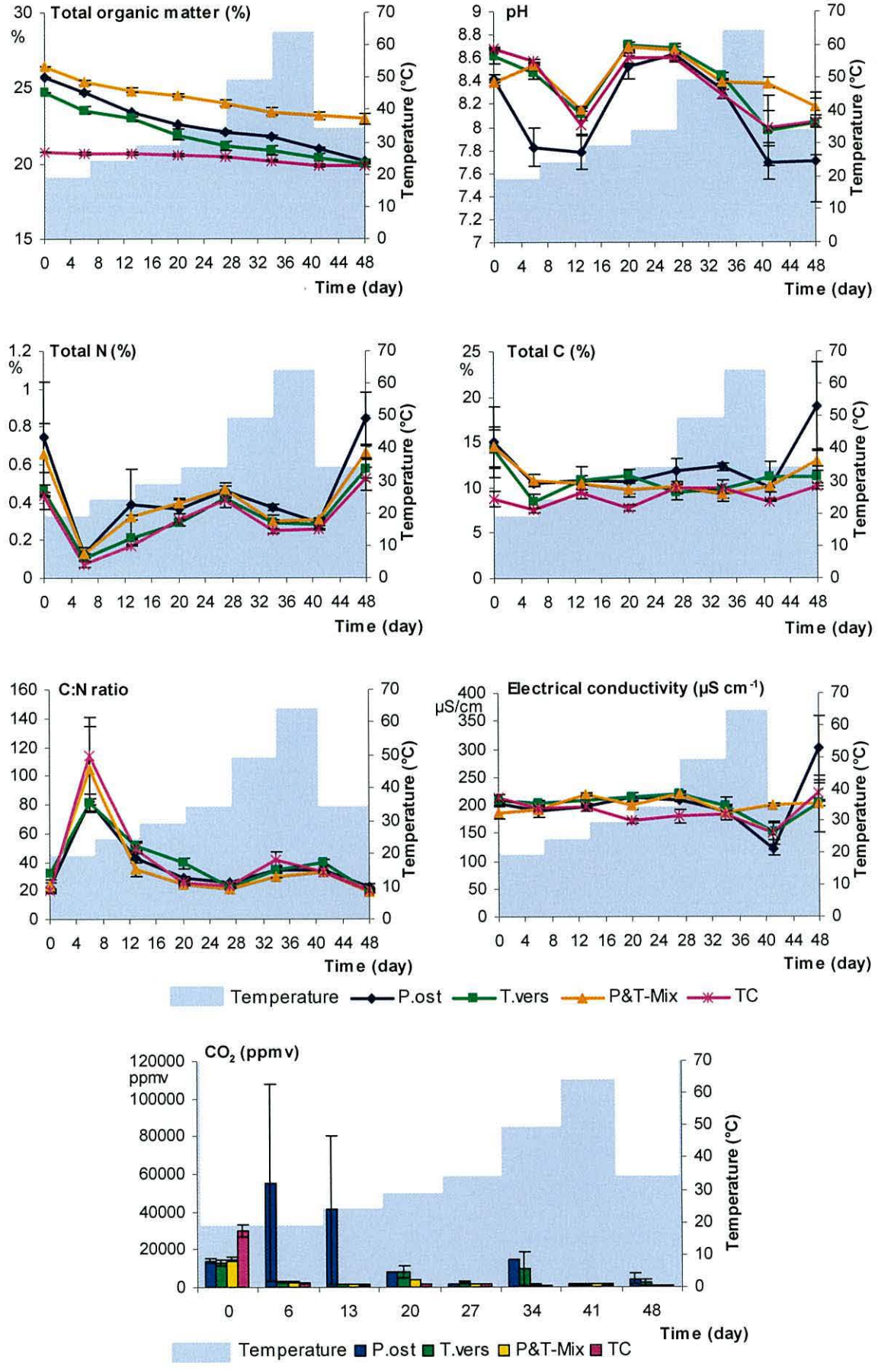

Figure 5.2: Mean values of the different physico-chemical analyses with the different treatments during the different stages of the 48 d experiment. Treatments are labelled as P.ost = blue, T.vers = green, P&T-Mix = yellow, TC = pink. The light blue background represents the applied temperature profile. Error bars represent mean ±S.E.; n=3

5.4.2. Microbial community changes

5.4.2.1. Total microbial and bacterial PLFAs

Total amounts of microbial PLFAs recovered from the soil (Figure 5.3) showed the same behaviour as amounts of bacterial PLFAs. After addition of the different additives total bacterial as well as microbial PLFA amounts were highest for the P.ost treatment, followed by the P&T-Mix, T.vers, and TC treatments.

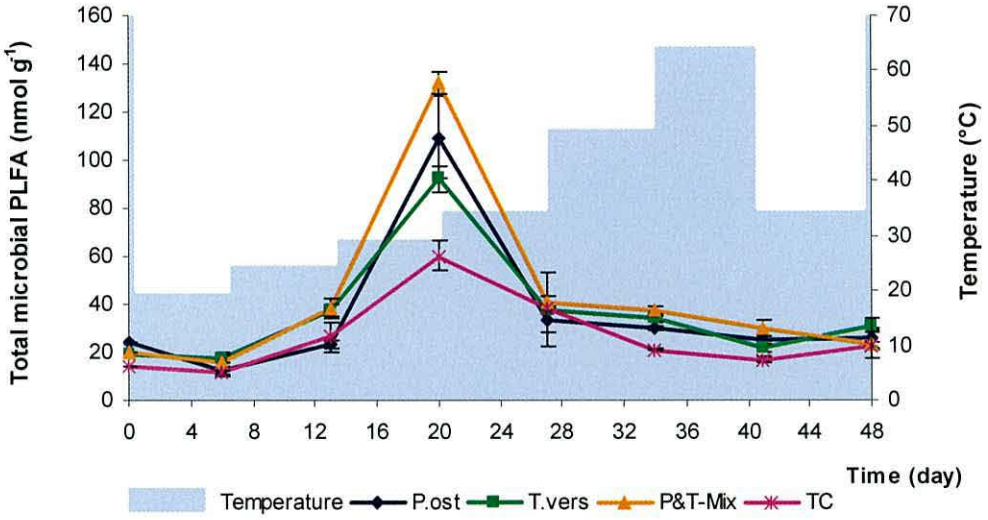

Figure 5.3: Mean values of total measured microbial PLFAs in nmol per g dry sample detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3

Total PLFA amounts were significantly lower for the TC treatment during most of the experimental period. They significantly increased for all treatments ($p < 0.0001$) during the first 3 temperatures steps and reached their highest values at day 20 followed by a subsequent decrease.

5.4.2.1.1. Gram-negative and Gram-positive bacteria indicating PLFAs

The ratios of Gram-negative-to-Gram-positive PLFAs were similar for all treatments over the whole course of the experiment (Figure 5.4). Rates decreased until day 20 followed by an increase during the following period form day 20 to 27 and a subsequent decrease during the remaining three weeks of the experiment. An exception to this behaviour is the TC treatment at day 13. The Gram-negative bacteria indicating PLFA cy19:0 increased significantly during the 25°C temperature step from day 6 to 13.

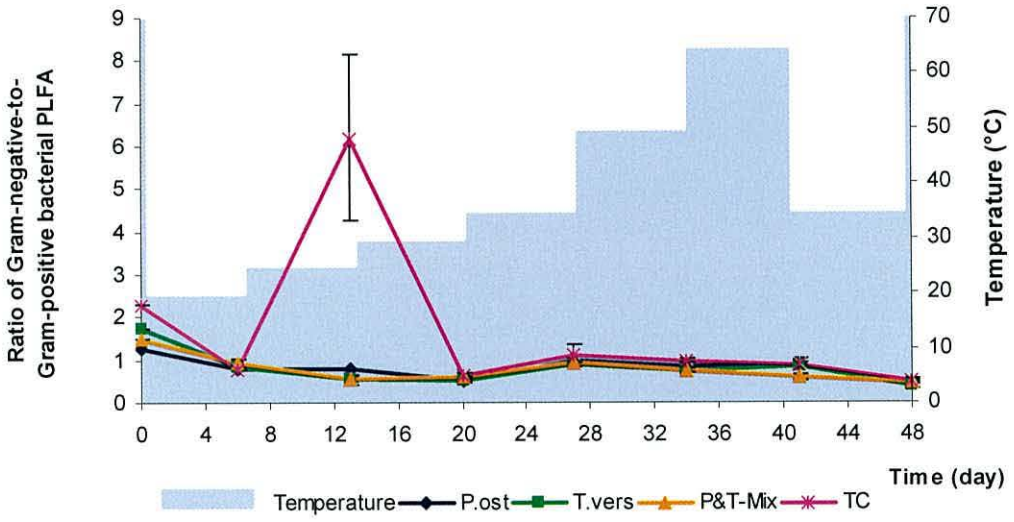

Figure 5.4: Mean values of the ratio of Gram-negative-to-Gram-positive bacteria indicating PLFAs detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3

Results of this study show definite differences between the fungi-amended and the TC treatment in the behaviour of bacterial PLFAs during the first steps of the experiment. In the TC treatment Gram-negative bacterial PLFAs increased during the 25°C temperature step to a larger extent, while Gram-positive bacterial PLFAs decreased for the TC treatment (Figure 5.5). On the other hand total Gram-positive bacterial PLFAs increased with the fungi-amended treatments.

Gram-negative bacteria are known to increase when easily utilised C is released in a bacterial community (Marielley and Agragano 1990). Easily utilised C could have derived from the added sawdust and made available by bacteria or fungal decomposition processes.

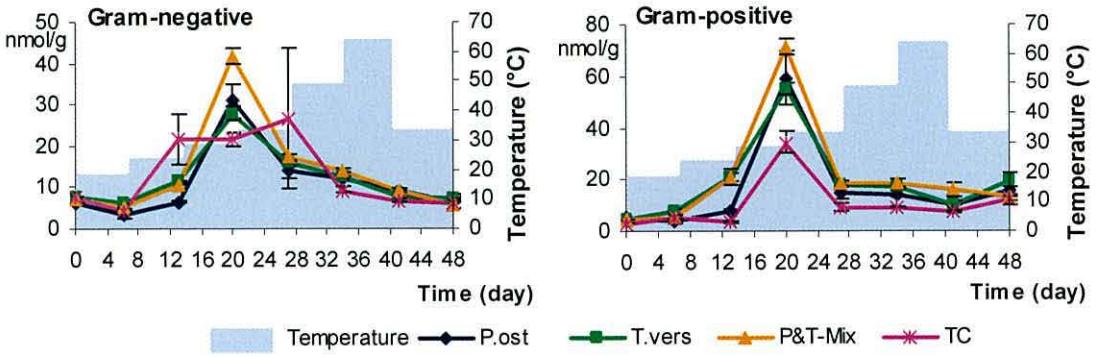

Figure 5.5: Mean values of the concentration of Gram-negative and Gram-positive bacteria indicating PLFAs in nmol per g dry sample detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3

Gram-negative bacteria have been found to usually dominate systems in hydrocarbon contaminated environments (MacNaughton *et al.* 1999) and in an ordinary composting process the PLFA cy19:0 indicating Gram-negative bacteria has been found to increase until temperatures reach 50°C, followed by a decrease at higher temperatures and a subsequent increase when temperatures decrease again (Klamer and Bååth 1998).

In this study, especially high increases in the Gram-negative bacteria indicating PLFA cy19:0 were found in the TC treatment. The increase also appeared earlier during the process than with the fungi-amended treatments. It has been found that the ratio of Gram-negative to Gram-positive bacteria increases under different stress conditions such as temperature increases and exposure to contaminants (Frostegard *et al.* 1993; Zelles *et al.* 1994; Kaur *et al.* 2005).

Cyclopropyl PLFAs (cy17:0 and cy19:0 – both Gram-negative bacteria indicating PLFAs) are formed by transmethylation of *cis*-monounsaturated fatty acids, as the cell enters the stationary phase. These PLFAs are more stable and help maintain a functional living membrane by minimising lipid losses or changes in membrane fluidity (Guckert *et al.* 1986; Kaur *et al.* 2005). Increases of cyclopropyl PLFAs have been detected during stress conditions including temperature increase, starvation, and low pH (Kaur *et al.* 2005) and a greater survival of Gram-negative bacteria might be due to those fatty acids in their membrane (Guckert *et al.* 1986).

5.4.2.2. Stress indicating ratio of iso-to-anteiso branched PLFAs

Bacteria are known to alter their membrane lipids in response to stress (White 1994; Kieft *et al.* 1997). Similar to increases in cyclopropyl PLFAs, increasing in the ratio of iso-to-anteiso branched PLFAs is one way in which bacteria respond to increasing stress levels (Kaneda 1991; Kieft *et al.* 1994; McKinley *et al.* 2005).

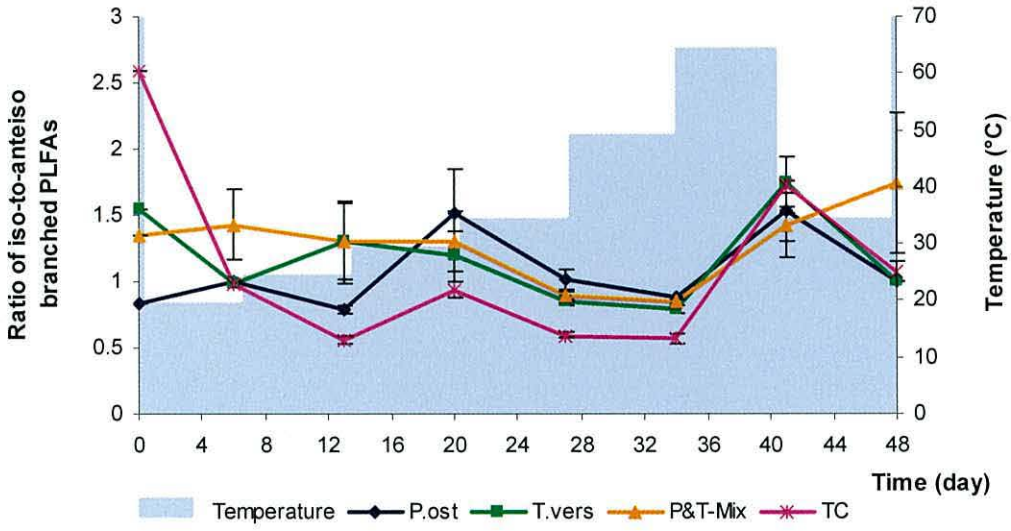

Figure 5.6: Mean values of the ratio of iso-to-anteiso branched PLFAs detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3

The ratio of iso-to-anteiso branched PLFAs decreased until the end of the 50°C temperature step with the P.ost and TC treatments showing an increase during the 30°C temperature phase from day 13 to 20 (Figure 5.6). The ratio increased in all the treatments during the 65°C temperature step indicating temperature stress on the microbial community. As with the other PLFAs no correlation could be detected with any other parameter measured. Correlations with single parameters might however, be difficult to detect due to complex interactions between all the factors.

5.4.2.2.1. Actinobacteria indicating PLFA

Total measured amounts of Actinobacteria indicating PLFAs changed in the same way as amounts of total microbial PLFAs. The percentage of bacterial PLFA attributable to Actinobacteria generally increased until day 20 (30°C) with all treatments (Figure 5.7).

The decrease in the Actinobacteria to bacterial PLFA ratio with the TC treatment at day 13 was again attributed to the sharp increase in PLFA cy19:0 which contributes to a rise in bacterial PLFAs.

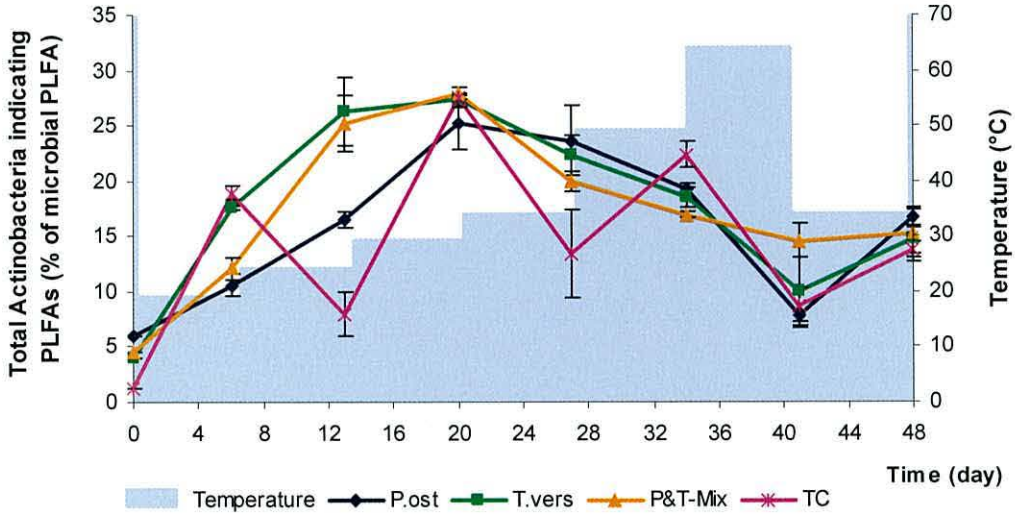

Figure 5.7: Mean values of the ratio of total Actinobacteria indicating PLFAs in % of total microbial PLFAs detected in the different treatments over the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3

With increasing temperatures, the ratios of Actinobacteria decreased further until the end of the heating phase. Only when the temperature was decreased during the last experimental period ratios of Actinobacteria increased again to higher levels.

5.4.2.3. Fungi indicating PLFA

Similar to the total microbial PLFA amounts total fungal PLFA detected in the treatment mixtures decreased during the first experimental period after mixing the inoculum with the soil and increased during the following two temperature steps (Figure 5.8).

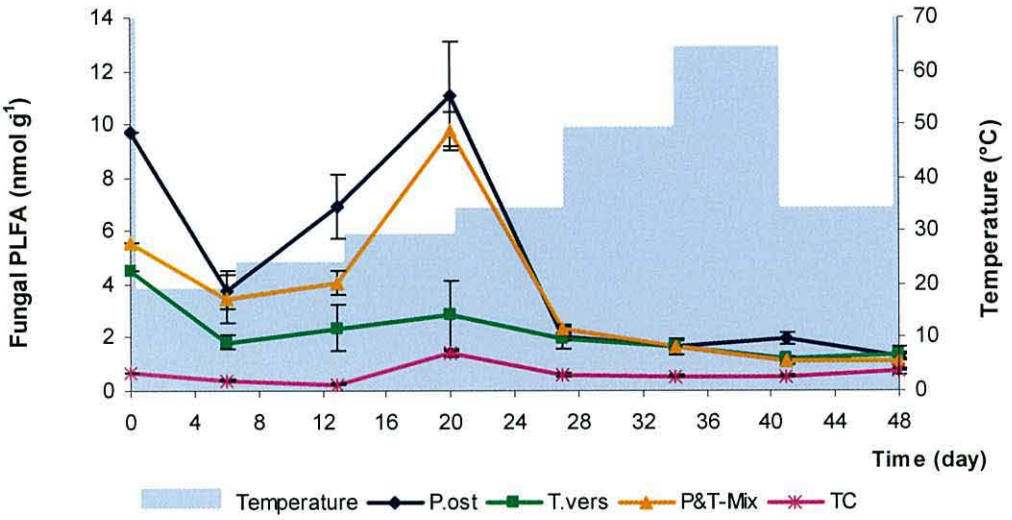

Figure 5.8: Mean values of the amount of fungi indicating PLFAs in nmol per g dry sample with the different treatments over the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3

With treatments containing the *P. ostreatus* inoculum (P.ost and P&T-Mix) the fungal PLFA increased about three-fold up to a total of 11.0 and 9.8 nmol per g dry mixture, while the amounts with the T.vers treatment only increased slightly staying below the initial amounts measured. The fungal PLFA amounts increased about two-fold with the TC treatment, reaching amounts of 1.4 nmol per g dry mixture.

Within all treatments, the ratio of fungal-to-bacterial PLFAs in the soil-mixtures decreased significantly towards the end of the experiment (Figure 5.9). Even though no significant differences in the total amounts of fungal PLFA could be detected between the fungi-amended treatments, the ratio of fungal-to-bacterial PLFA showed initial differences between the treatments.

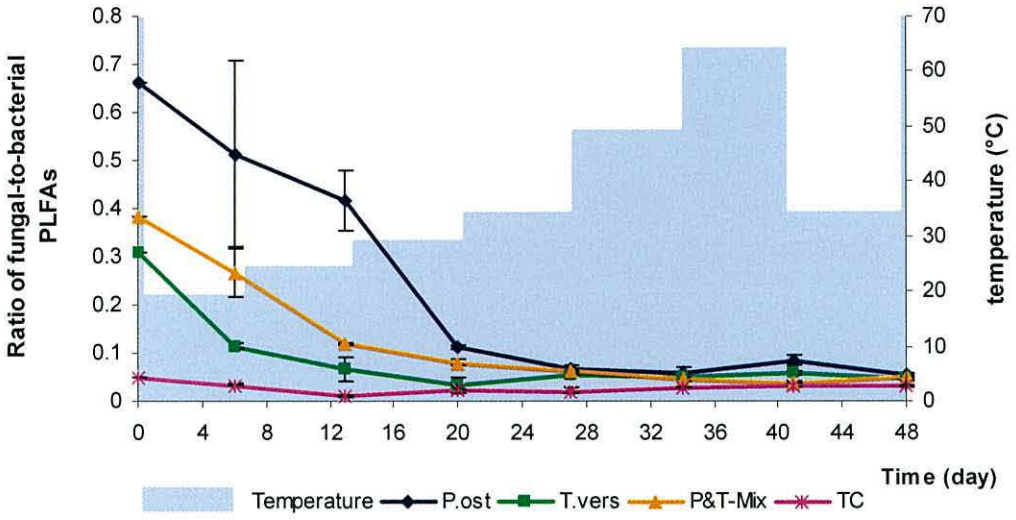

Figure 5.9: Mean values of the ratio of fungal-to bacterial PLFAs detected in the different treatments during the different stages of the 48 day experimental period. Error bars represent mean ±S.E.; n=3

The highest ratio was detected with the P.ost treatment with values around twice as high as with the other fungi-amended treatments and about ten times higher than in the TC treatment. However, for the fungi-amended treatments those ratios decreased quickly until day 20 before levelling out at values around 0.066, 0.053, and 0.049 within the P.ost, T.vers, and P&T-Mix treatments respectively, compared to values of around 0.031 with the TC treatment.

5.4.2.4. Differences in the microbiological profile of the different treatments

Interactions of treatment and temperature (time) were detected by split-plot ANOVA for the different groups of PLFAs. Those interactions were in all cases mainly caused by the TC treatment behaving differently to the fungi-amended treatments.

Differences between treatments were mainly detected for the fungi indicating PLFA (total amounts, ratio of fungal-to-bacterial PLFA). In the beginning of the experiment the TC treatment showed significantly lower values in these cases, while the fungi amended treatments were similar to each other. Those initial differences were attributed to the addition of the fungi inoculated sawdust. However, the amount of fungi indicating PLFA behaved similarly in all treatments including the TC treatment, suggesting that the introduced fungi did not grow well under the given conditions. Total amounts, as well as the ratio of fungal-to-bacterial PLFA remained significantly higher in the fungal inoculated treatments than in the TC treatment suggesting that the surviving fungi can maintain a status in which they can compete with the bacterial community. The higher ratios of fungal PLFA in the P.ost treatment additionally support findings that *P. ostreatus* is a better soil coloniser (Novotný *et al.* 1999) and can compete with the indigenous soil microbial community.

Surprisingly, Diagnostic Biplot analysis of the extracted PLFA data revealed distinct differences between the treatments right from the beginning of the experiment (Figure 5.10). After the fungi-inoculated sawdust had been mixed with the contaminated soil the P.ost treatment was mainly associated with the fungal PLFA 18:2w6 as well as the Gram-positive bacteria indicating PLFAs 15:0, a15:0, and 10Me18:0; the latter also indicative for Actinobacteria. The P&T-Mix treatment on the other hand was mainly associated with the Gram-positive bacteria indicating PLFAs i17:0 and 10Me16:0; the latter also indicative for Actinobacteria. Contrastingly, the T.vers treatment was closer associated with the Gram-negative bacteria indicating PLFA cy17:0. While the P.ost treatments remains closer associated with the fungal PLFA 18:2w6 until day 13, the other fungi-amended treatments become more similar during this time. The associations found by Bioplot analysis are partly reflected by the patterns detected for grouped PLFAs showing higher ratio of Actinobacteria indicating PLFAs in relation to total microbial PLFAs in the T.vers and P&T-Mix treatments (Figure 5.7) and a higher concentration of fungal PLFA in the P.ost treatment (Figures 5.8 and 5.9).

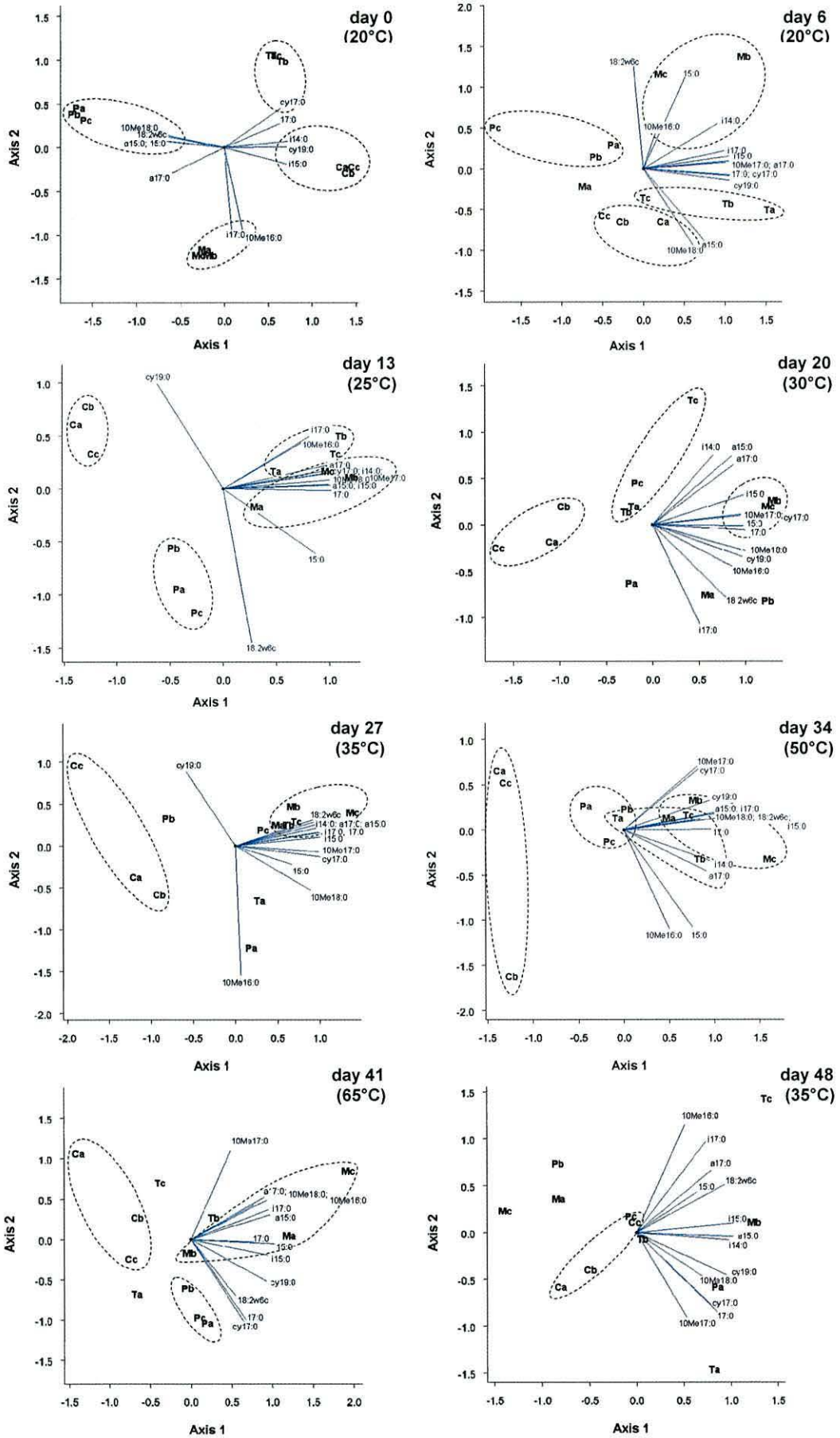

Figure 5.10: Diagnostic Biplots showing associations of PLFAs with the different treatments at each sampling point. The treatments are indicated as follows: P = P.ost, T = T.vers, M = P&T-Mix, C = TC, a-c indicate replicate vessels.

During the remaining weeks of the experiment treatments become more similar to each other with the TC treatment remaining to show more distinct differences to the other treatments. The fungi-amended treatments are most similar at the end of the 50°C temperature step (day 34) and different PLFAs become more abundant in different vessels during the 65°C temperature step and especially during the final cooling phase (35°C, days 41 to 48)

Differences in the treatments might be explained by a higher amount of fungal PLFA and especially a higher ratio of fungal-to-bacterial PLFAs that have been added to the P.ost treatment with the *P. ostreatus* inoculated sawdust (Table 5.1) resulting in higher initial fungal PLFA concentrations as well as ratios of fungal-to-bacterial PLFAs (Figures 5.8 and 5.9).

5.4.3. Changes of extractable PAH amounts

To take the weight loss of the soil/sawdust/fungi mix into account, the results for the extracted PAHs were corrected by referring to the amount of ash, as this is considered to be the most chemically stable parameter during the degradation process (Amir *et al.* 2005). Due to the inhomogeneous distribution of the contaminants in the aged PAH contaminated soil, as well as to investigate temperature effects on PAH removal, the results were looked at in three different ways ("percentage change", "rates of degradation", and "concentration") as detailed in Chapter 3 (Materials and methods)

5.4.3.1. Changes in percentages of PAH concentrations remaining in the treatment mixtures over the 48 day experiment

5.4.3.1.1. Effects of treatments on extractable USEPA PAHs

In comparison to the initial PAH concentration, significantly lower concentrations of extractable PAHs were detected at the end of the experimental period in all treatments ($p < 0.05$). The percentage removal of total measured USEPA PAHs was highest in the P.ost treatment ($p<0.05$) (Figure 5.11).

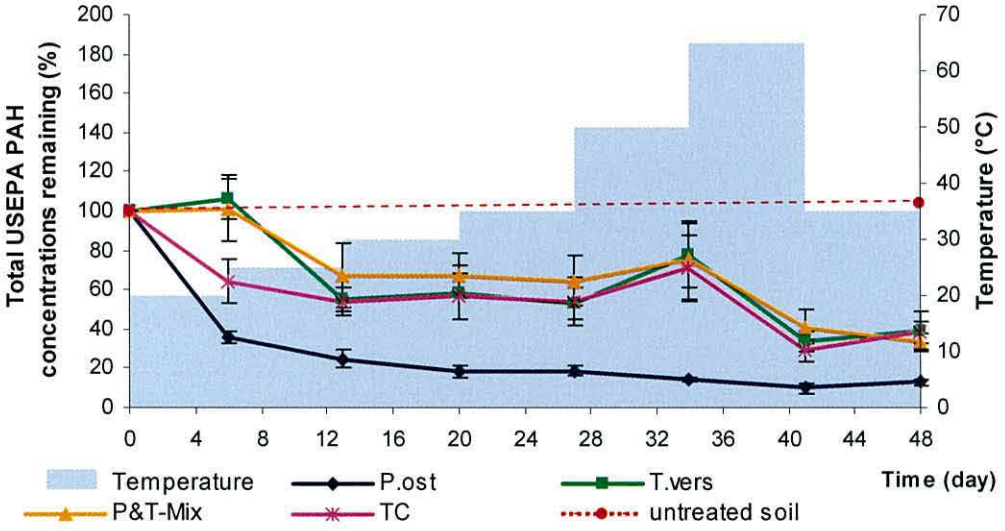

Figure 5.11: Mean percentages of concentrations of total USEPA PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining total PAHs detected in the untreated soil after 48 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=3

Concentrations of nearly all the individual PAHs were significantly lower at the end of the experiment for all treatments relative to that present at the start (Table 5.2). No significant decreases were detected for Phenanthrene, Anthracene, and Chrysene with the P.ost treatment and Phenanthrene, Anthracene, and Benzo[g,h,i]perylene with the T.vers treatment. The T.vers treatment was also the treatment without a significantly lesser percentage of LMW PAHs remaining at the end of the experiment. In the P&T-Mix

treatment, Anthracene concentrations had not declined while in the TC treatment Benz[a]anthracene also showed similar concentrations to those found at the beginning of the experiment.

Table 5.2: Percentage of individual PAHs remaining at the end of the 48 d experiment.

	P.ost	T.vers	P&T-Mix	TC
	% ±S.E.	% ±S.E.	% ±S.E.	% ±S.E.
LMW PAHs				
Naphthalene	24.5±16.4^{a*}	60.6±17.2^{a}	50.6±16.5^{a*}	61.8±18.8^{a}
Acenaphthylene	16.1±4.7^{a*}	44.8±10.8^{a*}	42.8±9.7^{a*}	41.7±3.2.^{a*}
Acenaphthene	9.6±4.3^{a*}	37.5±14.2^{a*}	42.1±6.0^{a*}	32.7±6.1^{a*}
Fluorene	15.0±8.1^{a*}	41.9±18.2^{a*}	34.3±9.5^{a*}	39.4±10.2^{a*}
Phenanthrene	69.4±24.8^{a}	124.6±44.8^{a}	64.5±10.5^{a*}	65.6±12.0^{a*}
Anthracene	111.4±37.6^{a}	89.3±18.7^{a}	65.2±22.1^{a}	39.2±18.8^{a*}
MMW PAHs				
Fluoranthene	9.3±1.0^{b*}	30.1±10.4^{ab*}	30.4±4.3^{a*}	29.2±2.7^{ab*}
Pyrene	12.7±1.3^{b*}	36.9±11.0^{a*}	32.8±6.1^{ab*}	42.2±3.4^{a*}
Benz[a]anthracene	0.5±0.2^{a*}	13.0±5.4^{a*}	5.2±3.3^{a*}	114.5±86.9^{a}
Chrysene	41.9±41.8^{a}	1.5±0.8^{a*}	0.0±0.0^{a*}	8.6±5.1^{a*}
HMW PAHs				
Benzo[b]fluoranthene	18.9±0.9^{b*}	45.3±7.9^{a*}	50.7±5.3^{a*}	63.3±9.5^{a*}
Benzo[a]pyrene	18.2±2.9^{b*}	55.5±9.2^{a*}	66.4±8.9^{a*}	62.5±8.1^{a*}
Indeno[1,2,3-c,d]pyrene	21.3±4.3^{b*}	61.5±11.7^{a*}	70.9±9.2^{a*}	65.3±9.8^{a*}
Dibenz[a,h]anthracene	11.8±1.8^{b*}	52.9±12.8^{a*}	61.1±6.4^{a*}	57.8±6.8^{a*}
Benzo[g,h,i]perylene	6.0±1.6^{a*}	139.6±105.4^{a}	33.3±3.6^{a*}	37.2±7.2^{a*}
Grouped PAHs				
Total LMW PAHs	63.9±11.8^{a*}	84.9±23.6^{a}	53.6±10.9^{a*}	41.9±14.6^{a*}
Total MMW PAHs	10.6±1.6^{b*}	31.6±9.2^{a*}	28.6±3.9^{a*}	37.1±3.3^{a*}
Total HMW PAHs	18.6±1.8^{b*}	53.9±10.6^{a*}	56.9±6.5^{a*}	62.5±9.2^{a*}
Total USEPA PAHs	14.2±1.7^{b*}	38.8±10.0^{a*}	33.4±4.1^{ab*}	38.4±5.8^{a*}

Values are means of the percentage of extractable PAHs remaining in % of the amount found in the soil at the beginning of the experiment ±S.E., n=3, values with the same letter are not significantly different between the treatments and * indicates a significant difference to the concentrations found at the beginning of the experiment at the $p < 0.05$ level as determined using a Tukey post-hoc test.

PAH concentrations in the untreated soil were not significantly different to those at the start by the end of the 48 day experimental period (94 to 117% of that present at the start). Increases and decreases in PAH concentrations varied between single PAHs and over the course of the experiment (Figures 5.12a and b).

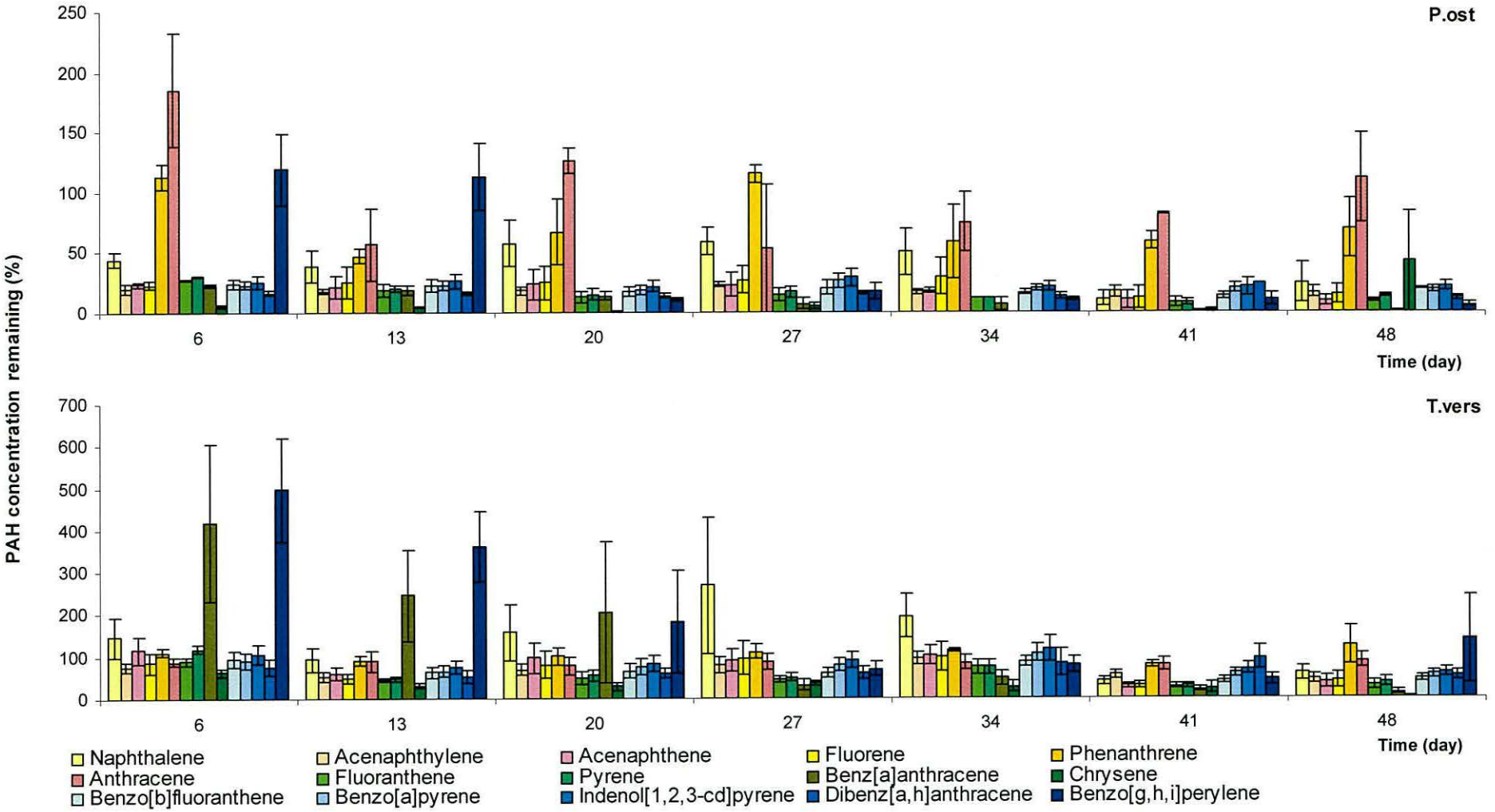

Figure 5.12a: Mean percentages of concentrations of individual PAHs remaining in the P.ost and T.vers treatments by the end of the different experimental periods. Error bars represent mean ±S.E.; n=3

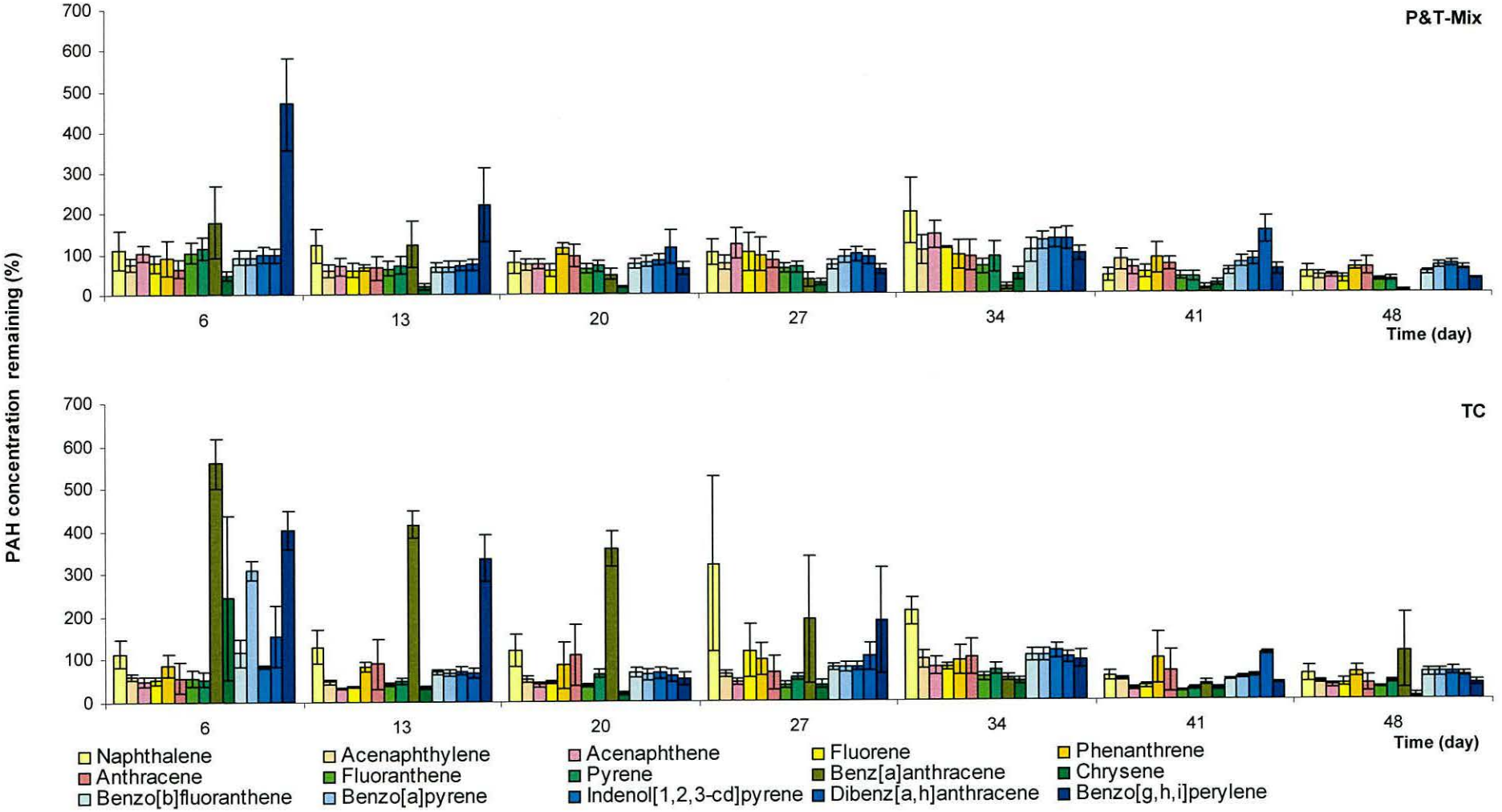

Figure 5.12b: Mean percentages of concentrations of individual PAHs remaining in the P&T-Mix and TC treatments by the end of the different experimental periods. Error bars represent mean ±S.E.; n=3

With the P.ost treatment the most significant decrease in concentration happened within the first 6 days for all HMW PAHs except Benzo[g,h,i]perylene, where the significant decrease was detected after the first 35°C temperature step (day 20). After this, concentrations declined further, with another significantly higher rate of Dibenz[a,h]anthracene loss during the last temperature step (35°C, day 41 to 48).

With the T.vers and P&T-Mix treatments significant changes in concentrations were only detected for Benzo[g,h,i]perylene. For this PAH concentrations increased initially before decreasing.

Besides Benzo[g,h,i]perylene, the PAHs Benzo[b]fluoranthene and Benzo[a]pyrene showed the same behaviour in the TC treatment. Indeno[1,2,3-c,d]pyrene concentrations on the other hand showed a variable trend with both increases and decreases during the experiment.

5.4.3.1.2. Treatment effects of PAHs group into low, middle, and high molecular weight categories

Temporal changes in the extractable concentrations of grouped PAHs present in the soil over the course of the experiment are presented below. With the untreated soil (red dotted line), the amounts of grouped PAHs only change slightly remaining at 112% for total measured USEPA PAHs (Figure 5.16), and 103%, 105%, and 105% for grouped LMW, MMW, and HMW PAHs respectively (Figures 5.13-5.15).

Effects of treatments on LMW PAHs

The variation in total LMW PAH concentrations between replicate vessels was relatively high as indicated by the error bars (Figure 5.13) and no significant changes in grouped LMW PAH concentrations were found during the experiment.

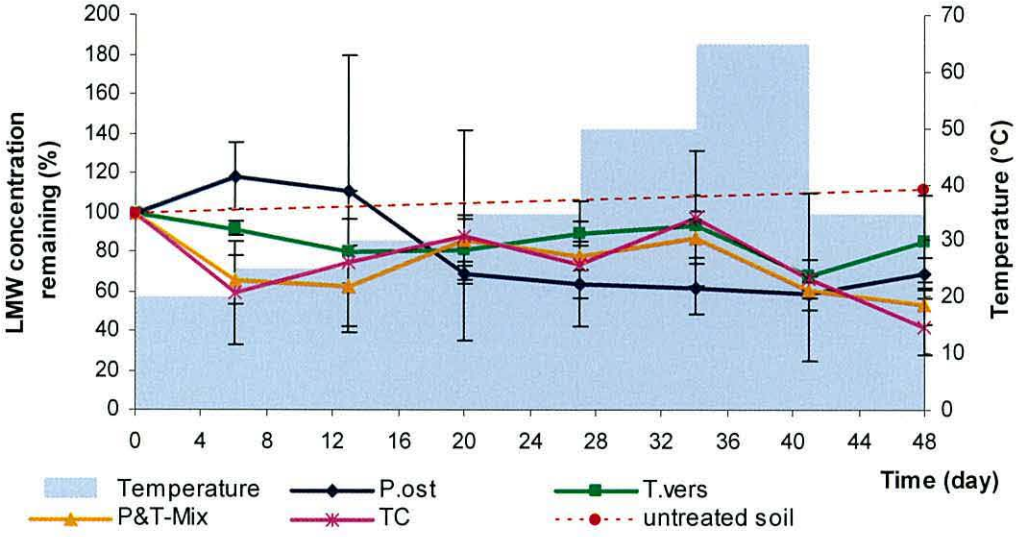

Figure 5.13: Mean percentages of concentrations of grouped LMW PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining LMW PAHs detected in the untreated soil after 48 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=3

Effects of treatments on MMW PAHs

The concentration of the grouped MMW PAHs was significantly lower at the end of the experimental period for all treatments ($p < 0.05$) (Figure 5.14). The highest decreases were detected for the P.ost treatment with only 11% of the initial extractable concentration of MMW PAHs remaining by the end.

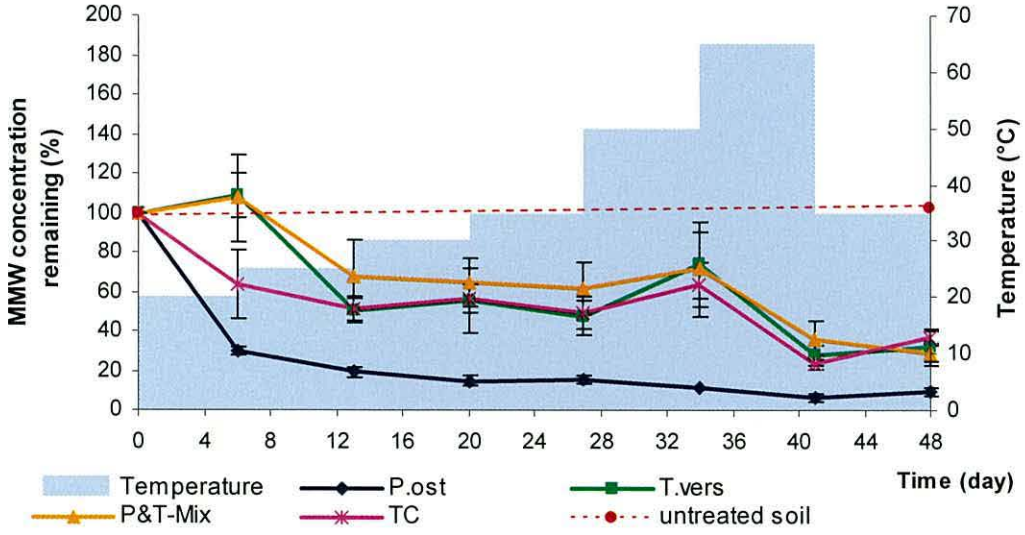

Figure 5.14: Mean percentages of concentrations of grouped MMW PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining MMW PAHs detected in the untreated soil after 48 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=3

With the P&T-Mix, T.vers, and TC treatment the amount of the extractable PAHs remaining were 29% (±4), 32% (±9), and 38% (±3) respectively. Biggest decreases were detected at the beginning and during the 65°C temperature step. This was also reflected in the degradation rates during single temperature steps (see below, part 5.4.3.2.). Split-plot ANOVA revealed an interaction of treatment and temperature (time) ($p < 0.05$) caused mainly by the P.ost treatment where, after an initial substantial decrease, concentrations between time points varied less than with the other treatments.

Effects of treatments on HMW PAHs

The total extractable HMW PAH concentrations (Figure 5.15) changed in a similar way to the total MMW PAHs concentrations. No significant changes were detected for the T.vers and P&T-Mix treatments during the experimental period. With the TC treatment concentrations increased initially before decreasing. At the end of the experimental period the total HMW PAH concentration with the TC treatment was not significantly different from the start.

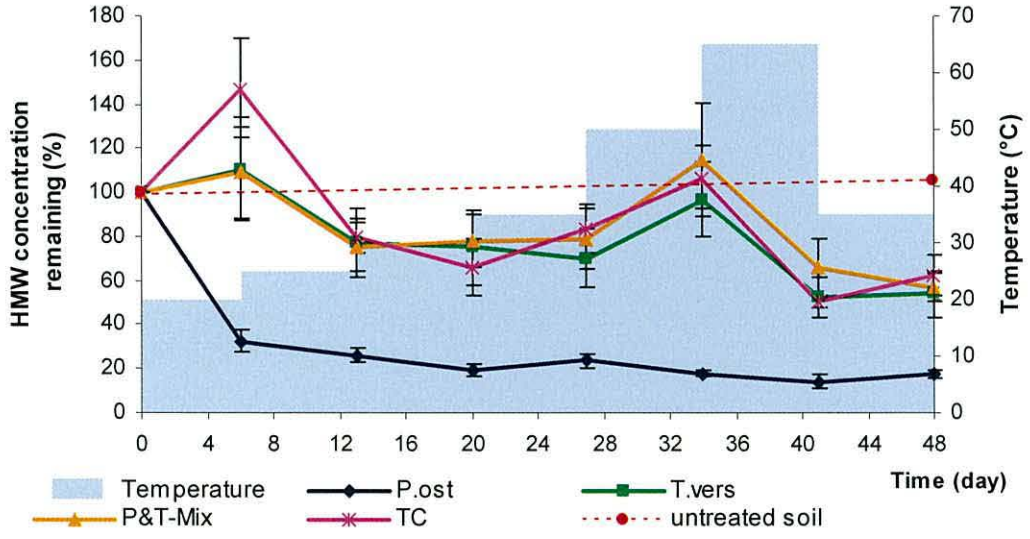

Figure 5.15: Mean percentages of concentrations of grouped HMW PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining HMW PAHs detected in the untreated soil after 48 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=3

With the P.ost treatment the total HMW PAH concentration decreased significantly during the first 6 days with a slight further decrease until day 41 ($p < 0.0001$). Across all treatments, the P.ost treatment decreased the extractable HMW PAH concentrations by the highest percentage during the experiment (17 ± 2% remaining). With the T.vers, P&T-Mix, and TC treatments 54 ± 11%, 57 ± 7%, and 63 ± 9% of the initial concentrations respectively, remained in the soil.

As for MMW PAHs split-plot ANOVA revealed an interaction of treatment and temperature (time) ($p < 0.05$) caused mainly by the P.ost treatment where, after an initial substantial decrease, concentrations between time-points varied less than with the other treatments.

5.4.3.2. Influence of incubation temperature on changes in PAH concentrations

Analysis of differences in degradation rates between the implied temperature steps revealed that highest loss of PAHs generally occurred during the first two (20° and 25°C) and the 65°C (penultimate) temperature steps. Degradation rates for single USEPA PAHs behaved similarly. With the T.vers, P&T-Mix, and TC treatments highest rates of loss mainly occurred during the 65°C temperature step.

The P.ost treatment resulted in highest degradation rates during the first temperature step for all detected PAHs. Degradation rates of Phenanthrene, Anthracene, Benz[a]anthracene, and Benzo[g,h,i]perylene did not differ significantly at any stage, while rates of Naphthalene and Chrysene loss were highest at 50-65°C.

With the T.vers treatment no difference in degradation rates could be detected with temperature for the LMW and MMW PAHs Acenaphthylene, Phenanthrene, Anthracene, Benz[a]anthracene, and Chrysene; the HMW PAHs Dibenz[a,h]anthracene and Benzo[g,h,i]perylene had highest degradation rates during the 25°C (day 13 to 20) and the last (35°C) temperature step.

The P&T-Mix treatment did not show significant differences in degradation rates for LMW and MMW PAHs in the MW range 166-202 and for Benz[a]anthracene (MW 228). For this treatment, the highest degradation rates for Acenaphthylene, Chrysene, and Dibenz[a,h]anthracene were detected during the last temperature step. Due to increases during the first temperature step and the first 35° and 50°C temperature steps Benzo[g,h,i]perylene degradation rates were highest during the remaining stages of the experiment.

With the TC treatment no significant differences in degradation rates could be detected for Fluorene, Phenanthrene, Anthracene, Chrysene, and Dibenz[a,h]anthracene. For Benz[a]anthracene the highest degradation rate was detected during the first 35°C temperature step, while Benzo[a]pyrene and Benzo[g,h,i]perylene had highest degradation rates during the previous week (30°C).

The degradation rates of summed LMW PAHs (Figure 5.16) did not differ significantly for any of the treatments during the experimental period.

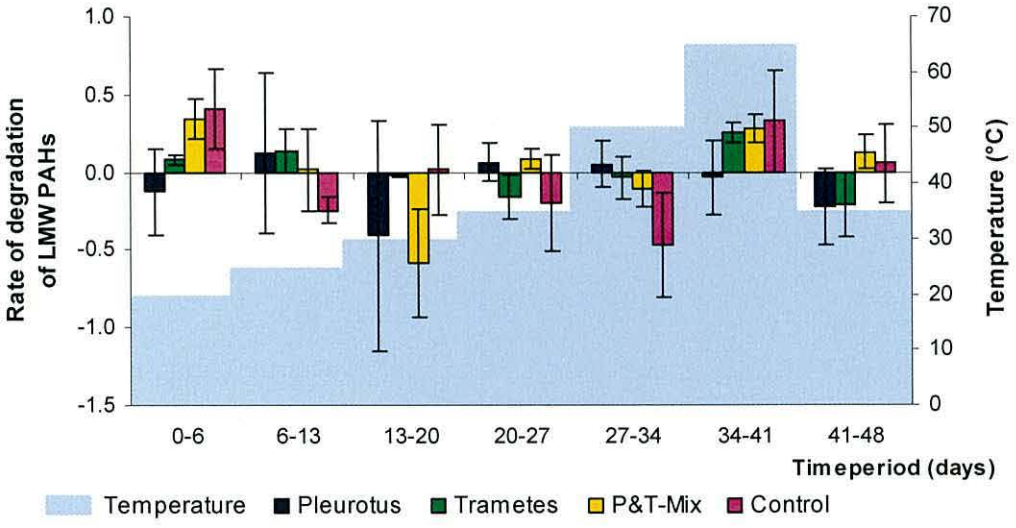

Figure 5.16: Mean rates of degradation of grouped LMW PAHs with the different treatments during the different temperature steps of the 48 day experiment. Error bars represent mean ±S.E.; n=3

The degradation rates for summed MMW PAHs were highest during the first 6 days for the P.ost treatment and during the 65°C temperature step (day 34 to 41) for the T.vers and TC treatments (Figure 5.17). Degradation rates were not significantly different between temperature steps with the P&T-Mix treatment.

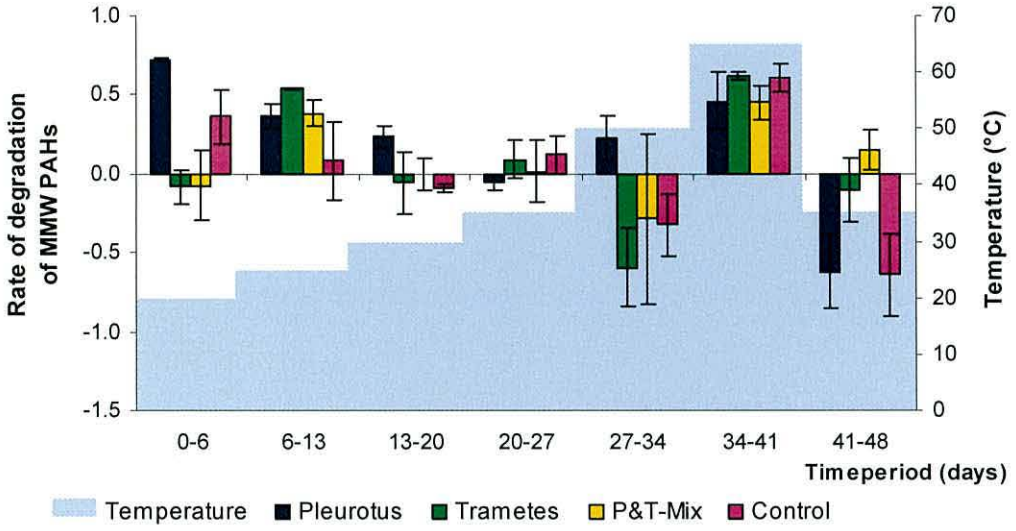

Figure 5.17: Mean rates of degradation of grouped MMW PAHs with the different treatments during the different temperature steps of the 48 day experiment. Error bars represent mean ±S.E.; n=3

Between treatments, no significant differences could be detected during any of the temperature steps, except during the first 6 days where degradation rates with the P.ost treatment were significantly higher than those with the other fungi-amended treatments ($p < 0.05$).

As with summed MMW PAHs, the degradation rates for summed HMW PAHs (Figure 5.18) were highest during the first 6 days for the P.ost treatment and during the 65°C temperature step (day 34 to 41) for the T.vers and TC treatments. With highest degradation rates during the 25°C (day 6 to 11) and the 65°C temperature step the P&T-Mix treatment behaved like the TC treatment.

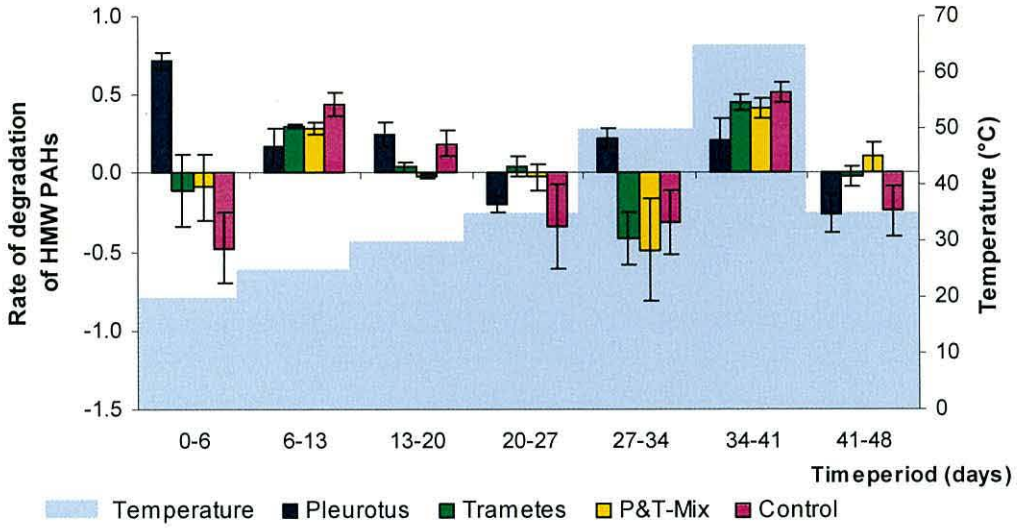

Figure 5.18: Mean rates of degradation of grouped HMW PAHs with the different treatments during the different temperature steps of the 48 day experiment. Error bars represent mean ±S.E.; n=3

Significant differences in degradation rates of total HMW PAHs were only detected between treatments during the first 6 days where degradation rates with the P.ost treatment were significantly higher than those with the other three treatments ($p < 0.01$).

5.4.3.3. Effects of extractable PAH concentrations

At the beginning of the experiment concentrations of extractable PAHs differed between the treatments (Table 5.3), with the P.ost treatment having higher extractable PAH concentrations per g ash than the other treatments. Looking at the degradation process purely by analysing degradation rates and concentrations expressed as a percentage of the initial amounts might distort results as differences between treatments might be attributed to the effect of the additive, overlooking the effects of the absolute PAH amounts.

Table 5.3: PAH concentrations of 15 detected USEPA PAHs at days 0 and 48 of the experiment after mixing the soil with the different additives. Values are averages (µg per g ash) of the amounts measured in the replicate vessels ±S.D.; n=3

		P.ost	T.vers	P&T-Mix	TC
	day	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.
LMW PAHs					
Naphthalene	0	12.4 ±2.0	5.0 ±1.8	7.7 ±3.5	4.1 ±0.7
	48	*2.1 ±0.8*	*2.5 ±0.5*	*2.3 ±0.2*	*2.7 ±0.2*
Acenaphthylene	0	10.2 ±1.2	3.4 ±0.8	3.5 ±1.1	3.6 ±0.4
	48	*1.4 ±0.1*	*1.4 ±0.1*	*1.5 ±0.0*	*1.3 ±0.1*
Acenaphthene	0	31.4 ±5.7	11.4 ±4.3	8.0 ±2.2	11.3 ±0.4
	48	*2.4 ±0.3*	*3.0 ±0.6*	*3.7 ±0.8*	*3.1 ±0.3*
Fluorene	0	44.9 ±9.8	21.2 ±8.8	17.9 ±6.2	18.9 ±1.2
	48	*5.2 ±0.7*	*5.8 ±1.9*	*7.4 ±1.8*	*5.0 ±0.3*
Phenanthrene	0	100.5 ±20.0	45.5 ±5.7	45.4 ±12.1	96.9 ±30.2
	48	*79.7 ±18.6*	*51.8 ±12.0*	*56.3 ±10.9*	*27.0 ±4.8*
Anthracene	0	122.4 ±9.0	107.0 ±20.2	87.8 ±20.7	382.1 ±205.1
	48	*129.0 ±16.5*	*88.6 ±6.2*	*79.3 ±6.9*	*48.3 ±3.5*
MMW PAHs					
Fluoranthene	0	2030.2 ±302.5	654.3 ±167.4	546.7 ±130.2	667.8 ±46.2
	48	*163.6 ±25.7*	*170.0 ±46.4*	*193.3 ±15.2*	*155.7 ±14.8*
Pyrene	0	2365.6 ±290.0	746.1 ±168.0	678.0 ±184.9	781.7 ±66.3
	48	*250.1 ±48.3*	*252.6 ±66.0*	*327.7 ±25.0*	*199.7 ±9.3*
Benz[a]anthracene	0	479.1 ±49.9	64.5 ±42.6	89.1 ±29.4	21.3 ±1.6
	48	*2.0 ±1.0*	*3.9 ±0.4*	*22.9 ±16.9*	*2.7 ±0.7*
Chrysene	0	68.8 ±7.6	16.9 ±4.2	17.8 ±4.6	17.1 ±1.7
	48	*15.6 ±15.5*	*0.3 ±0.2*	*1.4 ±0.8*	*0.0 ±0.0*
HMW PAHs					
Benz[b]fluoranthrene	0	264.7 ±25.5	90.6 ±22.1	80.1 ±16.2	87.8 ±7.0
	48	*47.2 ±4.2*	*38.0 ±4.6*	*54.3 ±4.9*	*39.0 ±3.7*
Benzo[a]pyrene	0	62.8 ±5.7	22.7 ±5.4	19.9 ±4.4	21.5 ±2.2
	48	*9.9 ±1.2*	*11.6 ±0.9*	*13.1 ±0.6*	*12.4 ±0.9*
Indeno[1,2,3-cd]pyrene	0	99.5 ±11.1	34.1 ±9.0	31.3 ±7.2	33.0 ±3.4
	48	*18.3 ±2.1*	*18.9 ±1.3*	*20.9 ±1.1*	*20.9 ±1.6*
Dibenz(a,h)anthracene	0	13.3 ±0.9	4.2 ±0.9	2.8 ±0.6	3.2 ±0.3
	48	*1.4 ±0.2*	*2.1 ±0.6*	*1.8 ±0.1*	*1.6 ±0.2*
Benzo[ghi]perylene	0	19.2 ±2.7	6.3 ±1.9	6.0 ±1.6	6.1 ±1.1
	48	*0.7 ±0.3*	*6.2 ±4.0*	*2.1 ±0.2*	*1.9 ±0.4*

Total amounts of PAHs can influence degradation rates for PAHs. In this experiment it emerged that the total initial concentrations of PAHs were up to three times higher with the P.ost treatment. Even though the soil was carefully mixed before separation and mixing with the different additives for the different treatments, it appears that the batch of the soil used for the P.ost treatment had a higher amount of PAHs. It has been found that the concentration of PAHs in soil influences degradation efforts and the existence of threshold values below which no further degradation can occur has been suggested (Dubourguier 2003).

A threshold value would result in PAH concentrations levelling out towards the end of the experimental period and no further changes in PAH concentrations being detectable. Significant changes in PAH concentrations could still be detected for the T.vers, P&T-Mix and TC treatments during the last temperature periods indicating that further reductions in extractable PAH concentrations may be possible. With the P.ost treatment, significant changes in PAH concentrations could not be detected after day 20 indicating that a threshold value might have been reached within this treatment.

Possible interactions of PAH amounts with physico-chemical properties, microbial community changes, PAH volatilisation, or PAH degradation were analysed using Spearman's rank correlation. Values of correlation coefficients (n=78, DF=76) were mainly < 0.4 representing only a weak correlation which might not have been caused by the PAH concentration but might be due to other factors influencing both variables in the same way.

However, some modest correlations ($|r|=0.6$ to 0.7) were found between Benz[a]anthracene as well as Benzo[g,h,i]perylene concentrations and the Gram-positive bacteria indicating PLFA a17:0 (r=-0.611, r=-0.683), % total N (r=-0.607, r=-0.659), and C to N ratio (r=0.610, r=0.667). Even though correlation coefficients were indicating a correlation those components were also highly correlated with time ($|r|>0.7$). Therefore it is suggested that the correlation between the PAHs and the PLFA or physico-chemical characteristic, respectively, is likely to be coincidental.

5.4.3.4. PAH volatilisation

The amounts of PAHs lost into the vapour phase were related to the amount of contaminated soil in the vessel. The losses of PAHs from the soil due to volatilisation were relatively low for all detected PAHs and represented an insignificant loss pathway until day 34 when temperatures were changed to 65°C. The rate of loss of PAHs by volatilisation increased significantly during the 65°C temperature step ($p < 0.01$) before decreasing again when the temperature was lowered to 35°C during the last week of the experiment.

The volatilisation rates of LMW PAHs (Figure 5.19) started to increase when temperatures reached 50°C. During the highest temperature step (65°C) up to 0.45% of the extractable LMW PAHs in the vessel were volatilised (TC) and 0.32%, 0.26%, 0.23% with the T.vers, P&T-Mix, and P.ost treatments, respectively.

As expected much lower total amounts of MMW PAHs (Figure 5.19) were volatilised than LMW PAHs. Volatilisation rates of MMW PAHs were over 10 times smaller than those for LMW PAHs. Early in the process the volatilisation rates of MMW PAHs increased (days 6 to 13) to around 100 ppm but then decreased to about 20 ppm until the end of the 50°C temperature step (day 34). During the 65°C temperature step rates increased 18-fold with the TC treatment to 430 ppm while rates with the fungi-amended treatments stayed significantly lower with amounts between 127 and 154 ppm.

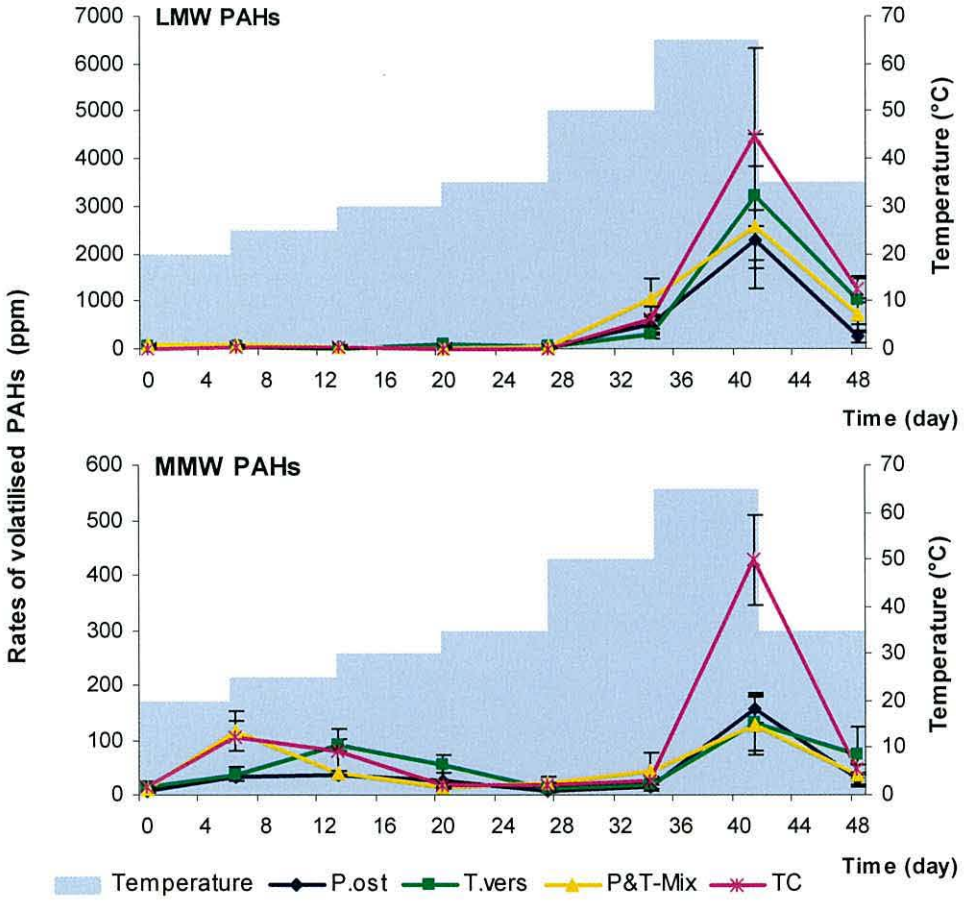

Figure 5.19: Mean rates of volatilised LMW and MMW PAHs with the different treatments during the different temperature steps of the 48 day experiment. Error bars represent mean ±S.E.; n=3

During the lower initial temperature steps (up to day 27), volatilisation rates of LMW and MMW PAHs were similar. Results show that enhanced temperature stimulated abiotic removal of PAHs via volatilisation. Volatilisation rates increased with both groups of PAHs and with all treatments. However, rates with the fungi-amended treatments stayed significantly lower than with the TC treatment, especially for the MMW PAHs. In addition rates were below 40 ppm for the P.ost treatment during the lower temperature steps, while values were much higher in the other treatments (~100 ppm). This suggests that the fungal treatments, and the P.ost treatment in particular, help to keep abiotic volatilisation of PAHs to a minimum.

5.4.4. Discussion of effects of different process characteristics on PAH removal

The soil used in this experiment came from the same source as the soil used in the manure experiment (Chapter 4) and was placed into a barrel and kept outdoors sheltered from rain for 33 days after the 2 months windrow pre-treatment (while the manure experiment was performed). As established within the manure experiment, extractable PAH concentrations did not change during this storage time and the soil still contained relatively high quantities of PAHs.

An important factor governing the success of bioremediation strategies is bioavailability of PAHs. Mechanisms resulting in increases of extractable PAH amounts are releases of PAHs from sorptive sites by: increasing temperatures (Gilot *et al.* 1997), other components taking their place (Kästner and Mahro 1996), or substances acting as surfactants (Wong *et al.* 2004). Decreases on the other hand can be caused by biodegradation due to microbial activity (direct use of the contaminant as C-source or co-metabolic degradation) and by sorption to the matrix of the added material. Although mineralisation decreases when covalent binding of PAHs with humic substances occurs, white-rot fungi have been found to degrade humic-bound pollutants (Haider and Martin 1988; Wunderwald *et al.* 2000).

No correlation was found between the amount of total fungal PLFA and absolute PAH concentrations, PAH concentrations remaining or degradation rates. The better performance of the P.ost treatment might have been due to the higher initial PAH concentration. This theory is supported by the fact that while total PAH amounts are significantly higher with the P.ost treatment at day 0; no significant differences could be detected in PAH amounts after day 11.

5.4.4.1. Effects of the treatments on the survival and activity of the introduced fungi

The most important factor for successful bioremediation of contaminated soil with specific organisms is that the environment offers suitable growth conditions for the inoculated organisms. Introducing the fungi within their natural habitat reduced the risk of competition and vulnerability towards predators and therefore increased the viability of the fungi. However, mixing with the contaminated soil changed the environmental conditions. This might have resulted in the introduced fungi only being able to be effective at the beginning of the process when the matrix they were introduced with, was still their primary growth matrix. A similar effect has been found by Atagana (2004) who found that organisms readily degraded PAHs whilst still growing on the poultry manure they were introduced with. The quick decline of the amount of fungi indicating PLFA in the fungi-amended treatments suggested that the introduced fungi were not able to maintain their high numbers under the new conditions.

Most white-rot fungi prefer an acidic pH, therefore, with pH values between 8.4 and 8.6 being observed after mixing the inocula with the soil, the fungi faced an environment that may not sustain favourable growth conditions. Additionally, other studies found that white-rot fungi generally compete poorly with soil micro-organisms and are inhibited in their growth by soil humic substances (Rayner and Boddy 1988; Steffen *et al.* 2000). However, *Pleurotus ostreatus* has been found to colonise soil and inhibit bacterial

growth by its ability to produce antibiotics and a highly hydrophobic mycelium, and by maintaining a low pH (Lang *et al.* 1997; Novotný *et al.* 1999; Tuomela 2002). *Trametes versicolor* on the other hand has been found to be a less successful soil coloniser (Novotný *et al.* 1999).

Results from this study seem to reflect those characteristics. The ratio of fungal-to-bacterial biomass declined quicker with *T. versicolor* addition than with *P. ostreatus*. This, however, might be due to the higher amount of bacterial PLFA that has been introduced with the *T. versicolor* inoculum compared to the *P. ostreatus* inoculum, especially as total amounts of fungal biomass decline faster with the P.ost treatment which had a higher total amount to start with. Further, the pH measured with the P.ost treatment showed a drop by 0.6 units within the first 6 days decreasing slightly over the next week, supporting the results found by other studies on the ability of *P. ostreatus* to increase acidity and maintain a lower pH. With the other treatments the pH dropped slower, reaching its lowest levels by day 13 being 0.4 units higher than with the P.ost treatment.

Another factor influencing fungal growth is temperature. The optimum temperature for *P. ostreatus* has been identified at 30°C (Zervakis *et al.* 2001). Although differences in optimal growth temperatures exist even between different strains, most white-rot fungi become inactive at temperatures above 35°C (Hale 2008, personal communication). During the experimental period total fungal PLFA decreased, however, when temperatures were increased from 20 to 25°C and even more noticeable, when they increased further to 30°C, a substantial increase in fungal biomass was detected especially for the *P. ostreatus* amended treatments. In a study by D'Annibale *et al.* (2005), the two white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus pulmonaris* were tested for their ability to bioremediate an aged PAH contamination. The authors found that during the 30 day experiment at 28°C both fungi survived under non-sterile conditions after being inoculated into the contaminated soil, but that they only exerted a limited impact on the concentration of the organopollutants. Those results suggest that the temperature profile introduced with this study might have been an additional factor for the low survival/growth rate of the introduced fungi.

The most obvious decrease in PAHs was found with the P.ost treatment during the first 6 days of the experiment, when the temperature was 20°C. White-rot fungi are able to degrade lignin by producing lignin peroxidases, manganese peroxidases, and/or laccases which are thought to be responsible for PAH degradation (Harayama 1997). In a study by Lang *et al.* (2000) it was found that *Pleurotus* sp. produced highest laccase activities in soil at 25 to 30°C and highest manganese peroxidase activities at 20°C, which peaked during the first week of their experiment, decreasing relatively quickly afterwards. This might explain the increased PAH loss with the P.ost treatment during the first week of the experiment, when temperatures stayed low and fungal biomass was highest.

The fact that the P&T-Mix treatment did not perform better in the way that PAH degradation would occur somewhere between the degradation rates detected with the single *P. ostreatus* and *T. versicolor,* might be explained by competition between the two introduced fungi inhibiting each others growth and therefore PAH degradation. Studies on interactions between those two fungi are scarce, however two studies by Tsujiyama and Minami (2005) and Radulovic (2006) investigated the effects those fungi have on each other. The final outcome of competition was seemingly contradictive between the two studies emphasising the fact that competition outcome is dependent on several factors such as environmental conditions and even species strain and difficult to predict. However, in both studies it was shown that the fungi inhibited each others growth or even halted it.

Initial PAH amounts were higher with the P.ost treatment. This can partly be the reason why degradation rates were higher during the initial step and it could be argued that the other treatments, especially the other two fungal treatments would have shown similarly high degradation rates would concentrations have been higher with them. However, the P.ost treatment also showed decreases during the 50°C temperature step when amounts increased with the other treatments, as well as less loss of PAHs through volatilisation.

5.5. CONCLUSIONS

Overall, this experiment examined the effects of the white-rot fungi *P. ostreatus* and *T. versicolor* (Thesis aim 2) and showed that organopollutant removal from contaminated soil is dependent upon the co-composting conditions. The key findings are summarised below:

- Significant differences in physico-chemical characteristics of treatments were found at the beginning of the experiment for pH and CO_2, both becoming more similar during the following temperature steps. No other significant differences in physico-chemical characteristics were detected.
- The addition of the fungal inocula increased the amounts of micro-organisms and changed the microbial community composition mainly by increasing the number of fungal PLFA resulting in significantly higher ratios of fungal-to-bacterial PLFAs. The total amounts of fungi and the ratio of fungal-to-bacterial PLFAs was highest for the P.ost treatment over the whole course of the experiment.
- No clear correlations between physico-chemical analysis or indicators of microbial community structure and concentrations of extractable PAHs could be detected.

Decreases as well as increases in extractable PAH concentrations were detected with all groups of PAHs and with all treatments.

- No change in PAH concentration was detected with the untreated soil. Significant changes in PAH concentrations were found for all analysed PAHs and for all the other treatments.
- For MMW and HMW PAHs, degradation rates were highest during the low temperature steps in the beginning of the experiment and during the 65°C temperature step.

- No accumulation of metabolites (PAH breakdown intermediates) could be detected.
- The T.vers and P&T-Mix treatments showed similar changes in PAH concentrations to the TC treatment.
- Addition of *P. ostreatus* inoculated sawdust decreased extractable PAH concentrations during the first experimental period and also resulted in a higher removal rate by the end of the experiment.
- Extractable PAH concentrations were highest with the P.ost treatment at the beginning of the experiment. This possibly influenced the higher rates of PAH removal.
- The loss of PAHs through volatilisation was insignificant; however differences between treatments and a temperature dependency were detected.

Overall, the results of this experiment indicate that the addition of *P. ostreatus* inoculated sawdust can enhance the degradation of an aged PAH contamination from soil. The hypothesis that white-rot fungi enhance the degradation of PAHs in soil during a co-composting process has to be accepted in the case of single addition of *P. ostreatus*, but rejected for *T. versicolor* and a combination of both fungi.

Considering the high degradation rates in the beginning of the experiment and formerly identified characteristics of *P. ostreatus* including its better ability to colonise soil, an addition of sawdust inoculated with this fungus seems recommendable for the remediation of an aged contaminated soil especially when high amounts of higher molecular weight PAHs are present. It also seems recommendable to add *P. ostreatus* as the only fungus, and not to mix it with other fungi, which might result in competition between the introduced fungi.

For a large scale application the influence of temperature and inoculum size should be further investigated by performing a pilot-scale experiment without external temperature application.

CHAPTER 6

Effects of *Pleurotus ostreatus* and chicken manure addition at different temperatures/times on the bioremediation of PAH contaminated soil

6.1. ABSTRACT

Contaminant availability, the presence of organisms able to degrade PAHs, and suitable conditions for their growth and metabolism are the main requirements for successful bioremediation of contaminated soil. Bacteria and fungi are the main organisms involved in contaminant degradation. While bacteria are considered to degrade PAHs more quickly they can have difficulties reaching the contaminants and their activity is restricted to smaller PAHs. Fungi however, can distribute themselves through the soil matrix and can degrade even the larger PAHs. A combination of bacteria and fungi should therefore lead to a more successful bioremediation process. This study investigates the effect of the addition of *Pleurotus ostreatus* inoculated sawdust and chicken manure on the degradation of a soil contaminated with mainly larger, weathered PAHs. Considering possible competition between the indigenous soil micro-fauna, the added fungus, and the micro-organisms added with the chicken manure, the chicken manure was added to the soil and *P. ostreatus* mixture at different stages during a simulated co-composting process. A soil/sawdust mix without *P. ostreatus* and manure addition served as a temperature-treated control and PAH concentrations were compared to those in a completely untreated soil. The results showed that addition of *P. ostreatus* inoculated sawdust increased PAH removal rates at the beginning of the process. Although manure addition changed the matrix conditions it did not induce significant losses of the measured PAHs. Of the tested treatments, a treatment with initial fungal addition and manure addition during a later stage of a co-composting process was the treatment with highest PAH removal rates.

6.2. INTRODUCTION

Successful bioremediation of PAH contaminated soil depends on the availability of the contaminant, the micro-organisms present (mainly bacteria and fungi), their community interactions and survival, and the environmental growth conditions. Bacteria have been found to be able to use several PAHs as a sole source for both carbon and energy and to mineralise some PAHs more quickly than fungi (Cerniglia 1997). However, they have limitations as they are mainly found in surface biofilms which may not allow them to reach the contaminant (Cases and de Lorenzo 2005). In addition many bacteria only produce intracellular enzymes which often limits them to the degradation of LMW PAHs (Grotenhuis *et al.* 1999). Fungi, on the other hand, build mycelia that permeate the soil and produce extracellular degradation systems which can reach contaminants that are less accessible to bacteria (Cerniglia 1997). Fungi can produce extracellular enzymes which can attack PAHs directly and fungi can produce free radical intermediates both degrading larger PAHs (Grotenhuis *et al.* 1999). However, fungi do not use PAHs as their sole carbon and energy source (Cerniglia 1997).

Previous studies have shown that soil additives such as fertiliser (Morgan and Watkinson 1990), composts (Kästner *et al.* 1995), manures (Wong *et al.* 2002; Atagana 2004), or microbial consortia (Guha *et al.* 1999; ŠaŠek *et al.* 2003) can stimulate PAH removal from hydrocarbon contaminated soil, particularly of smaller PAHs. Surfactants have also been shown to improve PAH degradation by releasing PAHs from adsorption sites in the soil (Bewley 1988; Bewley *et al.* 1989; Ellis *et al.* 1991; Madsen and Kristensen 1997).

As fungi, and white-rot fungi in particular, have been found to successfully degrade larger PAHs (Hestbjerg *et al.* 2003) better remediation results may be achieved if fungi attack the larger PAHs and bacteria further degrade the resulting metabolites.

The first two experiments of this thesis demonstrated the advantage of chicken manure addition compared to the other manures tested (Chapter 4), while *P. ostreatus* inoculation was more successful in PAH remediation in comparison to *T. versicolor* (Chapter 5).

Following those results, the aim of this part of the thesis is:

> To evaluate a complementary effect of fungus and manure addition on PAH removal from an aged contaminated soil.
> (Thesis aim 3a)

and

> To determine the importance of the time point at which manure is added on the effects of PAH removal.
> (Thesis aim 3b)

It is hypothesised that:

> A combination of the white-rot fungus *P. ostreatus* and chicken manure enhances the removal of PAHs from an aged contaminated soil;

and that, due to expected competition of the introduced micro-organisms with each other as well as soil indigenous microbial communities,

> The time-point at which manure is added to the fungus inoculated soil influences PAH removal from the aged contaminated soil.

6.3. MATERIALS AND METHODS

6.3.1. Soil

The soil used in this study was from the same source as the soil used in the previous experiments. For a description of the soil see Chapter 3 "Materials and Methods". For this experiment the soil was additionally contaminated with known amounts of deuterated PAHs D-Acenaphthene and D-Benz[a]anthracene (Sigma-Aldrich, Schnelldorf, Germany) giving a final concentration of 33 and 8 mg kg^{-1} dry soil, respectively.

Results concerning those deuterated PAHs are discussed in Chapter 7. The deuterated PAHs were added in an acetone solution. To ensure the survival of indigenous micro-organisms only 25% (7.5 kg) of the soil was additionally contaminated and later mixed with the remaining soil before being split up into the 4 treatments (see Chapter 7, point 7.3.1).

6.3.2. Additives

Following the outcome of the previous manure and fungal additive experiments (see Chapters 4 and 5) *P. ostreatus* inoculated sawdust and chicken manure where chosen for this part of the study. Before adding the additives to the soil they were analysed for physico-chemical characteristics and microbial-community profiles (Table 6.1).

Table 6.1. Physico-chemical characteristics and microbial community profiles of the *P. ostreatus*-inoculated sawdust and the chicken manure used for this experiment. Values represent mean ± S.D. where applicable.

	***P. ostreatus* inoculated sawdust**	**Chicken manure**
Physico-chemical characteristics		
Dry matter (%)	28.5	53.0
pH	4.42 ± 0.04	7.19 ± 0.06
Total C (% of dry matter)	46.7 ± 1.0	41.8 ± 2.1
Total N (% of dry matter)	1.10 ± 0.07	2.48 ± 0.16
C:N ratio	42.8 ± 3.8	16.9 ± 0.7
Electrical conductivity (µS cm^{-1})	91 ± 6	875 ± 42
Microbial community profile (PLFA)		
Total bacterial PLFA (nmol g^{-1} dry sample)	50.0	198.1
Total bacterial PLFA added to the mixture (nmol g^{-1} soil)	2.10	29.39
Total fungal PLFA (nmol g^{-1} dry sample)	19.2	30.9
Total fungal PLFA added to the mixture (nmol g^{-1} soil)	0.81	4.59
Bacteria in % of total PLFAs	50.0	43.3
Actinobacteria in % of total PLFAs	1.50	7.07
Ratio of fungal-to-bacterial PLFAs	0.38	0.16
Ratio of Gram-negative-to-Gram-positive bacterial PLFAs	5.90	0.19
Ratio of iso-to-anteiso monounsaturated PLFAs	0.26	0.46

6.3.3. Treatments

The contaminated and additionally spiked soil was mixed with *P. ostreatus* inoculated sawdust and chicken manure was added at different times during the experiment. Investigating effects of a combined influence of manure and fungus, for the first treatment chicken manure was added together with the *P. ostreatus* inoculum at day 0. Most white-rot fungi become inactive above 35°C additionally, due to the relatively low organic matter content it is unlikely that temperatures would rise naturally to those set in this experiment without additional organic matter addition. Therefore, with the second treatment, the chicken manure was added at day 21 when temperatures were changed from 35 to 40°C. To further investigate the time effect of manure addition, with the third treatment the chicken manure was added at the beginning of the highest temperature phase.

Treatments were as follows with a ratio of soil: *P. ostreatus* inoculum : chicken manure (25:10:15) (v:v:v) for the fungus/manure treatments.

- T-d01: with the chicken manure added at the start of the experiment
- T-d21: with the chicken manure added at day 21 of the experiment
- T-d35: with the chicken manure added at day 35 of the experiment
- Temperature treated control (TC) with soil : sawdust (1:1) (v:v)

After mixing of the treatments the moisture content of the soil mixtures was adjusted to 40 ± 4% on a dry weight basis by adding tap-water. The characteristics of the soil and the sawdust as well as the methods used are described in Chapter 3 "Materials and Methods".

The temperature profile was changed weekly as follows: days 0-7 22°C (room temperature), days 7-14 30°C, days 14-21 35°C, days 21-28 40°C, days 28-35 50°C, days 35-42 65°C, followed by longer periods at lower temperatures from days 42-63 35°C and days 63-91 27°C representing the cooling/maturation phase of a commercial scale composting process.

6.3.4. Statistical Analysis

Statistical analysis was performed as described in Chapter 3 "Materials and Methods". Additionally split-plot ANOVA was performed separately for the three phases of the experiment to investigate possible interactions of temperature (time) and treatment and to assess the effects of manure addition. Therefore data were split into three time series. The first series contained the data from day 0 to day 21. During this period the composition of treatments T-d21 and T-d35 was the same. The second time series contained the data from day 21 after manure was added to treatment T-d21 until day 35. The third time series contained the remaining data from day 35 after manure was added to treatment T-d35 to the end of the experiment (day 91).

6.4. RESULTS AND DISCUSSION

6.4.1. Physico-chemical analysis of the process

A change in the treatments, i.e. addition of fungus/manure, changed the conditions at the time of mixing. Results showed that manure addition influenced physico-chemical characteristics of the treatments to a larger extent than fungus addition. However, following this "disturbance", physico-chemical treatment characteristics changed in a similar way and combined effects of treatment and temperature/time were insignificant as found by split-plot analysis. Nevertheless, some combined effects of treatment and temperature (time) were detected. Most interactions were detected during the phase after manure addition and could be attributed to the addition of chicken manure. The following paragraphs describe the results of the different physico-chemical analyses in more detail.

6.4.1.1. Total organic matter content

The TOM content (Figure 6.1) was significantly lower ($p < 0.005$) for treatments T-d21 and T-d35 until the chicken manure was added (days 0-21 and 21-35). Once the manure was added those treatments had significantly higher TOM contents than treatments T-d01 and TC ($p < 0.001$). The TOM content increased by a higher extent the later the manure was added. This can be explained by the fact that, due to the experimental setup and the removal of samples for analysis, relatively larger amounts of manure were added the later the time-point of manure addition.

The steepest slopes in the graph indicate that the highest rates of decomposition were detected during the 40° and 50°C temperature steps (days 21 to 35), as well as during the 65°C temperature step (days 35 to 42) with the T-d35 treatment after manure addition.

6.4.1.2. pH

The pH values increased from the beginning of the experiment while temperatures increased until day 42 (Figure 6.1). With decreasing temperatures pH values decreased during the remaining experimental period.

The addition of the chicken manure caused substantial decreases in the pH values. Treatment T-d01 had a significantly lower pH than the other treatments ($p < 0.0001$), until chicken manure was added to treatment T-d21. From days 21 to 35 pH values in treatments T-d01 and T-d21 were significantly lower ($p < 0.01$) than in the two treatments without manure addition (TC and T-d35). After manure addition to T-d35 the pH of T-d35 declined by 0.9 units and the pH values of the three fungi/manure treatments were similar in their pH values, but significantly lower than in the TC treatment ($p < 0.05$).

6.4.1.3. Total N

The total N content in the compost mixtures ranged from 0.21% (TC treatment) to 1.47% (T-d35 treatment straight after manure addition) (Figure 6.1). Split-plot analysis did not reveal any significant differences in the changes in N contents between treatments over the temperature-periods. Manure addition increased total N contents of the treatment mixtures. Decreases in total N were detected during the 40°C step (days 21 to 28) with the fungi amended treatments and 65°C (days 35 to 42) with all treatments.

Elevated nitrogen concentrations have been suggested to be inhibitory to PAH degradation in some cases, as an expression of enzymes such as fungal peroxidases are brought about by nutritional starvation (Fernando and Aust 1994). On the other hand it has been found that in some white-rot fungi peroxidase enzyme production is induced by high carbon and nitrogen concentrations (Collins and Dobson 1995; Kaal *et al.* 1995). In a review by Fog (2008) it was summarised that adding N to decomposing matter has no or a negative effect on microbial activity and that the negative effect is mainly found with recalcitrant organic matter with a high C:N ratio (e.g. straw, wood). The negative effects were explained by disturbing the outcome of competition between potent and less potent decomposers, blocking enzyme production, and formation of toxic or inhibitory substances (e.g. amino compounds condensing with decomposition products and ammonia being toxic to fungi (DePasquale and Montville 1990)).

6.4.1.4. Total C

Not surprisingly, the total C content of the mixtures increased with manure addition (Figure 6.1). After manure was added the C content was high, but declined quickly. With the T-d21 treatment total C content decreased significantly during the 40°C temperature step ($p < 0.005$) following the manure addition after which no further significant changes could be detected. When manure was added at a later stage (T-d35 treatment) the C content decreased more slowly over the following 4 weeks during the 65°C

and 35°C temperature steps, and after this little further change was apparent. However, when manure was added total C remained higher until the end of the experiment than without manure addition. The TC treatment showed little change in total C content.

6.4.1.5. C to N Ratio

The C:N ratio (Figure 6.1) was greatest in the TC treatment throughout the experiment with the highest ratio of 62.6 measured at the end of the 65°C temperature step. As neither fungi nor manure were added this was expected. The C:N ratios were lowest when both additives (fungi and manure) had been added. As expected from total N and total C analysis manure addition influenced the C:N ratio more than fungus addition.

6.4.1.6. Electrical conductivity

Electrical conductivity (EC) measurements showed higher values, at each point when the manure was added (Figure 6.1). With manure addition, EC values increased initially indicating an increase in soluble ion/salt concentrations in the treatment mixtures caused by manure addition, before decreasing again.

As with other physico-chemical analysis EC varied with temperature resulting in an increase of values which were maintained at a higher level for 2 weeks when manure was added at medium temperatures (day 21, T-d21). An interaction of treatment and temperature-period was detected during the first two experimental periods (days 0-21, $p < 0.005$; days 21-35, $p < 0.05$). During both phases the treatment with recent manure addition showed EC values changing differently to those measured with the other treatments. From days 0 to 21 EC values decreased with the T-d01 treatment while no changes were detected with the other treatments. When manure had been added the T-d21 treatment demonstrated increasing conductivity values, while values with the other treatments did not change significantly.

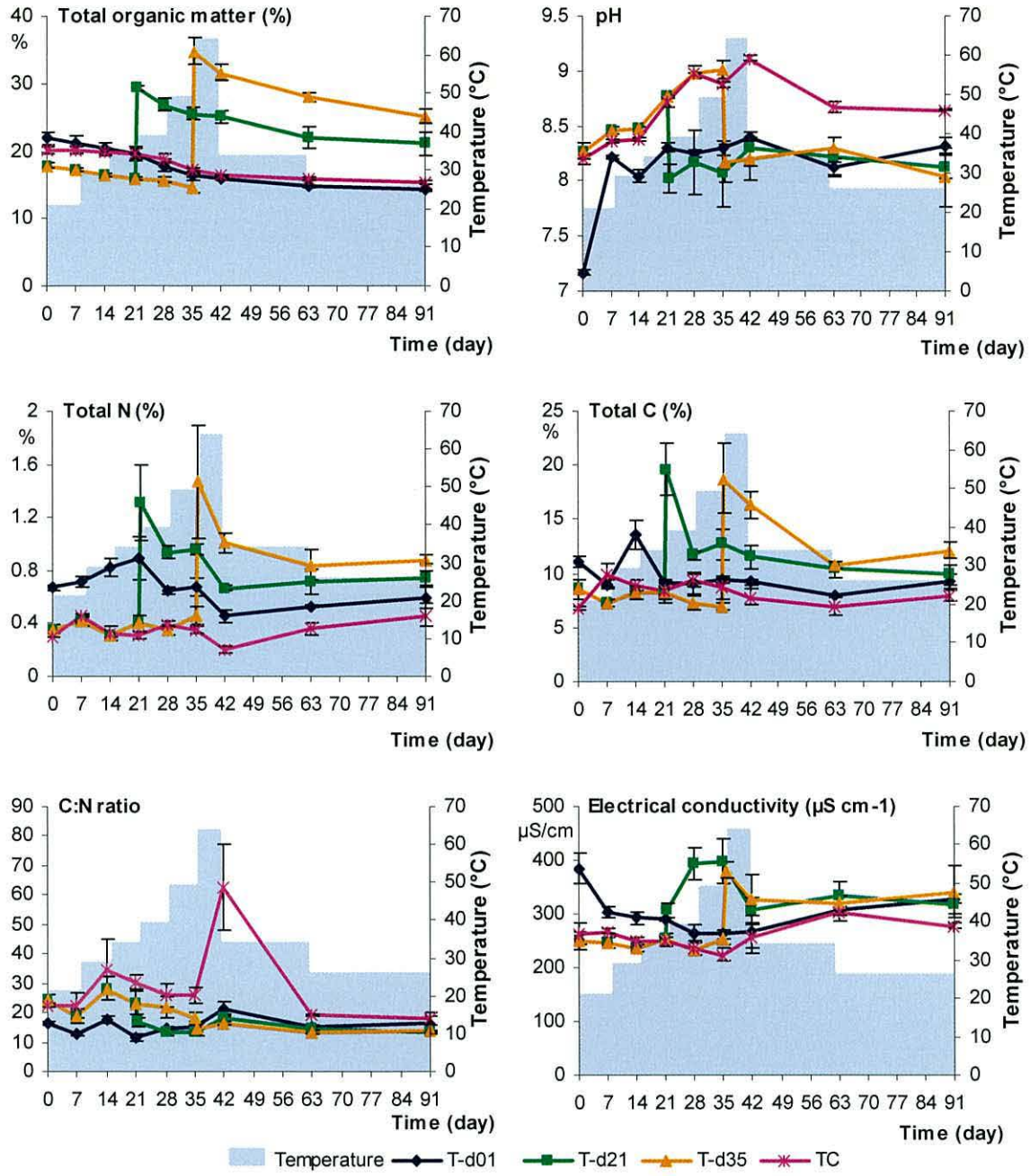

Figure 6.1: Mean values of different physico-chemical analyses with the different treatments during the different stages of the 91 d experiment. Treatments are labelled as T-d01 = blue, T-d21 = green, T-d35 = yellow, TC = pink. The light blue background represents the applied temperature profile. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

6.4.1.7. Ammonia emissions

Similar to total N contents, the ammonia emissions were significantly higher when manure was added (Figure 6.2). When manure was added at the start of the experiment (treatment T-d01), NH_3 emissions were detected at day 7. After manure addition, temperature additionally stimulated further loss of NH_3 by volatilisation. NH_3 emissions increased with the treatments that had

manure added during the 40°, 50°, and 65°C temperature steps. Highest NH_3 emissions were detected during the 65°C temperature step and when manure was added at day 21. When manure was added at day 35 (T-d35) emissions were lower than with the treatment where the manure had been added at day 21 (T-d21).

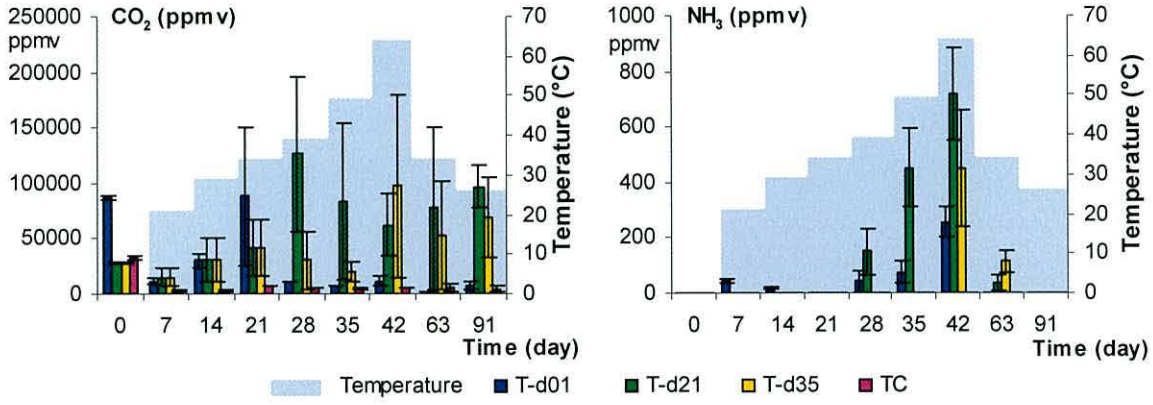

Figure 6.2: Mean values of CO_2 and NH_3 emissions with the different treatments during the different stages of the 91 d experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

6.4.1.8. Carbon dioxide emissions

CO_2 emissions decrease within the first 7 days of the experiment for all treatments (Figure 6.2). With all fungus/manure treatments CO_2 emissions increased during the following two weeks (until day 21). During the next temperature steps CO_2 emissions decreased when no manure was added. With manure addition to T-d21 the CO_2 emissions increased, however, when the temperatures were increased to 50°C and above the values decreased. Subsequently when the temperatures were lowered the CO_2 emissions increased again.

With T-d35, the temperatures were already at 50°C when manure was added. CO_2 emissions measured directly after manure addition were measured at 187500 ppmv (±120000) (data not included in the figure), measured CO_2 levels rapidly decreased during the following two weeks becoming similar to those measured with T-d21 and increasing again during the last week of the experiment.

The treatments did not behave significantly differently from each other during any of the experimental periods, but the results show high variation.

CO_2 is emitted by microbial respiration and increases in CO_2 emissions indicate an increase in microbial activity (Osborne *et al.* 1980) explaining the higher CO_2 emissions found with the fungus/manure amended treatments.

6.4.2. Microbial community changes

6.4.2.1. Total microbial and bacterial PLFAs

The total bacteria indicating PLFAs showed the same changes as the total microbial PLFAs (Figure 6.3) as well as the same relationships between treatments. Differences in total amounts of microbial PLFAs between treatments were due to fungi and manure addition. With additive addition the PLFA concentrations were initially higher than without (TC). However, after 21 days those differences could no longer be detected as long as no additional manure was added. When manure was added at this stage (day 21) microbial numbers increased 5-fold (T-d21) and stayed higher during the following two temperature steps, before decreasing substantially during the week at the highest temperature (65°C).

Total microbial PLFA concentrations were influenced by the addition of fungi and chicken manure. As expected, the highest amounts of PLFAs were found when the treatment was amended with both additives and lowest when only fungi were added.

By day 21 total microbial PLFA concentrations were not significantly different between the treatments. Manure addition to T-d21 increased microbial PLFA concentrations which remained significantly higher ($p < 0.001$) than in the other treatments until the beginning of the 65°C temperature step. While total microbial PLFA amounts in treatments T-d01, T-d35, and TC have not been significantly different from day 21 to 35, manure addition to treatment T-d35 caused an increase in total microbial PLFAs. However, those increased amounts declined significantly in treatment T-d35 as well as in treatment T-d21.

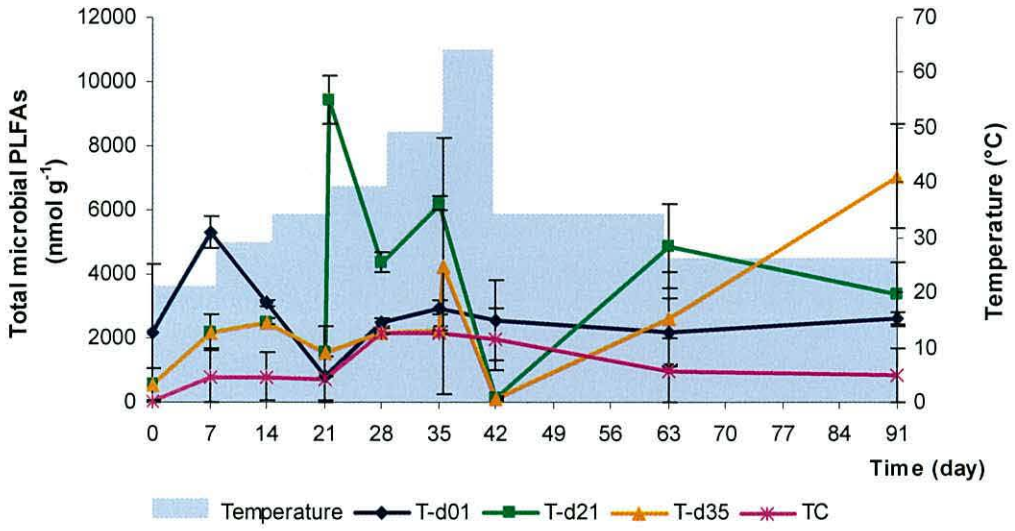

Figure 6.3: Mean values of the amount of total measured microbial PLFAs in nmol per g dry sample detected with the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

Those decreases might indicate that the introduced microbial community might have initially out-competed the indigenous microbial community, but that it was not well adapted to the increased temperatures (65°C). In treatments T-d01 and TC no significant changes in total microbial PLFA concentrations could be detected until the end of the experiment. In the T-d21 and T-d35 treatments, however, microbial PLFA concentrations recovered and increased significantly ($p < 0.01$) until the end of the experiment

6.4.2.2. Gram-negative and Gram-positive bacteria indicating PLFAs

Manure addition decreased the ratio of Gram-negative to Gram-positive bacterial PLFA significantly by increasing the amounts of Gram-positive bacteria to a larger extend than the amounts of Gram-negative bacteria (Figure 6.4). Split plot analysis did not reveal any significant differences in the changes of Gram-negative to Gram-positive PLFA ratios of treatments over time. However, while all treatments behaved similarly, the ratio stayed significantly lower when manure was added than without its addition.

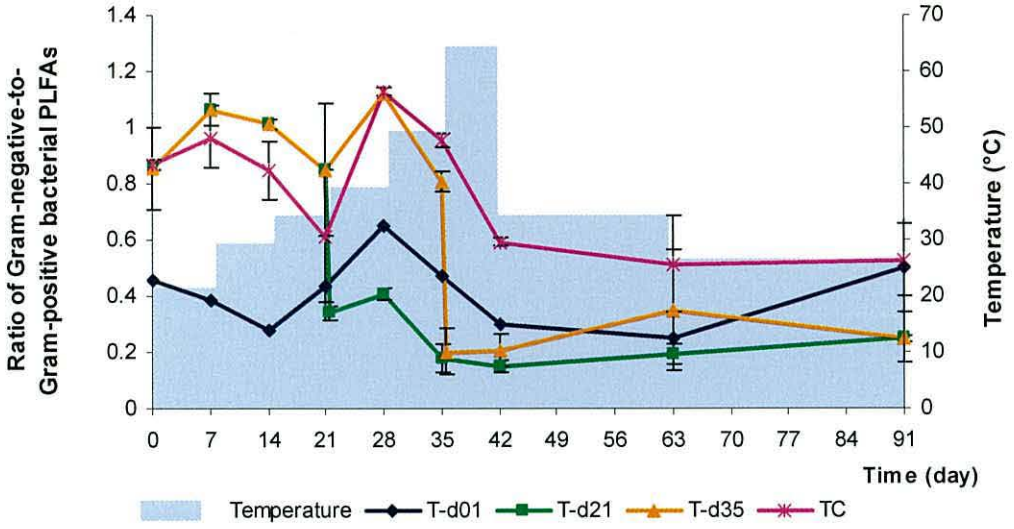

Figure 6.4: Mean values of the ratio of Gram-negative-to-Gram-positive bacteria indicating PLFAs in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

Lower concentrations of Gram-positive bacterial PLFA were detected when no manure was added to the contaminated soil. Manure addition increased the concentration of Gram-positive bacteria and therefore decreased the ratio of Gram-negative bacteria (Figure 6.5). This was expected, as manure is dominated by Gram-positive bacteria.

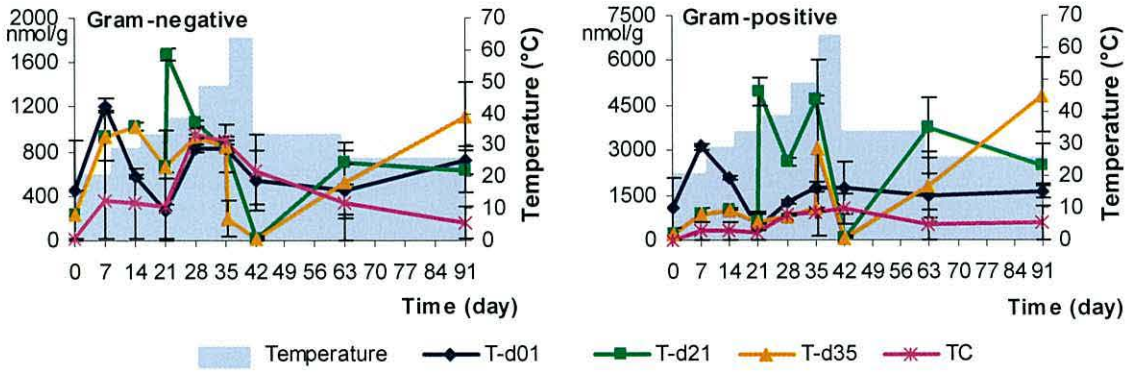

Figure 6.5: Mean values of the concentrations of Gram-negative and Gram-positive bacteria indicating PLFAs in nmol per g dry sample detected in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

Gram-negative bacteria have been found to dominate systems in hydrocarbon contaminated environments (MacNaughton *et al.* 1999) and ratios of Gram-negative-to-Gram-positive bacteria were found to increase under stress situations (Frostegard *et al.* 1993; Zelles *et al.* 1994; Kaur *et al.* 2005). The increases of the ratio of Gram-negative-to-Gram-positive PLFAs could therefore be attributed to the contamination as well as to the increase in temperature, both favouring Gram-negative bacteria.

As Gram-negative and Gram-positive PLFAs in the TC treatment changed in a similar way as the fungi and manure amended treatments it is more likely that bacterial communities were reacting to the change in temperature rather than being influenced by the contamination.

6.4.2.3. Stress indicating PLFAs

The ratio of iso-to-anteiso branched PLFAs generally increased with all treatments over the course of the experiment (Figure 6.6). Increases in the ratio iso-to-anteiso branched PLFAs typically show increases in stress levels (Kaneda 1991; Kieft *et al.* 1994; McKinley *et al.* 2005). Decreases were only detected during the 35°C temperature step from day 14 to 21 and during the 65°C temperature step. While manure addition only very slightly decreased the ratio when added at day 21 (T-d21), a more pronounced decrease was detected when added at day 35 (T-d35). Due to the experimental setup a larger (in relation to the compost mixture in the vessel) amount of chicken manure was added to the T-d35 treatment at day 35 than had been at day 21 with the T-d21 treatment. The stronger decrease in the stress indicating PLFA ratios with manure addition at day 35 can be explained by a higher relative amount of organisms introduced with the chicken manure shifting the ratio closer to the one found in the pure chicken manure.

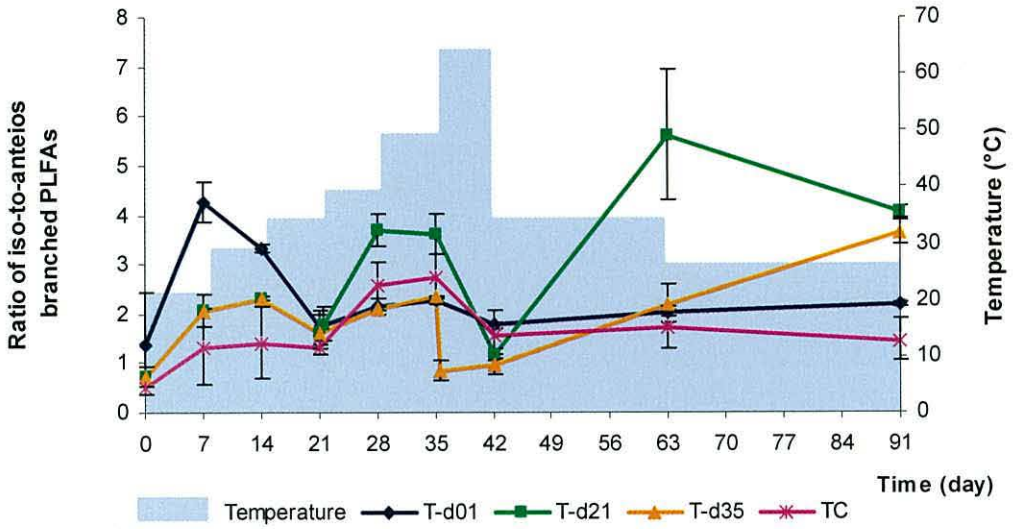

Figure 6.6: Mean values of the ratio of iso-to-anteiso branched PLFAs in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

6.4.2.4. Actinobacteria indicating PLFA

The ratio of Actinobacteria indicating PLFAs of total bacterial PLFAs (Figure 6.7) declined with all treatments during the first two weeks of the experiment. This decline was more pronounced with the fungi/manure treatments (T-d01, T-d21, T-d35) where the ratio increased again with increasing temperatures.

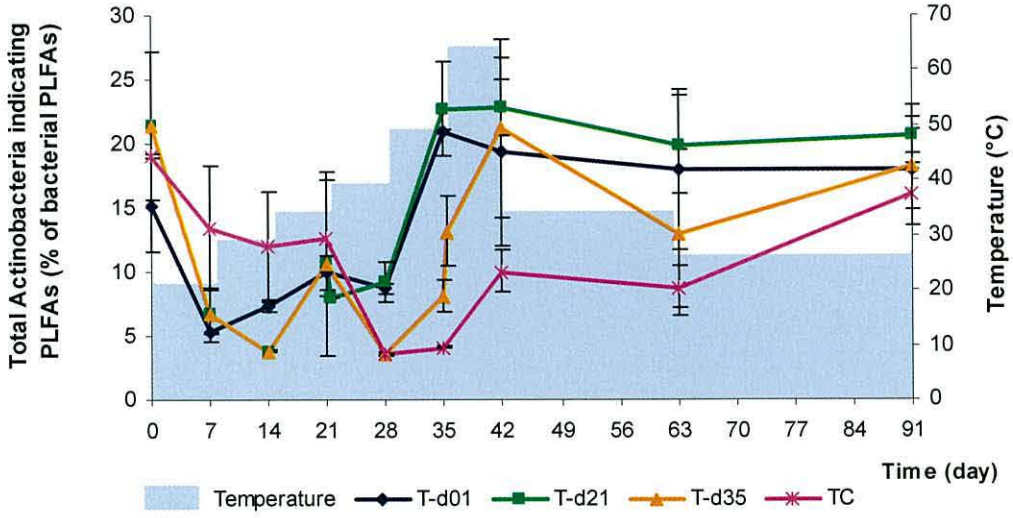

Figure 6.7: Mean values of the amounts of total Actinobacteria indicating PLFAs in % of the total amounts of bacterial PLFAs detected in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

Manure addition at day 21 (T-d21) decreased the ratio of Actinobacteria indicating PLFAs of total bacterial PLFAs, however, the ratio increased with increasing temperatures with all treatments, whereby the manure amended treatments showed a bigger increase than the T-d35 and TC treatment. The T-d35 treatment behaved similar to the TC treatment until manure was added at day 35 (when the temperature was changed from 50° to 60°C). Following manure addition at day 35 changes of Actinobacteria indicating PLFAs in the T-d35 treatment showed the same pattern as the T-d01 and T-d21 did during the previous week. In detail: with T-d01 and T-d21 the ratios of Actinobacteria increased during the 50°C temperature step and remained at high levels during the 65°C temperature step before decreasing during the following week. The same effect was detected with T-d35 during the 65°C and the first and following 2 weeks of the 35°C temperature step.

As expected, total amounts of Actinobacteria indicating PLFAs (Figure 6.8) increased during the second half of the heating phase (days 21 to 42). This change in Actinobacteria indicating PLFA concentrations is more pronounced for treatments T-d01 and TC while treatments T-d21 and T-d35 show the same substantial decrease in Actinobacteria indicating PLFA concentrations as was found for total microbial PLFA concentrations.

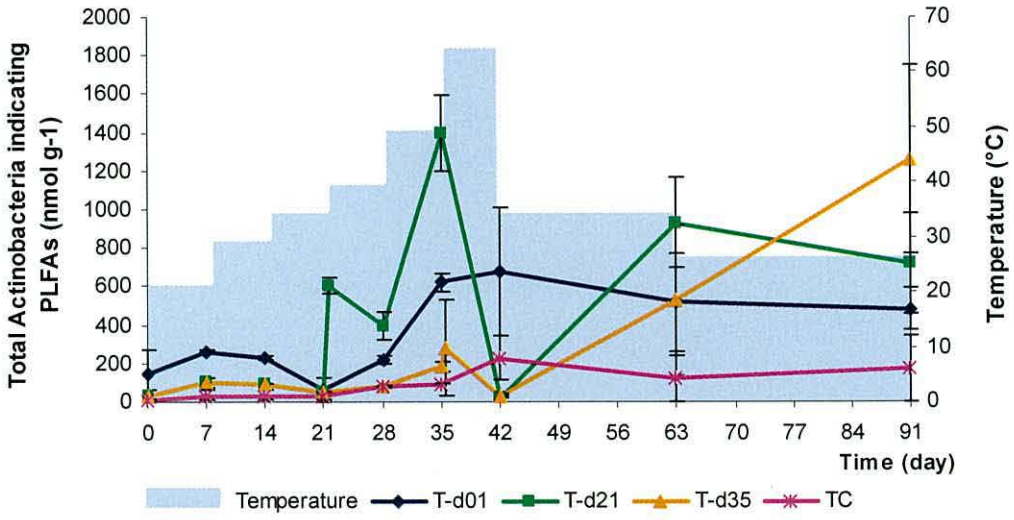

Figure 6.8: Mean values of the amounts of total Actinobacteria indicating PLFAs in nmol per g dry sample in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

6.4.2.5. Fungi indicating PLFA

Figure 6.9 shows the quick decline of fungal PLFA with the fungi/manure treatments. Numbers of fungal PLFA fell below the detection limit by day 14 with all fungal amended treatments; however, they increased again during the 35°C temperature step (days 14 to 21). Manure addition after this stage (T-d21) increased the ratio of fungal PLFA additionally. However, the amounts of fungal PLFA which showed similar changes as the ratio of fungal to bacterial PLFA (Figure 6.10), decreased quickly during the following 40°C temperature step with all treatments, falling below the detection limit at the end of the 50°C temperature step.

The concentration of fungal PLFA per g dry sample and the ratio of fungal-to-bacterial PLFA subsequently increased again during the 65°C temperature period with all but the T-d35 treatment. With the T-d35 treatment manure has been added at the beginning of this temperature step increasing fungal PLFA levels. During the following temperature step numbers decline to levels similar to those detected with the other treatments.

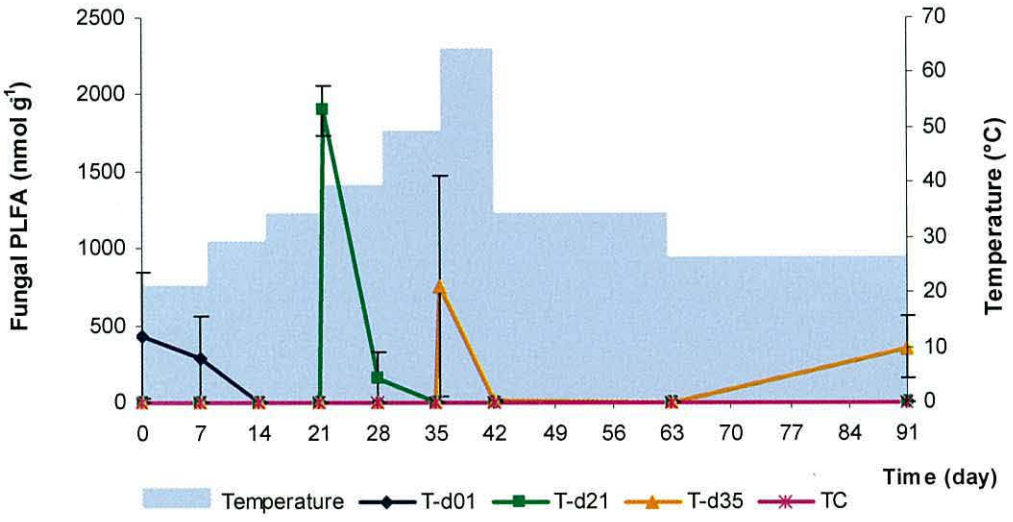

Figure 6.9: Mean values of the amounts of total Actinobacteria indicating PLFAs in nmol per g dry sample in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

This increase of the ratio of fungal-to-bacterial PLFA with manure addition is due to an increase in fungal PLFA. While large numbers of bacteria were added with the chicken manure, it also supplied high numbers of fungi increasing fungal PLFA from below the detection limit to 1900 and 750 nmol per g dry sample for the T-d21 and T-d35 treatments, respectively.

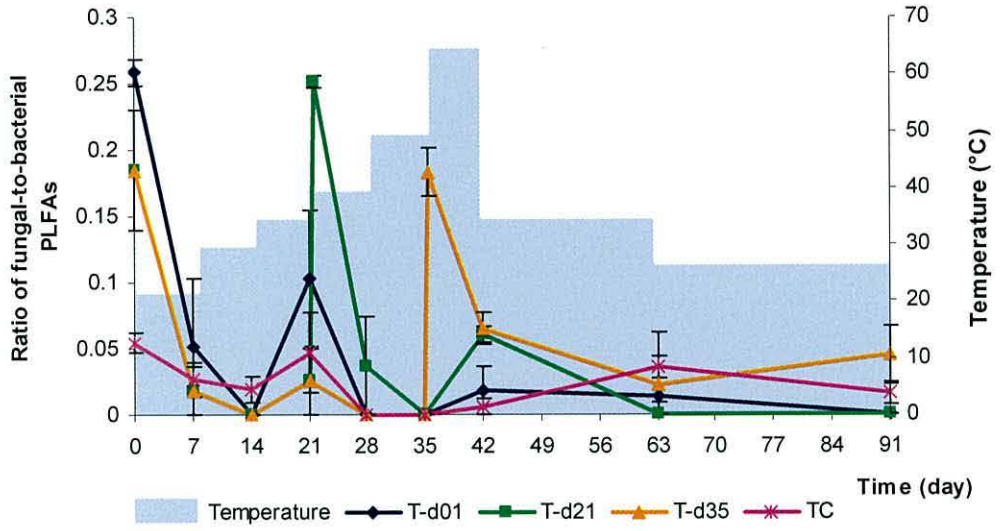

Figure 6.10: Mean values of the ratio of fungi-to-bacteria indicating PLFAs in the different treatments during the different stages of the 91 day experimental period. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

All but the T-d35 treatment showed an increase in fungal PLFA which was most notable with the T-d21 treatment. Temperatures above 35°C are considered unfavourable for growth of white-rot fungi. Even though the inoculum consisted of the white-rot fungus *P. ostreatus* PLFA analysis cannot distinguish between different fungi. Fungi growing at the higher temperatures were expected to be thermophilic fungi such as *Mucor* sp. or *Aspergillus* sp. which have been found during composting (Kane and Mullins 1973; Atagana 2004).

6.4.2.6. Influence of additives on the PLFA profile development

No differences were detected in the changes of PLFA profiles between the treatments over time for any of the microbial groups during the first three weeks of the experiment. During the second phase of the experiment treatment T-d21 showed changes in PLFAs which were different to those found with the other treatments. The manure addition increased the total amounts of microbial PLFAs as well as the ratio of fungal-to-bacterial PLFAs, however, those values quickly decreased again during the following temperature step (while increases were detected with the other treatments) before changing in the same way as with the other treatments. The opposite effect of manure addition was found for Actinobacteria indicating PLFAs. Here manure addition decreased the ratio followed by an increase during the following temperature step before the treatment subsequently showed the same changes in those PLFAs as the other treatments.

During the last experimental phase differences in the changes of PLFA profiles over time were again found for the treatment with recent manure addition. Manure addition increased the total amounts of bacteria but the amounts significantly decreased during the following temperature step for both treatments with recent manure addition (T-d35 as well as T-d21) while amounts remained unchanged with the other treatments. The significant interaction of treatment and temperature (time) on the amounts of fungal PLFA was mainly identified for treatment T-d35. Manure addition increased the amounts of fungal PLFA which then decreased significantly during the following temperature step (day 35 to 42, 65°C) while amounts increased with the other treatments.

Overall, manure addition changed the PLFA profiles with its addition. An influence of the manure addition on a microbial community profile becomes most obvious at the end of the temperature period following the manure addition. By this time microbial communities had time to adapt to the changed conditions.

Diagnostic Biplot analysis revealed associations of treatments and PLFAs (Figures 6.11 to 6.13). Treatments where manure had been added at the beginning of the investigated temperature period (T-d01 at day 7, T-d21 at day 28, T-d35 at day 42) were closely associated with the Gram-positive bacteria indicating iso-branched PLFAs i17:0 and i15:0 and with the Actinobacteria indicating methyl branched PLFA 10Me18:0. Vessels only inoculated with fungi (T-d21 day 7 and T-d35 days 7 and 28) were mainly associated with the Actinobacteria indicating methyl branched PLFA 10Me17:0.

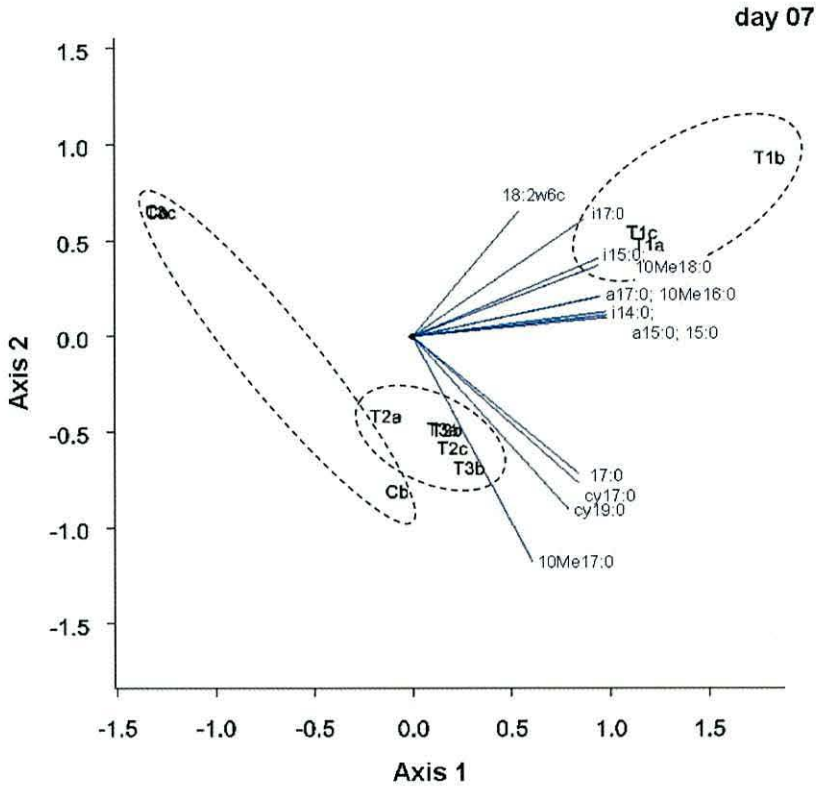

Figure 6.11: Diagnostic Biplot showing association of PLFAs with the different treatments at day 07. T1=T-d01, T2=T-d21, T3=T-d35, C=TC, a-c indicating replicate vessel.

During the subsequent temperature steps individual PLFA concentrations developed in different directions following no clear pattern. The influence of manure addition became clear again by day 28 after manure had been added to treatment T-d21. Diagnostic Biplot analysis (Figure 6.12) showed an association with the formerly mentioned PLFAs i17:0, i15:0, 10Me18:0, 18:1w9c as well as with the Gram-positive bacteria indicating PLFAs i14:0, a15:0, 15:0, 10Me17:0 (Actinobacteria) and the fungi indicating PLFA 18:2w6c.

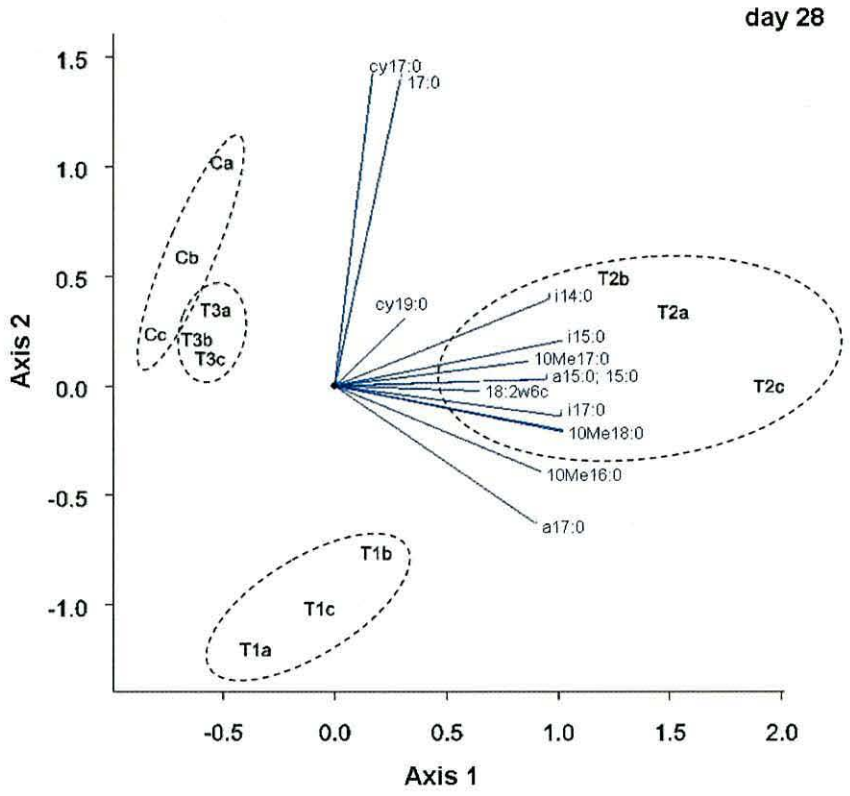

Figure 6.12: Diagnostic Biplot showing association of PLFAs with the different treatments at day 28. T1=T-d01, T2=T-d21, T3=T-d35, C=TC, a-c indicating replicate vessel.

While manure addition seemed to favour the development of the microbial communities in a certain way, temperature also played an important role. As Diagnostic Biplot analysis for day 42 shows, no clear association of treatment with PLFAs could be made. Therefore no influence of manure addition on microbial community profile development could be detected (Figure 6.13). The only association found was two replicate vessels of treatment T-d01 with the PLFAs a17:0 and 10Me16:0 and two replicate vessels of the treatment TC with the PLFAs cy17:0 and 17:0 indicating Gram-negative bacteria and bacteria in general.

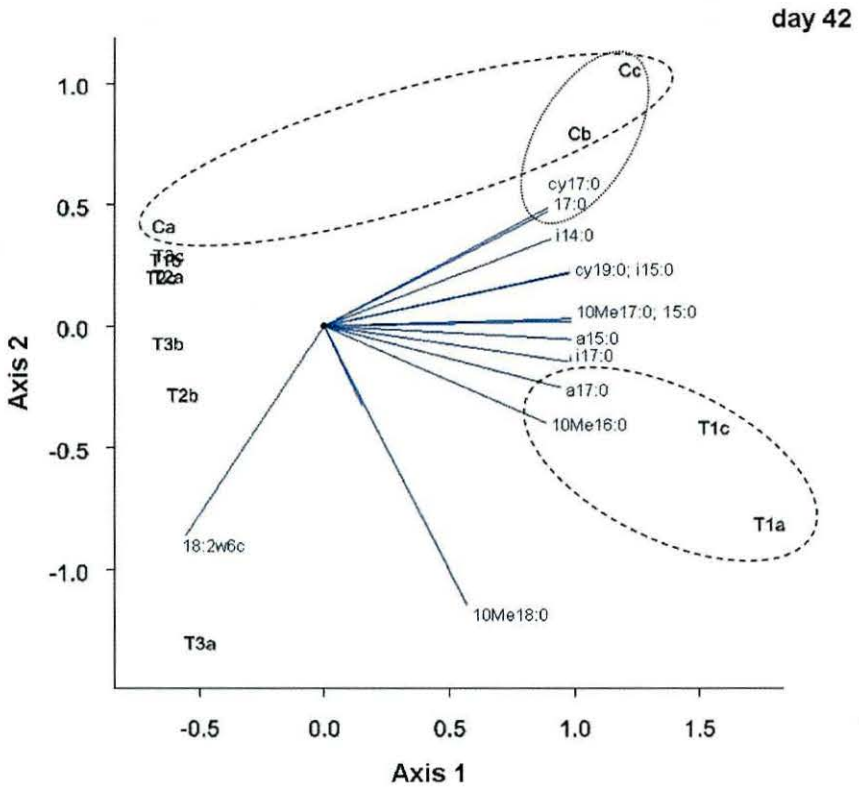

Figure 6.13: Diagnostic Biplot showing association of PLFAs with the different treatments at day 42. T1=T-d01, T2=T-d21, T3=T-d35, C=TC, a-c indicating replicate vessel.

However, manure addition only seemed to have had a limited effect on microbial community development. Within the PLFA profile significant interactions of treatment over time were mainly due to the treatment with recent manure addition. This treatment became more similar to the other treatments during the temperature step following manure addition, while the PLFA profile with the other treatments might have changed in a different way. Only during the first step of the cooling phase did the influence of manure addition at later stages of the experiment seem apparent. However, no correlation could be found between PLFA analysis and physico-chemical characteristics of the compost mixtures. On the other hand, correlation analysis revealed a positive correlation ($r > 0.5$) between all measured PLFAs for all treatments except the fungi indicating PLFA 18:2w6c which was negatively correlated to the other PLFAs.

The similarity of the development of microbial profiles as indicated by PLFA analysis of all treatments and the lack of correlation with physico-chemical analyses as well as with PAH analysis suggests that the development of the microbial community composition is mostly influenced by the temperature/time effect, with temperature being assumed to be the more important factor, especially as associations of certain PLFAs with treatments change depending on when manure was added, i.e. what temperature was applied during the time-period after manure addition.

6.4.3. Changes in extractable weathered PAH amounts

Taking the weight loss of the soil/sawdust/fungi/manure mix into account the PAH data were corrected by referring to the amount of ash which is considered the most chemically stable parameter during the degradation process (Amir *et al.* 2005). Due to the heterogeneous distribution of the contaminants in the aged PAH contaminated soil, as well as to investigate temperature effects on PAH removal, the results have been looked at in three different ways ("percentage change", "rates of degradation" and "concentration") as described fully in Chapter 3 (Materials and methods).

6.4.3.1. Changes in percentages of PAH concentrations remaining in the treatment mixtures over the 91 day experiment

For comparative purposes the amount of PAHs measured at the different periods were related to the values measured with each vessel at the beginning of the experiment. This takes initial differences in PAH distribution into account. Normalising the data like this enables an overall comparison of the effects of the different treatments.

6.4.3.1.1. Effects of treatments on extractable weathered USEPA PAHs

For the purpose of this study total aged PAHs (as identified by the USEPA and listed in Table 6.2) do not include the added deuterated PAHs (Acenaphthene-D10 and Benz[a]anthracene-D12). Results for those PAHs are presented in Chapter 7. Total aged PAH concentrations per g ash were significantly lower at the end of the experimental period compared to the start for all fungi/manure treatments ($p < 0.005$) (Figure 6.14).

The replicates of the TC treatment varied much more than the replicates of the fungi/manure treatments. Even though final amounts were 35% lower than initial amounts, due to the large variation between the replicates statistically no significant difference could be detected for the TC treatment between the start and endpoint of the experiment.

Analysis of the combined effect of treatment and temperature (time) revealed that changes of total extractable USEPA PAH concentrations were only significantly different between all treatments over the first experimental period (days 0-21, $p < 0.001$).

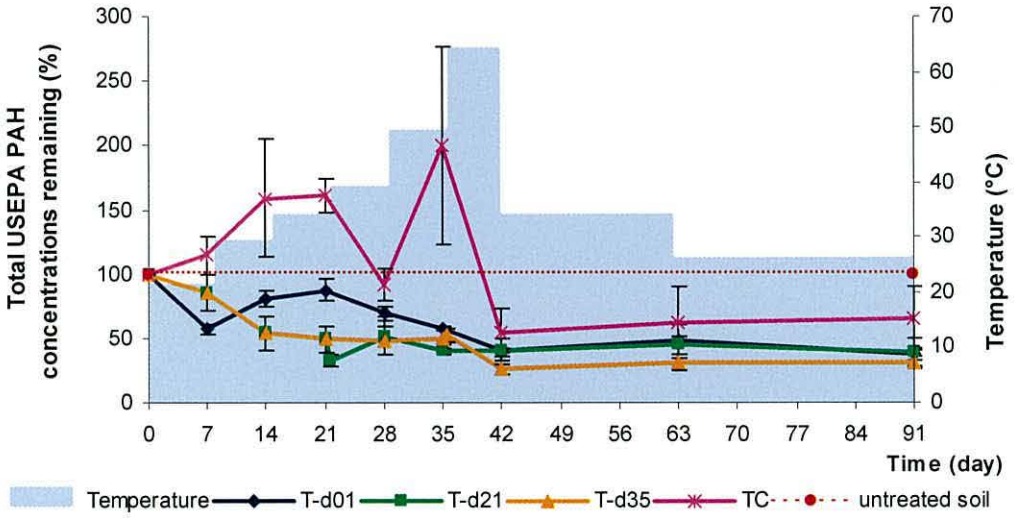

Figure 6.14: Mean percentages of concentrations of total aged USEPA PAHs remaining in the different treatments by the end of different temperature steps. The red dotted line connects the percentage of the remaining total PAHs detected in the untreated soil after 91 days with the 100% in the beginning of the experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

For all fungi/manure treatments, concentrations of most measured weathered PAHs (Table 6.2 and Figures 6.15a/b) were significantly lower at the end of the experimental period than at the beginning ($p < 0.05$). The TC treatment on the other hand only had significantly lower PAH concentrations for the LMW PAHs Naphthalene and Acenaphthylene ($p < 0.05$). In contrast, most of the other LMW and MMW PAH concentrations increased above the initial starting concentrations (by day 7) and remained high until the end of the experiment. The HMW PAH concentrations increased in the TC treatment until day 35 (50°C). Even though concentrations decreased during the following 2 weeks, values increased again resulting in amounts not significantly different to the amounts measured at the beginning of the experiment. One exception is the HMW PAH Benzo[g,h,i]perylene whose extractable concentration increased.

PAH concentrations in the untreated soil were not significantly different by the end of the 91 day experimental period. Final remaining percentages were between 90.0 and 136.5% for all measured PAHs except Naphthalene and Acenaphthene where final concentrations were reduced to 75.6% and 83.2% of the initial concentrations, respectively. The final concentrations were not significantly different to the initial ones except for Naphthalene and Acenaphthene.

Table 6.2.: Percentage of individual PAHs remaining in the treatment mixtures at the end of the experiment.

	T-d01	T-d21	T-d35	TC
	%±S.E.	% ±S.E.	%±S.E.	%±S.E.
LMW PAHs				
Naphthalene	22.7±8.5^{a*}	24.8 ±13.1^{a*}	8.3±4.8^{a*}	19.9±10.7^{a*}
Acenaphthylene	15.7±3.4^{a*}	18.2 ±5.8^{a*}	23.3±8.3^{a*}	29.9±10.6^{a*}
Acenaphthene	38.0±13.2^{a*}	33.6 ±1.8^{a*}	44.6±16.7^{a*}	102.0±39.6^{a}
Fluorene	23.9±4.2^{a*}	22.5 ±7.6^{a*}	31.1±7.3^{a*}	50.1±30.7^{a}
Phenanthrene	49.4±4.4^{a*}	56.3 ±22.2^{a}	49.5±13.7^{a*}	85.6±26.9^{a}
Anthracene	48.1±3.8^{a*}	56.9 ±22.5^{a}	48.0±12.5^{a*}	86.5±28.9^{a}
MMW PAHs				
Fluoranthene	31.5±5.8^{a*}	31.3 ±9.6^{a*}	27.7±2.0^{a*}	54.3±21.6^{a}
Pyrene	31.3±5.4^{a*}	34.9 ±9.8^{a*}	27.7±2.0^{a*}	54.9±22.2^{a}
Benz[a]anthracene	41.3±5.5^{a*}	27.3 ±7.3^{a*}	18.9±6.0^{a*}	57.9±46.8^{a}
Chrysene	41.3±5.4^{a*}	25.9 ±7.1^{a*}	18.6±3.4^{a*}	38.5±26.8^{a*}
HMW PAHs				
Benzo[b]fluoranthene	64.7±11.2^{a*}	54.9 ±12.6^{a*}	42.8±4.1^{a*}	117.1±46.5^{a}
Benzo[a]pyrene	68.1±11.6^{a*}	60.0 ±11.6^{a*}	45.0±3.4^{a*}	125.2±43.8^{a}
Indeno[1,2,3-c,d]pyrene	55.2±8.6^{ab*}	46.5 ±8.6^{ab*}	35.7±1.5^{b*}	109.6±34.6^{a}
Dibenz[a,h]anthracene	64.9±2.5^{ab*}	49.1 ±2.5^{ab*}	33.4±2.0^{b*}	92.7±26.0^{a}
Benzo[g,h,i]perylene	45.2±3.2^{a*}	148.8 ±3.2^{a}	76.8±53.6^{a}	228.0±116.5^{a}
grouped aged PAHs				
Total LMW PAHs	46.1±3.7^{a*}	52.7 ±20.4^{a*}	46.2±12.0^{a*}	81.3±28.0^{a}
Total MMW PAHs	32.0±5.6^{a*}	34.4 ±9.5^{a*}	27.2±2.1^{a*}	54.2±22.7^{a}
Total HMW PAHs	61.1±9.4^{ab*}	59.6 ±13.4^{ab*}	42.7±6.4^{b*}	123.1±28.5^{a}
Total USEPA PAHs	37.5±4.4^{a*}	38.5 ±11.4^{a*}	31.7±4.1^{a*}	64.8±25.1^{a}
D-Acenaphthene	2.0±1.3^{a*}	5.0 ±2.3^{a*}	1.3±0.2^{a*}	3.5±1.4^{a*}
D-Benz[a]anthracene	14.5±4.8^{a*}	31.2 ±12.7^{a*}	11.8±1.0^{a*}	19.7±6.5^{a*}

Values are means of the percentage of extractable PAHs (nmol) remaining in % of the amount found in the soil at the beginning of the experiment ±S.E., values with the same letter are not significantly different between the treatments and * indicates a significant difference to the concentrations found at the beginning of the experiment at the $p < 0.05$ level as determined using a Tukey post-hoc test., n=6 for T-d21/T-d35 from day 0 to 21, else n=3

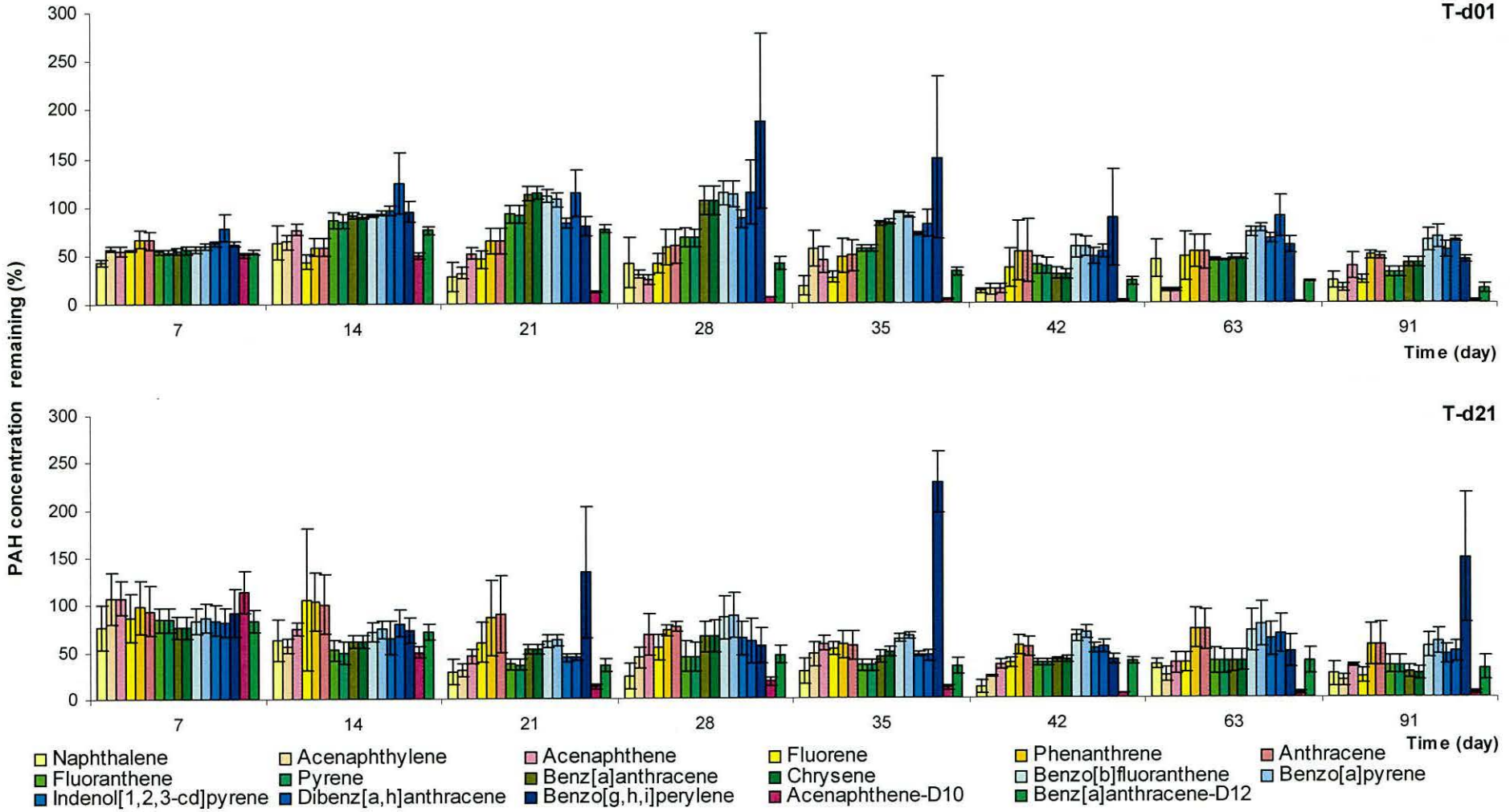

Figure 6.15a: concentrations of measured PAHs per g ash (% of start amounts) at the end of different temperature steps of treatments T-d01 and T-d21 during the 91 day experiment. Bars represent ±S.E. The behaviour of the deuterated PAHs Acenaphthene-D10 and Benz[a]anthracene-D12 are discussed in Chapter 7; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

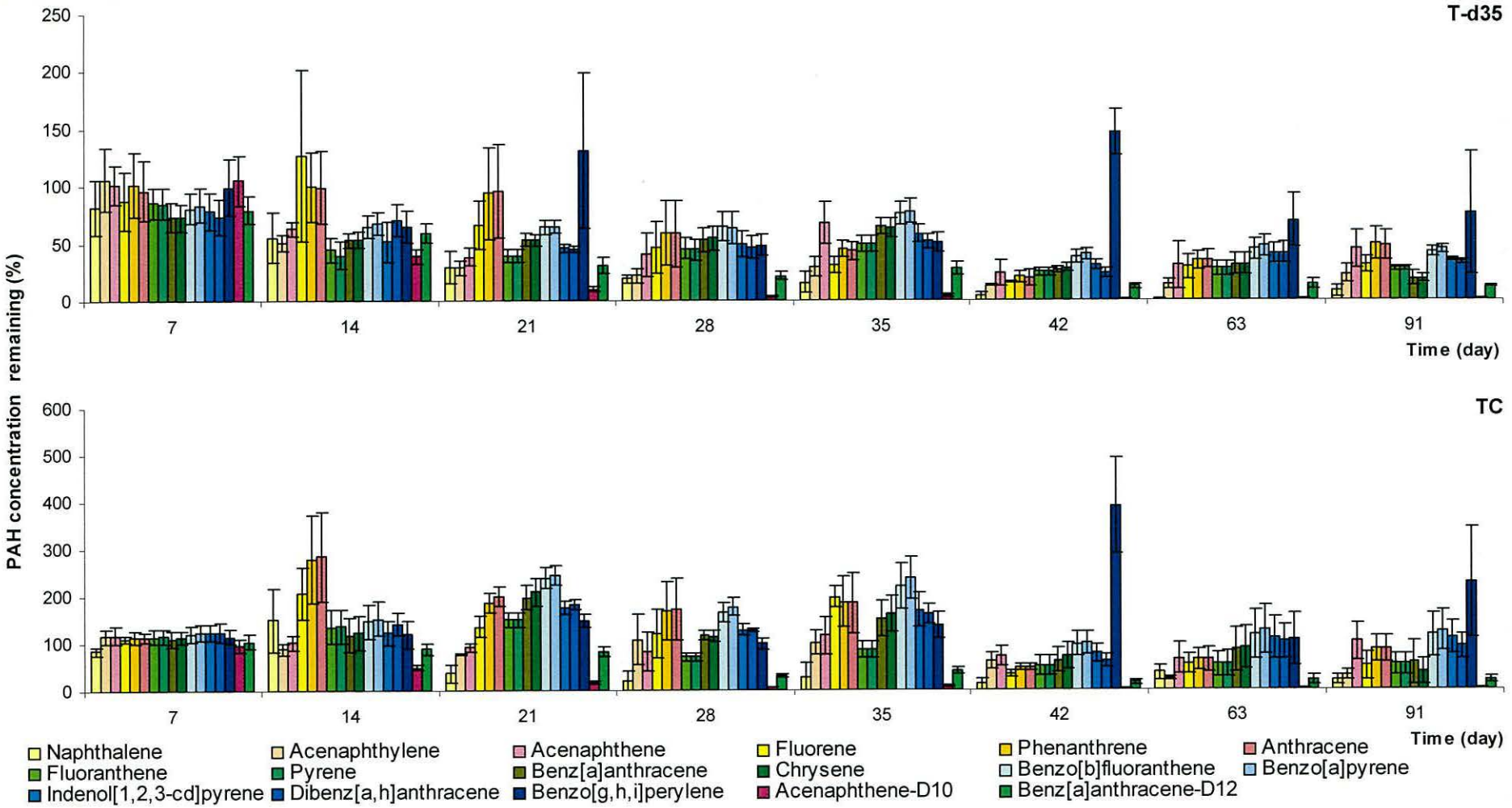

Figure 6.15b: concentrations of measured PAHs per g ash (% of start amounts) at the end of different temperature steps of treatments T-d35 and TC during the 91 day experiment. Bars represent ±S.E. The behaviour of the deuterated PAHs Acenaphthene-D10 and Benz[a]anthracene-D12 are discussed in Chapter 7; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

6.4.3.1.2. Effects of treatments on extractable grouped LMW PAHs

The weathered LMW PAHs showed the highest variability between the replicate vessels. While their concentrations increased with the TC treatment concentrations stayed below the initially measured ones with the other treatments (Figure 6.16). In the T-d01 treatment, when fungi and chicken manure were added together at the start of the experiment, total LMW PAH concentrations decreased by 35% during the first week and showed further decreases until the end of the experiment.

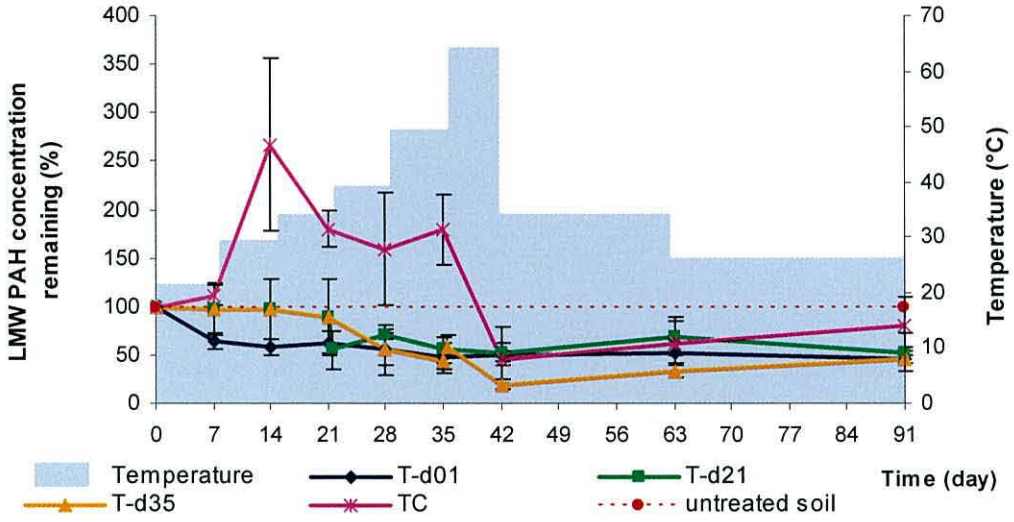

Figure 6.16: Mean percentages of concentrations of grouped weathered LMW PAHs remaining by the end of different temperature steps. The red dotted line connects the percentage of the remaining LMW PAHs detected with the untreated soil after 91 days with the 100% at the beginning of the experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

With the treatments where the manure was added at a later stage total LMW PAH concentrations did not change until day 21 and decreased subsequently until day 42. Manure addition did not change total LMW PAH concentrations significantly and due to a larger variation between the replicate vessels changes in LMW PAH concentrations were not statistically significant. Percentages of PAH concentrations remaining at the end of the experiment of total LMW PAHs were lower with the fungi/manure treatments (46, 53, 46% for T-d01, T-d21, T-d35) than the TC treatment (81%) and the untreated soil (100%). Split-plot analysis revealed no significant interaction of treatment and temperature/time during any of the phases of the experiment.

6.4.3.1.3. Effects of treatments on extractable grouped MMW and HMW PAHs

With the exception of Benzo[g,h,i]perylene, concentrations of all weathered MMW and HMW PAHs changed in a similar way and their behaviour is reflected by the behaviour of total MMW (Figures 6.17) and total HMW PAH (Figures 6.18) concentrations for each of the treatments. In the T-d01 treatment concentrations of grouped MMW and HMW PAHs decreased significantly during the first 7 days of the experiment. During the following 3 weeks concentrations increased again until day 28, when temperatures reached 40°C. However, at the end of the 65°C temperature step (day 42) concentrations were significantly lower than those at the start ($p < 0.0001$ for MMW and HMW PAHs). Even though a slight increase in concentrations was detectable during the first step of the cooling phase (day 63, 35°C) the concentrations stayed at significantly lower levels in comparison to the start, reaching lowest concentrations at day 91.

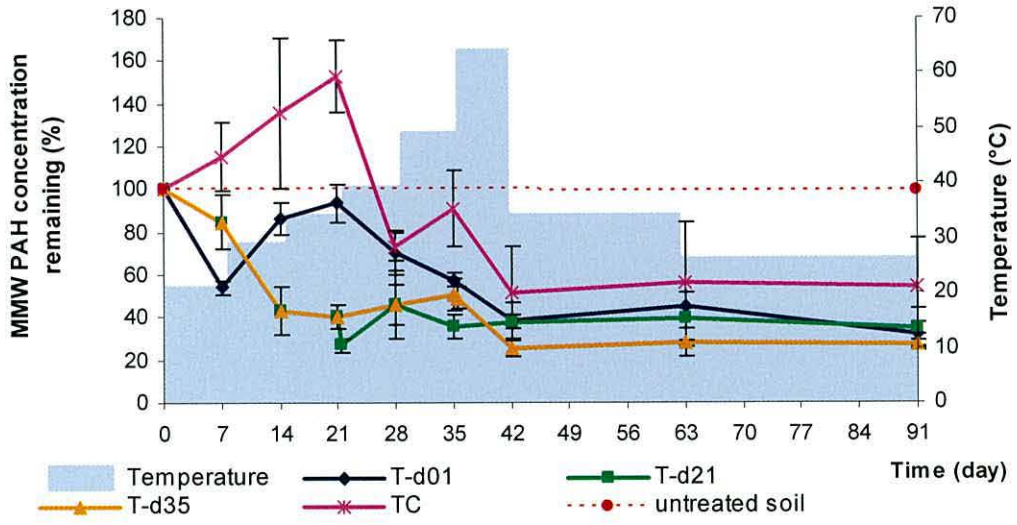

Figure 6.17: Mean percentages of concentrations of grouped weathered MMW PAHs remaining by the end of different temperature steps. The red dotted line connects the percentage of the remaining MMW PAHs detected with the untreated soil after 91 days with the 100% at the beginning of the experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

MMW PAH concentrations with treatments T-d21 and T-d35, where only the fungal inoculum was initially added, decreased more slowly but over a longer period resulting in lowest concentrations by day 14 (T-d35) and 21

(T-d21). From these points onwards no significant changes in weathered MMW PAH concentrations could be detected during the remaining experimental period. In the TC treatment, however, total weathered MMW PAH concentrations increased before decreasing again and percentages of PAH concentrations remaining in the treatment mixture were still higher, but not significantly different from those with the fungi/manure treatments.

Split-plot analysis of amounts of grouped MMW PAHs in ng g^{-1} ash revealed significant interactions of treatment and temperature/time during the first two phases of the experiment ($p < 0.0001$ days 0-21; $p < 0.005$ days 21-35). During the first phase (days 0 to 21) percentages of PAH concentrations remaining decreased for treatments T-d21 and T-d35 while treatments T-d01 and TC showed increases. During the second phase (days 21 to 35) percentages of PAH concentrations remaining changed differently with all treatments.

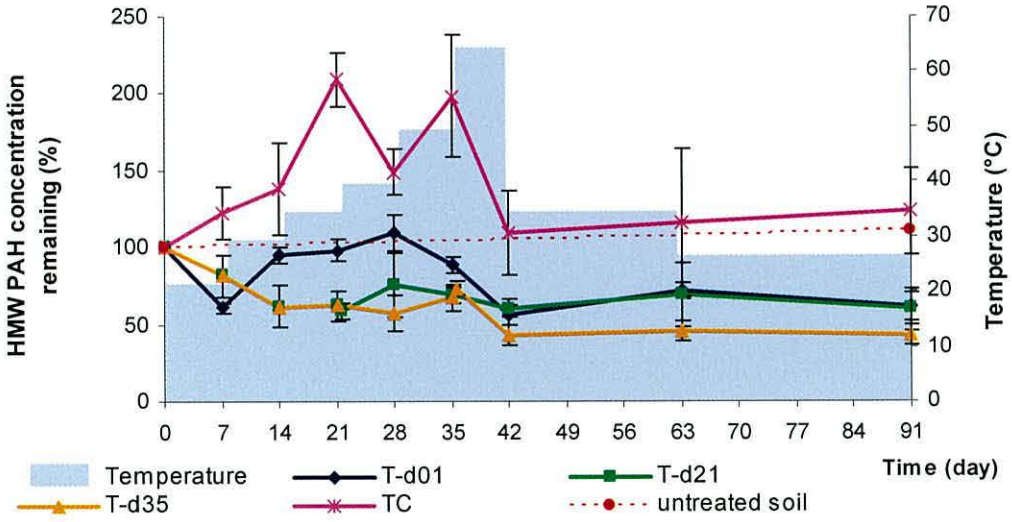

Figure 6.18: Mean percentages of concentrations of grouped weathered HMW PAHs remaining by the end of different temperature steps. The red dotted line connects the percentage of the remaining HMW PAHs detected with the untreated soil after 91 days with the 100% at the beginning of the experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

As with the MMW PAHs, HMW PAH concentrations also decreased more slowly, but for a longer period when manure was added to the fungi treatments at a later stage (T-d21 and T-d35) than at day 0 (T-d01). At the

end of the experimental period, concentrations of total HMW PAHs in the TC treatment were 123 ±29% of that initially present, while correspondingly, concentrations in the untreated soil increased to 111%. With the fungi/manure treatments, HMW PAH concentrations remaining were decreased to 61 ±9%, 60 ±13% and 43 ±6% within the T-d01, T-d21, and T-d35 treatments, respectively.

Split-plot analysis of the amount of grouped HMW PAHs (in ng g^{-1} ash) revealed significant interactions of treatment and temperature/time but only during the first phase (days 0-21) of the experiment ($p < 0.001$). During this phase the percentage of PAH concentrations remaining changed in different ways with all treatments.

6.4.3.2. Influence of incubation temperature on changes in PAH concentrations

Analysis of PAH degradation at each of the imposed temperature steps failed to show any clear temperature-dependent PAH removal patterns (Figures 6.19 and 6.20). This was probably due to the large inherent variability between the replicate vessels.

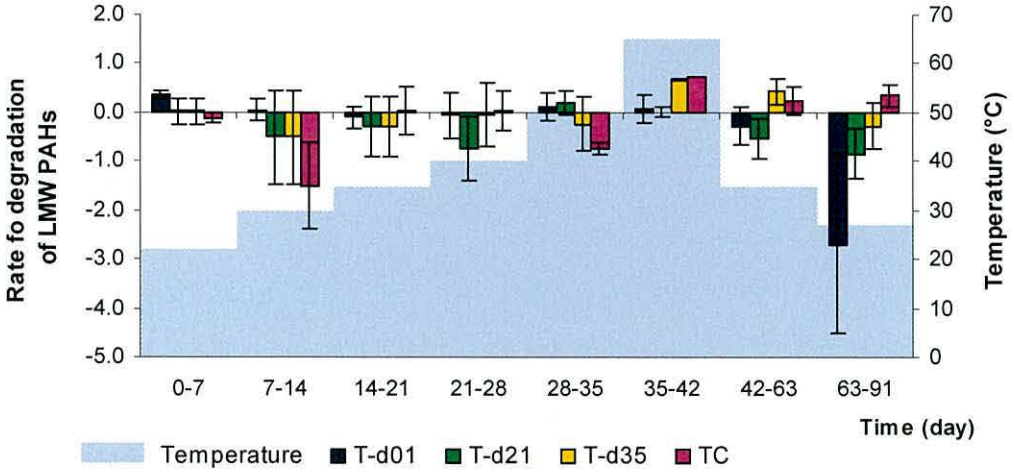

Figure 6.19: Mean rates of degradation of grouped MMW and HMW PAHs with the different treatments during the different temperature steps of the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

The rates of degradation of MMW and HMW PAHs were similar (Figure 6.24) and not significantly different between temperature-periods within the T-d21, T-d35, and TC treatments. Within the T-d01 treatment rates were significantly higher during the initial 22°C and the 65°C (days 35-42) temperature steps than during the first 35°C (days 7-14) and last temperature period (days 63-91, 27°C). This was due to an increase in concentrations during the latter periods. Overall, no clear pattern of degradation rates was detectable for either MMW PAHs or HMW PAHs.

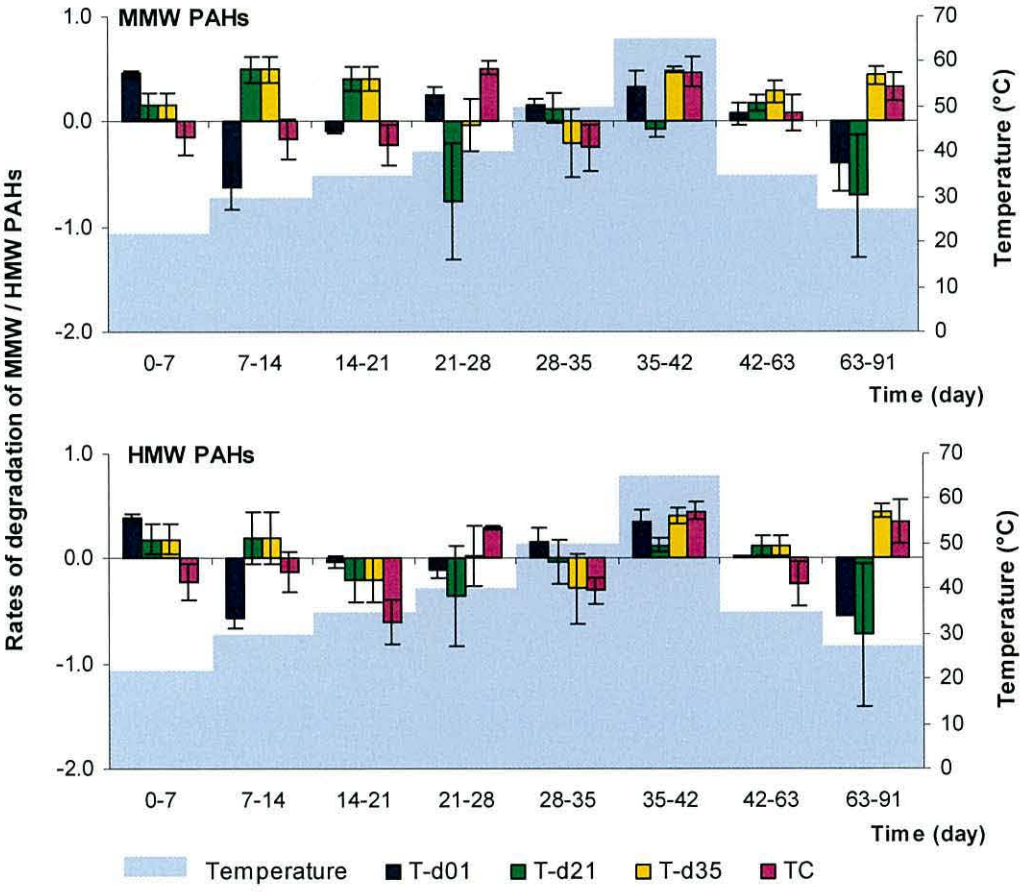

Figure 6.20: Mean rates of degradation of grouped MMW and HMW PAHs with the different treatments during the different temperature steps of the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

No significant correlation of PAH degradation rates with any physico-chemical or microbiological analysis were found.

6.4.3.3. Effects of extractable PAH concentrations

Differences in PAH concentrations (in µg g^{-1} ash) were detected between treatments and their replicates (Table 6.3). PAH concentrations have been shown to be one factor influencing degradation. While high pollutant concentrations enhance sorption to soil and thus enhance ageing, a minimum threshold concentration is required for catabolic induction in degrading organisms (Semple *et al.* 2001). Contrastingly, a very high concentration can be toxic to degrading organisms (Ahtiainen *et al.* 2002). Analysing a degradation process purely by looking at degradation rates and concentrations expressed as a percentage of initial amounts might therefore distort results.

Possible interactions of relative PAH amounts with physico-chemical properties, microbial community changes, PAH volatilisation, or PAH degradation were analysed using Spearman's rank correlation. Values of correlation coefficients (n=83, DF=81) were below 0.3 representing only a weak correlation which might not have been caused by the relative PAH amounts but might be due to other factors influencing both variables in the same way.

The possibility that concentrations detected at day 91 might represent a threshold value below which further degradation was not possible under the present circumstances was investigated. A treatment reaching a threshold value would result in PAH concentrations levelling out at a certain amount. Extractable PAH concentrations did not change significantly during the cooling phase of the experiment for any PAH group with any of the treatments, suggesting that a threshold value might have been reached below which a further reduction of PAH concentrations is not possible under the present conditions. A threshold value independent of the treatment on the other hand would result in PAH concentrations being similar within all vessels. At the start of the experiment the vessel with the highest level of contamination had between 3.7 (Dibenz[a,h]anthracene) to 4.9 (Naphthalene) times the amount of the PAH (in µg g^{-1} ash) as was found in

the vessel with the lowest level of contamination. At the end of the experimental period these ratios of highest to lowest PAH concentration had increased for all PAHs except Fluoranthene, Pyrene, and Indeno[1,2,3-c,d]pyrene and ranged from 3.7 (Indeno[1,2,3-c,d]pyrene) to 28.0 (Benzo[g,h,i]perylene). This increasing difference between relative amounts of PAHs in the vessels indicates that the soil mixtures still have the potential for further PAH degradation.

Table 6.3: PAH concentrations of 15 detected USEPA PAHs at day 0 of the experiment after mixing the soil with the different additives. Values are averages (µg per g ash) of the amounts measured in the replicate vessels ±S.D.; n=6 for T-d21/T-d35 day 0, else n=3

		T-d01	**T-d21**	**T-d35**	**TC**
	day	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.	µg g^{-1} ±S.D.
LMW PAHs					
Naphthalene	0	3.1 ±1.1	2.1 ±0.2	3.1 ±0.9	1.5 ±0.8
	91	*0.7 ±0.4*	*0.5 ±0.4*	*0.3 ±0.2*	*0.2 ±0.1*
Acenaphthylene	0	2.4 ±0.3	1.8 ±0.3	2.5 ±0.3	1.1 ±0.3
	91	*0.4 ±0.2*	*0.3 ±0.2*	*0.6 ±0.3*	*0.3 ±0.1*
Acenaphthene	0	1.5 ±0.2	0.9 ±0.2	1.6 ±0.1	0.7 ±0.3
	91	*0.5 ±0.3*	*0.3 ±0.1*	*0.7 ±0.5*	*0.4 ±0.2*
Fluorene	0	6.2 ±2.2	5.5 ±1.1	5.6 ±0.5	2.3 ±0.4
	91	*1.6 ±1.1*	*1.2 ±0.6*	*1.8 ±0.8*	*0.8 ±0.7*
Phenanthrene	0	64.1 ±24.2	56.4 ±23.8	58.8 ±7.4	23.1 ±3.2
	91	*32.3 ±15.8*	*25.7 ±12.6*	*30.3 ±18.7*	*13.4 ±10.5*
Anthracene	0	64.0 ±24.1	57.4 ±25.3	58.7 ±7.6	23.0 ±3.3
	91	*31.6 ±15.7*	*26.1 ±12.9*	*29.3 ±17.2*	*13.4 ±10.5*
MMW PAHs					
Fluoranthene	0	295.1 ±22.0	300.6 ±60.1	327.9 ±30.8	136.8 ±46.0
	91	*92.3 ±25.6*	*109.0 ±59.6*	*91.4 ±17.4*	*49.9 ±14.5*
Pyrene	0	296.9 ±20.3	301.5 ±58.1	328.4 ±30.4	136.5 ±46.6
	91	*92.5 ±25.4*	*109.2 ±59.8*	*91.3 ±17.2*	*50.0 ±14.7*
Benz[a]anthracene	0	22.2 ±1.2	22.4 ±2.5	22.3 ±2.4	9.3 ±3.2
	91	*9.2 ±2.2*	*6.3 ±3.3*	*4.4 ±2.7*	*2.9 ±2.6*
Chrysene	0	22.2 ±1.3	22.3 ±2.5	22.2 ±2.4	8.9 ±2.9
	91	*9.2 ±2.2*	*6.0 ±3.2*	*4.2 ±1.7*	*2.1 ±1.4*
HMW PAHs					
Benz[b]fluoranthrene	0	21.8 ±1.1	20.6 ±2.7	22.2 ±2.3	9.5 ±3.2
	91	*14.2 ±4.6*	*11.6 ±5.5*	*9.5 ±2.0*	*7.0 ±2.9*
Benzo[a]pyrene	0	25.2 ±1.5	22.9 ±2.8	26.3 ±2.5	11.1 ±3.6
	91	*17.3 ±5.5*	*14.1 ±6.4*	*11.8 ±2.1*	*8.9 ±3.7*
Indeno[1,2,3-cd]pyrene	0	31.1 ±2.3	31.2 ±4.0	34.1 ±3.0	14.6 ±4.6
	91	*17.3 ±5.2*	*15.0 ±6.9*	*12.2 ±1.4*	*10.4 ±4.3*
Dibenz(a,h)anthracene	0	5.3 ±1.5	6.0 ±1.0	7.2 ±0.5	2.9 ±0.8
	91	*3.5 ±1.2*	*3.1 ±1.5*	*2.4 ±0.3*	*1.9 ±0.8*
Benzo[ghi]perylene	0	6.4 ±1.1	6.6 ±1.6	7.3 ±0.6	3.0 ±1.0
	91	*2.9 ±0.7*	*9.6 ±8.6*	*5.6 ±6.8*	*4.9 ±6.2*

6.4.3.4. Combined influences of the different treatments and temperature (time) on the behaviour of grouped PAHs

Significant differences between the behaviour of the treatments were detected by split plot ANOVA for all grouped PAHs except the LMW PAHs during the initial 3 weeks of the experiment with all treatments behaving differently. Total weathered PAH concentrations seem to be decreased by addition of the fungal inoculum (T-d01, T-d21, T-d35), while additional amendment with chicken manure (T-d01) seemed to increase concentrations after an initial decrease. Without additives (TC), PAH concentrations increased constantly during the first 3 weeks. Those developments were significantly different between the treatments during the first three weeks ($p < 0.0005$). No significant effects of treatments over temperature (time) were detectable during the rest of the experiment.

The degradation behaviour of LMW PAHs was not significantly different between any of the treatments. Due to a high variability between replicate vessels LMW PAHs also failed to show significant decreases in concentrations. As suggested in previous parts of the study, this might be due to the pre-treatment of the soil used. During the initial soil pre-treatment and mixing phase, the introduction of O_2 induced the loss of mainly small PAHs (LMW PAHs and partly MMW PAHs) leaving the more strongly bound and less available PAH fractions in the soil.

For MMW PAHs the TC treatment continued to show a different behaviour to the fungi/manure treatments until day 35 ($p < 0.0001$ day 0-21; $p < 0.005$ day 21-35). MMW PAHs concentrations were significantly lower by the end of the experiment with all treatments; however, results show that treatments T-d21 and T-d35 (fungal amendment with manure addition at a later stage) decreased PAH concentrations more quickly. For those two treatments the lowest concentrations of weathered MMW PAHs were reached by day 14, whereas treatments TC and T-d01 only reached their lowest concentrations by day 42.

An advantage of a fungi/manure amended treatment was shown for the HMW PAHs. While at the end of the 91 day experimental period concentrations of HMW PAHs were not significantly different to initial concentrations with the TC treatment, significantly lower concentrations were detected with the fungi/manure amended treatments. As with MMW PAHs the treatments amended with fungi but without manure (T-d21 and T-d35) behaved significantly differently ($p < 0.001$) to the other treatments during the first 3 weeks of the experiment with constantly decreasing HMW PAH concentrations.

6.4.3.5. PAH volatilisation

The amounts of PAHs lost in the vapour phase were related to the amount in the contaminated soil in the vessel. Overall, the loss of PAHs from the soil due to volatilisation was relatively low and represented an insignificant loss pathway in comparison to the total amount remaining in the soil. However, a temperature dependency of PAH volatilisation could be detected (Figure 6.21).

The LMW PAHs volatilisation rates were highest at the end of the 50°C temperature step for all but the T-d01 treatment, decreasing until the end of the experiment. This indicates that volatilisable LMW PAHs have been released at 50°C and that a further temperature increase did not result in further LMW PAH release.

Volatilisation rates of MMW PAHs were highest at the end of the first 35°C temperature step (day 21) for the treatments with only fungi addition (T-d21 and T-d35). Those volatilisation rates decreased during the following temperature step and with all treatments an increase was detected after the temperature was increased to 65°C (day 42).

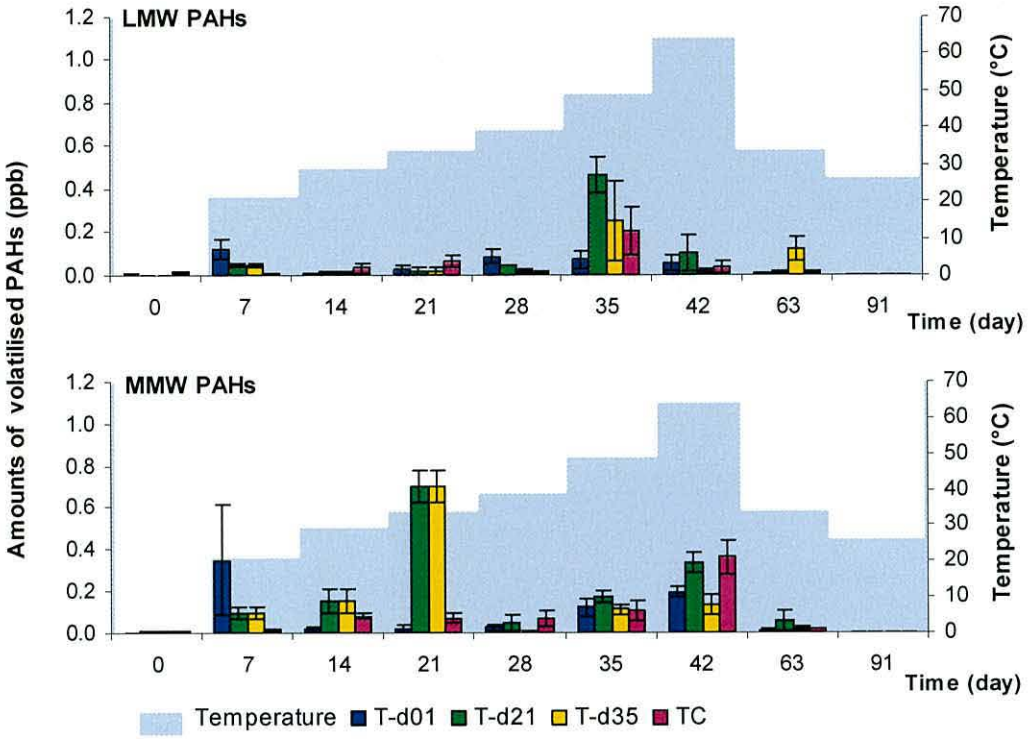

Figure 6.21: Mean volatilisation rates of the amounts of PAHs measured in relation to the amount of those PAHs detected in the soil in the vessel at any time. Rates are given for grouped aged LMW and MMW PAHs during the different temperature steps with the different treatments during the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

Figure 6.21 shows PAH volatilisation rates as ppb of the total amount of the respective extractable PAHs in the treatment mixture in the soil. In relation to the extractable amounts present in the soil-mixtures, more MMW PAHs were released than LMW PAHs. However, total amounts of LMW PAHs detected in the gas phase were up to 30 times higher than MMW PAHs (Figure 6.22). No HMW PAHs were detected within the gas phase.

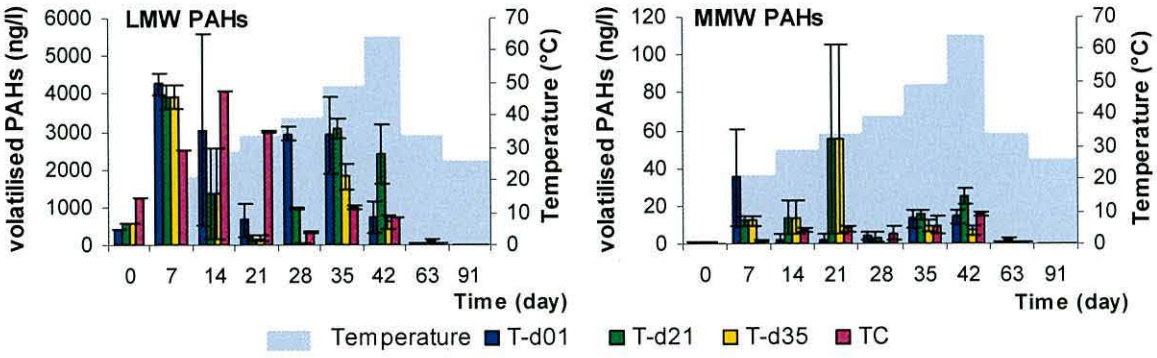

Figure 6.22: Mean PAH concentrations measured in ng per litre air taken from the headspace of the treatment vessels. Concentrations are given for grouped weathered LMW and MMW PAHs during the different temperature steps with the different treatments during the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

6.4.4. Discussion of effects of different process characteristics on PAH removal

The soil used in this experiment came from the same source as the soil used in the manure and fungi experiments (Chapters 4 and 5) and was placed into a barrel and kept outdoors sheltered from rain for 3 months after the 2 months windrow pre-treatment (while the manure and fungi experiments were performed). As established within the manure experiment extractable PAH concentrations did not change significantly during this storage time and the soil still contained relatively high quantities of PAHs.

An increase in extractable PAHs was detectable with the TC treatment during the heating phase of the experiment. An increase in extractable PAH amounts also indicates increased bioavailability, as does the increase in volatilisation rates at higher temperatures. PAH bioavailability is considered one of the main factors influencing biodegradation of contaminated soil (Pignatello and Xing 1996; Carmichael and Pfaender 1997; Semple *et al.* 2001).

With increasing contact time (ageing) with soil, PAHs exhibit decreasing extractability and bioavailability to soil organisms (Brusseau *et al.* 1991; Northcott and Jones 2001). Factors further affecting bioavailability are temperature (Gilot *et al.* 1997), substitution of PAHs by other components at binding sites (Kästner and Mahro 1996) and surfactants which can increase availability (Wong *et al.* 2004), and sorption to organic matter which can decrease bioavailability (Vogel 1996).

Overall results of this study indicate that bioavailability is the main factor influencing the bioremediation effort. High clay and organic matter contents have been shown to restrict the mass transfer of the contaminants to the micro-organisms (Vogel 1996). PAH concentrations were not correlated to any other analysis undertaken and no clear evidence has been found that the micro-organisms introduced with the fungal inoculum and chicken manure are responsible for PAH removal. However, differences between the

treatments were detected. Those differences were insignificant for the removal of the fresh contamination with the deuterated PAHs (see Chapter 7), but more pronounced with the aged contamination, emphasising the importance of bioavailability on bioremediation.

6.4.4.1. The role of physico-chemical characteristics and microbial communities

Differences in PAH removal during the first experimental weeks between the treatment that had fungi and manure added at day 0 (T-d01) and the fungi amended treatments where chicken manure had not been added so far (T-d21, T-d35) might be attributable to competition between the introduced fungi and the micro-organisms introduced with the chicken manure, as well as the physico-chemical characteristics of the chicken manure.

The chicken manure had a high N content. It has been found that the expression of lignin-degrading peroxidases is inhibited in some white-rot fungi by high N availability (Sinsabaugh 2005) and ligninolytic enzyme production has been found to be induced under nutrient limiting conditions with some strains (Faison and Kirk 1985; Buswell 1991). Conversely, a positive effect of nitrogen rich conditions on ligninolytic enzyme activities of other white rot fungi including *P. ostreatus* has been reported by Kaal *et al.* (1995). However, in their study peroxidase activity was reported to have started between day 17 and 20 depending on the medium used. In the present study changes in temperature occurred too quickly for such a lag-phase. Here the MMW and HMW PAH amounts increased with the T-d01 treatment after an initial decrease. This later increase might be due to a combined effect of increasing temperatures and the added organic matrix substituting PAHs from bound sites.

Changes in the microbial community profiles as well as in physico-chemical characteristics however, seemed to have been influenced to a larger extent by temperature/time than by the treatments. Microbial communities as analysed by PLFA extraction were not correlated to PAH degradation or

physico-chemical characteristics. Nevertheless, micro-organisms might still profit from nutrients introduced with additive addition. The organic matrix also supplies ecological niches which might be used by PAH degrading organisms (Kästner and Mahro 1996).

Similar to findings by Kästner and Mahro (1996), the lack of correlation between PAH removal, and microbial communities suggests that the indigenous microbial community might have had a greater effect on PAH removal than the introduced organisms. Even though no statistical evidence was found, the micro-organisms introduced with the additives might have a positive effect on PAH degradation during a shorter period which might not be statistically detectable.

6.4.4.2. Advantages of different treatments

In contrast to treatment TC, the treatments initially only amended with the *P. ostreatus* inoculum (T -d21 and T-d35) did not show an increase in PAH amounts. Assuming that temperature had a similar effect on PAH release with these treatments as with the TC treatment and that volatilisation only had a limited effect on PAH loss, the lower PAH concentrations were either caused by biodegradation and/or binding by the organic matrix introduced with the fungal inoculum. Sorption of organic compounds in soil is well known and increases with the organic matter content (Means *et al.* 1980). However, in the present study extractable PAH concentrations were not correlated with the organic matter content of the mixtures and it is unlikely that the difference in PAH concentrations between the treatments could be simply caused by sorption to the added organic matrix. Therefore results imply that the loss of PAHs was caused by real biodegradation.

Compared to treatments TC and T-d01 the treatments with initially only fungi addition seem to behave in a more predictable way. With final PAH concentrations being not significantly different between the fungi/manure amended treatments a treatment of a combination of fungal additive with

additional manure addition after day 21 seemed recommendable. In case of treatment T-d21 the initial PAH removal was found to be higher and more predictable with only fungus addition than with additional manure amendment (T-d01). Manure was added at day 21 when the temperature was increased from 35° to 40°C. In a natural co-composting process the manure addition would induce a rise in temperature. In case of treatment T-d35 manure was added when temperatures were changed from 50° to 65°C. It is very unlikely that temperatures would have increased to these values without manure addition. Therefore, even though all fungus/manure amended treatments showed similar effects on final PAH removal treatment T-d21 is recommendable.

6.5. CONCLUSIONS

This Chapter investigated the complementary effect of fungus and manure addition on PAH removal from the soil (Thesis aim 3a) and determined the importance of the time point at which manure is added (Thesis aim 3b). Differences in the co-composting processes were found to be dependent on the treatments. The major findings of this work are summarised as follows:

- Physico-chemical characteristics were significantly influenced by the addition of manure but not by the addition of the fungal inoculum, however, this difference had little effect on PAH removal rates.
- The addition of the fungal inoculum increased the amounts of fungal PLFA.
- Chicken manure addition increased the amount of fungal PLFA and decreased the ratio of Gram-negative-to-Gram-positive bacteria by increasing Gram-positive bacteria to a larger extent.
- After changes in the PLFA profile by additive addition, microbial communities still developed in similar ways during the experiment.

Decreases as well as increases in extractable PAH concentrations were detected with all groups of PAHs and with all treatments.

- No changes in weathered PAH concentrations were detected within the untreated soil.
- Fungi and manure amended treatments decreased PAH concentrations more than the TC treatment.
- A higher variability of the extractable PAH fraction was detected with TC treatment which was also the only treatment in which concentrations increased well above their starting amounts.
- Manure addition at the beginning of the experiment caused the PAH concentrations with treatment T-d01 to change in a different way than treatments T-d21 and T-d35 with only fungus addition, during the first 3 weeks.
- Highest removal rates of weathered PAH were found during the heating phase with treatments T-d21 and T-d35. T-d35 showed increasing concentrations during the cooling phase.
- No accumulation of PAH degradation products could be detected.
- The loss of PAHs through volatilisation was insignificant; however a temperature dependency was detectable.
- None of the treatments appeared to have reached a threshold value suggesting that the imposed composting regime failed to completely remove all the potentially available PAHs.

Out of the treatments tested, the addition of *P. ostreatus* inoculated sawdust followed by the addition of chicken manure at day 21 had the greatest potential to enhance PAH removal from an aged contaminated soil. In this case the fungus inoculated into the soil can grow without having to compete with additional organisms introduced with the chicken manure, or with having to adapt to significantly changed environmental conditions. The

hypothesis that the time-point at which manure is added to the fungus inoculated soil, influences PAH removal from the aged contaminated soil, has to be accepted.

In the T-d35 treatment PAH concentrations have been reduced to a similar extent as in the T-d21. However, in a large scale composting process the temperatures would probably have to be changed artificially if manure was to be added during the thermophilic phase, as due to the lower amount of organic matter, a temperature profile would not develop. To avoid this, chicken manure should therefore be added during the lower temperature phases, as was with treatment T-d21. However, even though the amended treatments showed higher PAH removal than the un-amended TC treatment, significant PAH removal following manure addition was not obvious.

The hypothesis that a combination of the white-rot fungus *P. ostreatus* and chicken manure enhances the removal of PAHs from an aged contaminated soil has to be accepted if compared to the TC treatment. If it has to be rejected compared to single amendments of fungus and manure will be analysed in the following Chapter 8. For a large scale application the influence of temperature and inoculum size should be further investigated.

CHAPTER 7

Differences in degradation behaviour between a fresh and aged source of contamination

7.1. ABSTRACT

As an expansion of the experiment described in Chapter 6 the soil was additionally spiked with the deuterated LMW PAH Anthracene-D10 and MMW PAH Benz[a]anthracene-D12. Differences in the degradation of the freshly added deuterated PAHs and the aged contamination were analysed. Analysis revealed a significantly higher degradation of the deuterated PAHs than of their aged counterparts showing the importance of bioavailability on removal rates. Comparison of different treatments with and without *Pleurotus ostreatus* and chicken manure amendment during different stages of the experiment implies a better suitability of indigenous microbial communities to degrade PAHs that are readily available. Additionally it was found that temperature did not seem to have as great an effect on PAH removal of a fresh contamination than of an aged one. On the other hand *P. ostreatus* amendment proved beneficial for PAH removal when PAHs were not readily available, as was the case for the weathered PAHs.

7.2. INTRODUCTION

Contaminant availability is one of the main requirements for successful bioremediation of contaminated soil. It has been found that the longer an organic chemical remains in contact with soil, the less likely it is that the chemical can be degraded by micro-organisms (Hatzinger and Alexander 1995; Semple *et al.* 2001).

The nature and extent of "ageing", which is the decrease in compound bioavailability with time, depends on the pollutant and its characteristics (e.g. hydrophobicity, vapour pressure) (Cerniglia 1992) as well as soil properties (e.g. organic matter content, particle size) (Hatzinger and Alexander 1995). It is widely accepted that sorption is the controlling factor in the ageing process. It can occur as sorption to soil organic matter and inorganic soil constituents. Another important factor is diffusion of compounds into spatially remote areas (soil macro and micro pores as well as within soil organic matter).

Ageing results in the movement of chemicals from accessible soil compartments into less accessible or inaccessible compartments, resulting in a reduction of bioavailability. After ageing three soil-associated pools of contaminants exist: [1] a fraction that can be rapidly desorbed, [2] a fraction which is desorbed more slowly, and [3] a fraction of "bound residue" (Semple *et al.* 2001). The third faction has been defined as compounds that persist in the sample matrix after extraction, whereby the extraction method does not substantially change the compounds themselves or the structure of the matrix (Semple *et al.* 2001). The size of the "bound residue"-fraction is therefore dependent on the extraction method used.

As compound uptake by micro-organisms is far more extensive from fluid than from sorbed states it has been proposed that pollutant mass transfer governs bioavailability (Semple *et al.* 2003). Successful bioremediation of PAH contaminated soil further depends on the micro-organisms present (mainly bacteria and fungi), their community interactions and survival, and their growth conditions. Both groups of organisms have advantages and limitations as mentioned before and a remediation process involving a combination of both has potentially the best prospect of success.

The experiment described in Chapter 6 had been extended to additionally investigate the effects of ageing on the removal of PAHs. The PAHs contained within the soil used here have been subjected to ageing processes over several decades, lowering their bioavailability (White *et al.* 1999; Volkering and Breure 2003).

The aim of this part of the thesis is

> To assess the influence of "ageing" on PAH availability and removal from contaminated soil.
> (Thesis aim 4)

The soil used in the experiment was therefore additionally spiked with the deuterated LMW PAH Anthracene-D10 and MMW PAH Benz[a]anthracene-D12. Those added D-PAHs have not been subjected to ageing processes and therefore represent fresh contamination. It is hypothesised that:

> Ageing influences the removal of PAHs from soil due to different removal-rate limiting processes;

and that

> The effectiveness of different treatments changes with ageing of the PAHs.

7.3. MATERIALS AND METHODS

7.3.1. Soil

The aged contaminated soil (see Chapter 3 "Materials and methods") was additionally contaminated with known amounts of the deuterated LMW PAH D-Acenaphthene and MMW PAH D-Benz[a]anthracene (Sigma-Aldrich, Schnelldorf, Germany) to give a final concentration of 33 and 8 mg kg^{-1} dry soil, respectively. This was to directly compare the decomposition rates of newly added contaminants with those intrinsically present which are largely bound to the soil's solid phase.

The soil was spiked following a method recommended by Brinch *et al.* (2002). To ensure the survival of indigenous micro-organisms only 25% (7.5 kg) of the soil was additionally contaminated and later mixed with the remaining soil before being split up into the 4 treatments. The deuterated compounds were prepared by dissolving 1 g Acenaphthene-D10 and 250 mg Benz[a]anthracene-D12 in 750 ml acetone. To ensure a more homogenous mixing, the 7.5 kg soil were split into 5 batches of 1500 g. 150 ml of the acetone solution was added to each batch and the soil was thoroughly mixed by shaking in a vessel. Following this first mixing, the 5 batches were mixed together and stored in a box in a fume cupboard for the acetone to evaporate. During the 48 hour evaporation period the soil was frequently mixed. After 48 hours no acetone smell could be detected and the spiked soil was thoroughly mixed with 22.5 kg of the un-spiked soil and divided into batches of 7.5 kg for each treatment.

7.3.2. Additives and Treatments

The additives used and the treatments and temperature profile applied are described in Chapter 6, while a description of soil and sawdust characteristics as well as the methods can be found in Chapter 3 "Materials and methods".

7.3.3. Statistical Analysis

Statistical analysis was performed as described in Chapter 3 "Materials and Methods". Additionally split-plot ANOVA was performed as described in Chapter 6 (point 6.3.4).

7.4. RESULTS

7.4.1. Comparison of changes of extractable deuterated and weathered PAH concentrations

Data from PAH extractions of the freshly added deuterated PAHs Acnaphthene-D10 and Benz[a]anthracene-D12 and their weathered counterparts Acenaphthene and Benz[a]anthracene have been analysed by looking at their concentrations (µg g^{-1} ash), percentage change (% remaining concentration of initial concentration), and rates of degradation (factor change in concentration during individual temperature steps) as described in Chapter 6 (point 6.4.3.)

7.4.1.1. Total amounts and concentrations over the 91 day experiment

Initial amounts as well as the behaviour of the added deuterated PAHs (Acenaphthene-D10 and Benz[a]anthracene-D12) were different to their weathered counterparts (Acenaphthene and Benz[a]anthracene) in the experiment. The initial amounts of Acenaphthene-D10 in the soil was 15 to 18 times higher than amounts of weathered Acenaphthene, while the amounts of Benz[a]anthracene-D12 were similar to the amount of weathered Benz[a]anthracene amounts (Table 7.1).

Table 7.1: Initial concentrations (µg g^{-1} ash) of the added deuterated PAHs and their weathered counterparts present in the treatments. Letters a,b,c represent the replicate vessels.

Vessel	Acenaphthene-D10	Acenaphthene	Benz[a]anthracene-D12	Benz[a]anthracene
T-d01-a	32.7	1.7	27.3	21.3
T-d01-b	26.2	1.3	23.2	21.8
T-d01-c	28.2	1.5	23.8	23.6
T-d21-a	10.5	0.8	13.9	19.8
T-d21-b	12.4	1.0	15.4	22.8
T-d21-c	13.0	1.1	16.5	24.8
T-d35-a	27.6	1.6	21.4	23.1
T-d35-b	28.2	1.7	23.9	24.2
T-d35-c	22.0	1.4	20.9	19.5
TC-a	7.5	0.4	6.2	5.6
TC-b	13.1	0.7	10.5	10.7
TC-c	14.7	0.9	11.3	11.6

Within all treatments, concentrations of Acenapththene-D10 decreased quickly during the first 3 weeks of the experiment with only 1.9, 4.7, 1.8, and 2.9% remaining after 42 days in the T-d01, T-d21, T-d35, and the TC treatments, respectively (Figure 7.1). Even though the starting concentrations of Acenaphthene-D10 were up to 18 times higher than those of the weathered Acenaphthene, after the 91 days Acenapthene-D10 concentrations were only 1.7 times higher with the T-d21 treatment, but 1.2 (T-d01), 1.9 (T-d35), and 1.6 (TC) times lower with the other treatments.

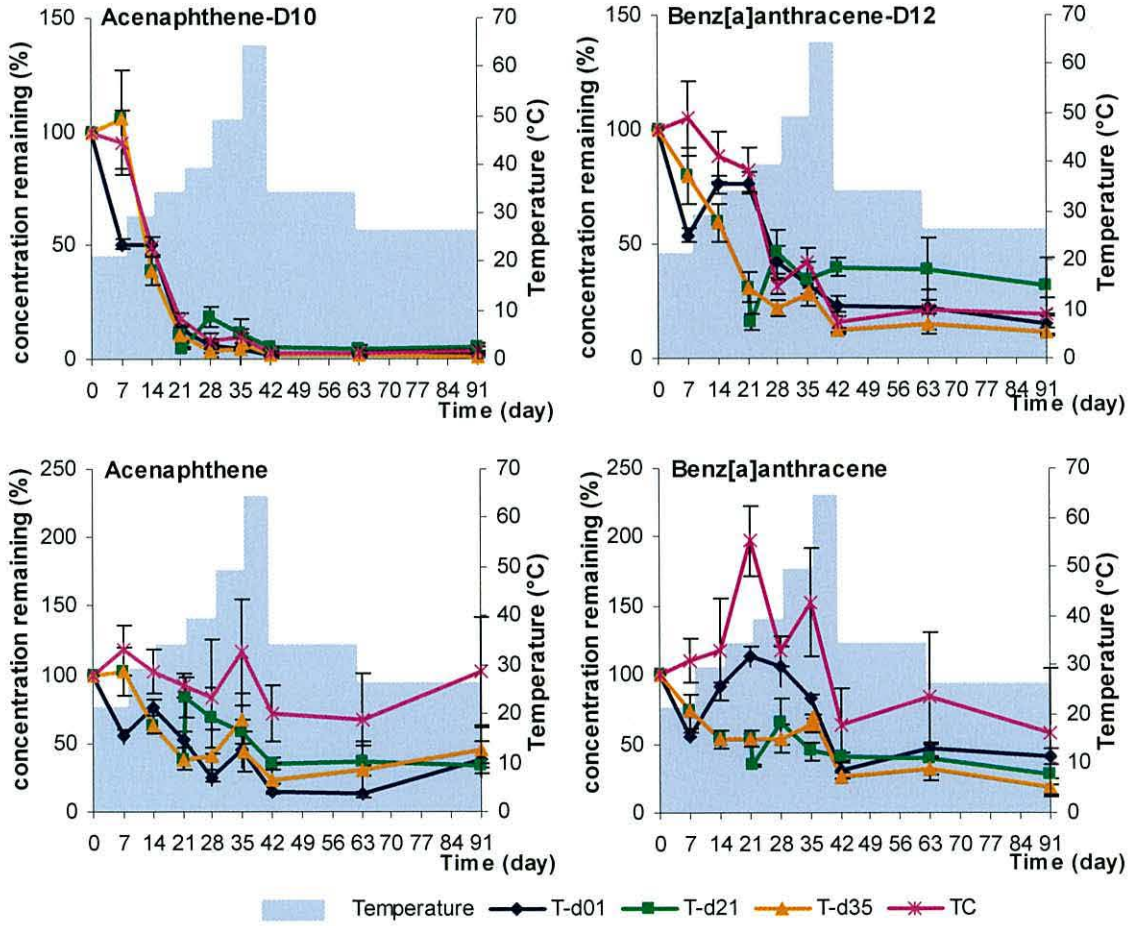

Figure 7.1: Mean percentages of the added deuterated PAHs Acenaphthene-D10 and Benz[a]anthracene-D12 and their weathered counterparts Acenaphthene and Benz[a]anthracene remaining in the different treatments by the end of different temperature steps of the 91 day experiment. Error bars represent mean ±S.E.; n=6 for T-d21/T-d35 from day 0 to 21, else n=3

Acenaphthene-D10 concentrations generally decreased during each temperature step of the heating phase (day 0 to 42) for all treatments. The only exemption is treatment T-d21. During the temperature step following manure addition (day 21 to 28) an increase in extractable Acenaphthene-D10 concentrations was detected. No significant changes in Acenaphthene-D10 concentrations were detected during the cooling phases for any of the treatments.

The Benz[a]anthracene-D12 concentrations also decreased faster than the weathered Benz[a]anthracene for all but the T-d21 treatment with most degradation occurring in the first 42 days (Figure 7.1). The removal-patterns of the weathered and deuterated Benz[a]anthracene were similar within the treatments. However, weathered Benz[a]anthracene concentrations increased to a larger extent than concentrations of the deuterated form resulting in higher amounts of the aged Benz[a]anthracene being extractable by the end of the experiment for all but T-d21.

Similarly to Acenaphthene-D10, Benz[a]anthracene-D12 concentrations decreased or showed no significant change during each temperature step of the heating phase (day 0 to 42) for all treatments and extractable Benz[a]anthracene-D12 concentrations increased in the T-d21 treatment during the temperature step following manure addition at day 21. No significant changes in Acenaphthene-D10 concentrations were detected during the cooling phases for any of the treatments.

7.4.1.2. Degradation rates of weathered and deuterated PAHs during each temperature step

Generally, degradation rates did not differ significantly between different temperature steps with all four investigated PAHs within each treatment, but were higher during the heating phase of the experiment especially when the contamination was fresh (deuterated-PAHs). With the temperatures changing, degradation rates of Acenaphthene-D10 and Acenaphthene changed in a similar way. However, degradation rates were generally higher

with the added Acenaphthene-D10. Degradation rates of Benz[a]anthracene-D12 and Benz[a]anthracene also changed in a similar way with generally higher degradation rates of Benz[a]anthracene-D12.

Kinetic models have been used to describe PAH removal from soil (Antizar-Ladislao et al. 2005b; Thiele-Bruhn and Brümmer 2005). As the temperature has been changed during this study a kinetic model relying on constant temperatures is not applicable. However, similar degradation rates during different temperature steps suggest the possibility of a degradation following an exponential equation of the general form

$$C_t = C_0 * e^{-r*t} \quad \text{(E.7.1)}$$

where C_t is the extractable PAH concentration at time t, C_0 is the extractable PAH concentration at the start of the experiment, r is the factor by which the PAH concentration is reduced per unit of time, t is time in days.

Rearrangement of equation 7.1 gives the linear integrated form:

$$\ln (C_t/C_0) = -r * t \quad \text{(E.7.2)}$$

Using equation 7.2, r can be obtained by linear regression analysis. Analysing the data in this way can present a way to compare PAH removal between the different treatments.

Regression analysis using data from the whole experimental period showed poor fittings (Table 7.2) which implied that degradation rates were not similar during all temperature steps and that temperature and time did have an influence on those degradation rates.

Table 7.2: Factor of PAH removal (r) and R^2 values obtained by linear regression to calculate average reduction of extractable PAH concentrations for the added deuterated PAHs and their aged counterparts.

	T-d01		T-d21		T-d35		TC	
	r	R^2	r	R^2	r	R^2	r	R^2
Acenaphthene-D10	0.048	0.74	0.034	0.66	0.052	0.72	0.048	0.78
Acenaphthene	0.015	0.38	0.012	0.56	0.009	0.29	0.008	0.61
Benz[a]anthracene-D12	0.022	0.85	0.010	0.48	0.024	0.74	0.027	0.80
Benz[a]anthracene	0.010	0.38	0.012	0.83	0.016	0.81	0.014	0.59

Degradation rates during single temperature steps showed that a reduction in PAH concentrations mainly occurred during the heating phase of the experiment. Taking this into consideration a multi-phase approach with separate regression analyses was additionally tested. Differences in the removal rates during the first 6 weeks and during the cooling phase (day 42 to 91) were investigated. The two-phase model was then further adapted taking the addition of chicken manure with treatments T-d21 and T-d35 into account (Figures 7.2a/b) as described for the groups of aged PAHs.

Splitting the data into separate phases improved the fitting of the regression models for the heating phases. The fitting for the cooling phases appeared very poor in some cases. A good fit indicates that an exponential equation can be used to describe PAH removal during the respective temperature period and that removal rates can be expressed as removal per day. A poor fit in cases like treatments T-d01, T-d21 and T-d35 for Acenaphthalene-D10 during the cooling phase is due to the fact that PAH concentrations were not showing further larger changes and an exponential equation to express removal of PAHs was not applicable.

Regression analysis showed good fits for Acenaphthene-D10 during the heating phase for all treatments (R^2 = 0.83 to 0.97). Under the given conditions removal rates were between 0.071 and 0.096 d^{-1} depending on the treatment. Over the whole heating phase, fits were worse for the weathered Acenaphthene than for the deuterated forms for all treatments.

With the T-d21 treatment splitting the heating phase into before- and after-manure-addition improved the fitting of the regression lines further, while with the T-d35 treatment a better fit was observed with no splitting of the heating phase. Removal rates of all four PAHs were highest during the heating phases (h1) when only fungal additives were added to treatments T-d21 and T-d35. Results of treatment T-d21 indicate an influence of manure addition on the removal rates when manure is added at day 21.

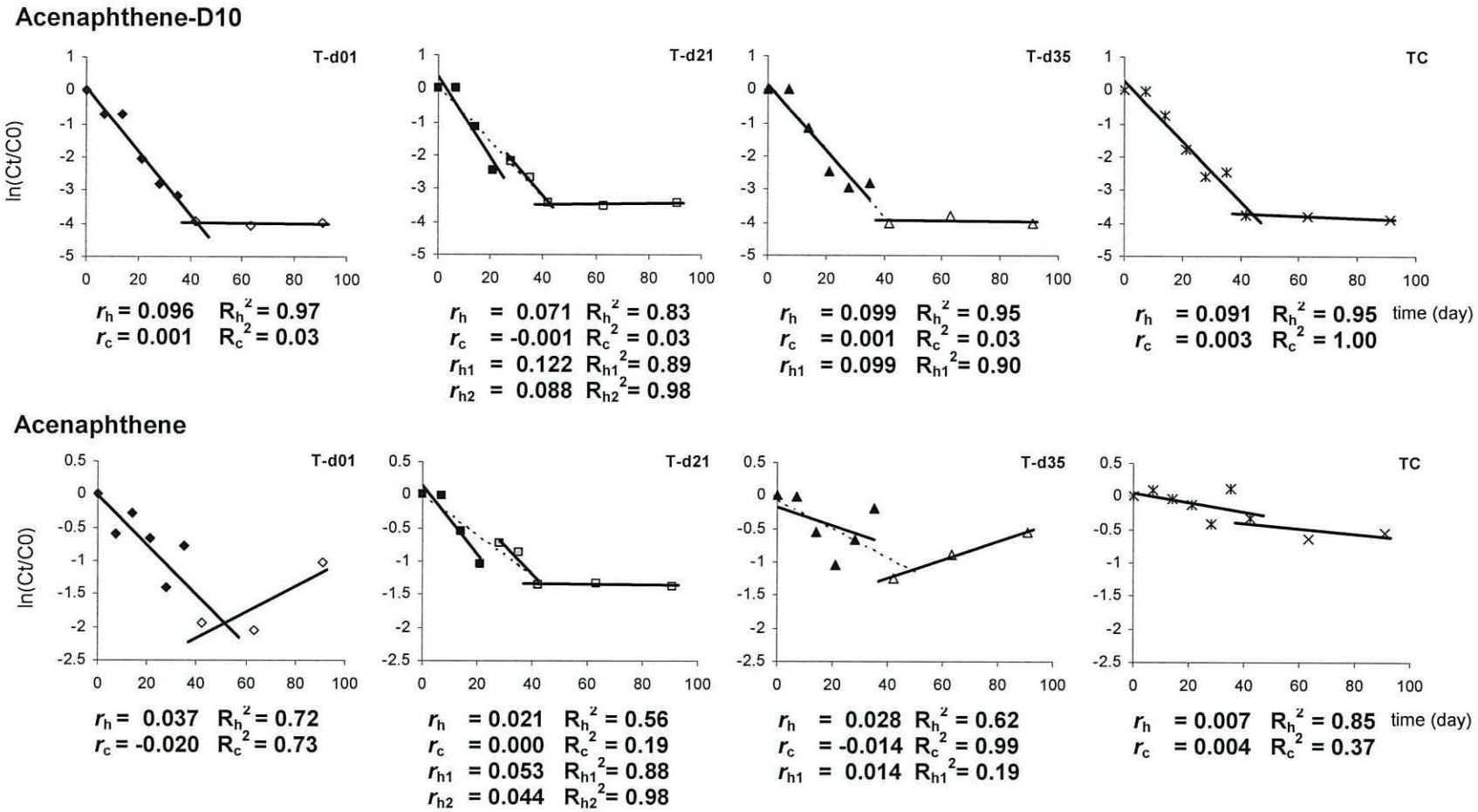

Figure 7.2a: Analysis of the average removal per day of Acenaphthalene-D10 and its aged counterpart Acenaphthene with the different treatments over the 91 day experiment. *r* represents average removal rate per day, subscripts indicate h= heating phase (dashed line, day 0 to 42), c=cooling phase (day 42 to 91), h1=heating phase before manure addition, h2=heating phase after manure addition.

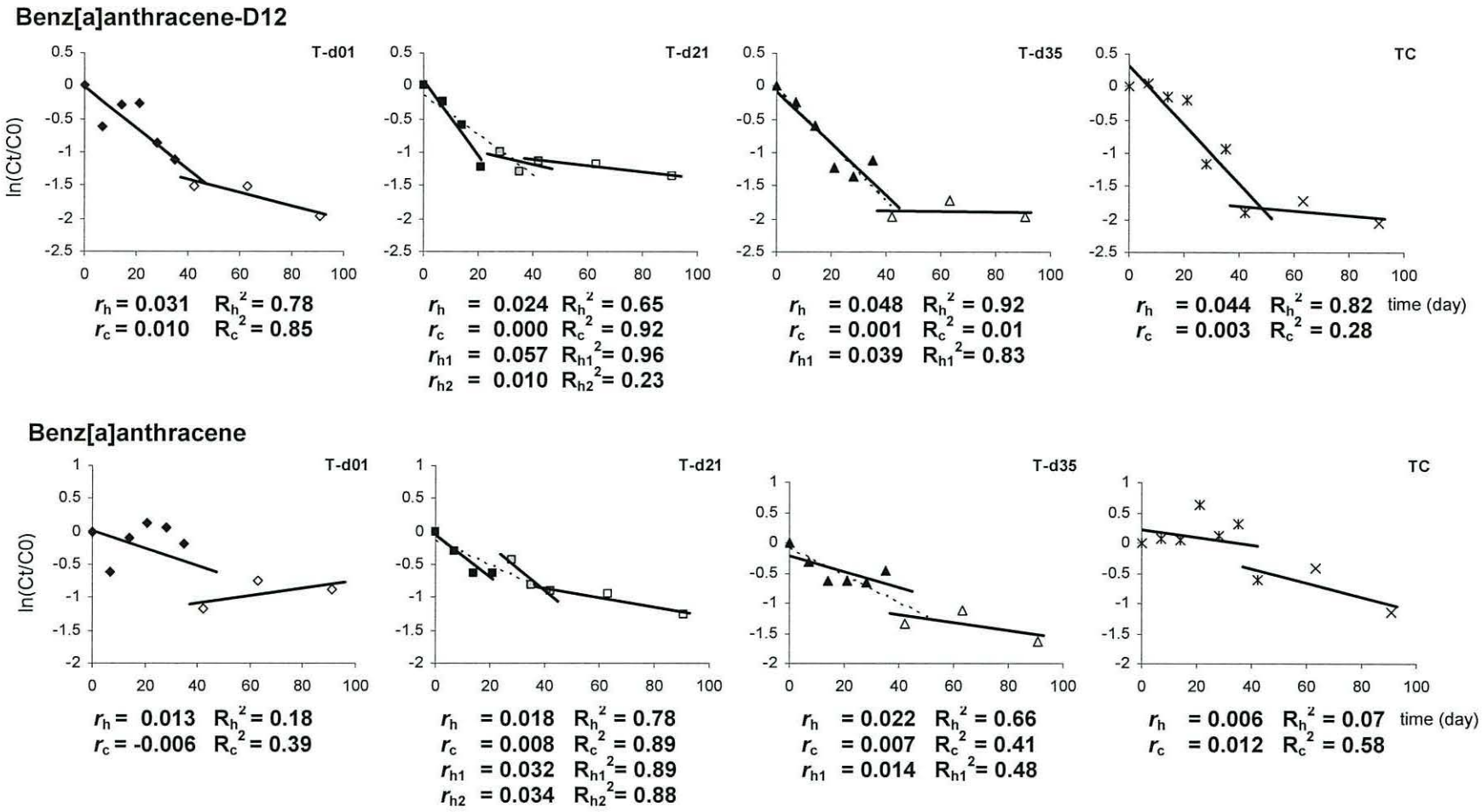

Figure 7.2b: Analysis of the average removal per day of Benz[a]anthracene-D12 and its aged counterpart Benz[a]anthracene with the different treatments over the 91 day experiment. *r* represents average removal rate per day, subscripts indicate h= heating phase (day 0 to 42), c=cooling phase (day 42 to 91), h1=heating phase before manure addition, h2=heating phase after manure addition.

7.5. DISCUSSION

7.5.1. Ageing - the importance of bioavailability

7.5.1.1. The influence of adsorption

Results of this study showed differences between the removal of weathered and freshly added PAHs. Removal rates of freshly added PAHs were higher than those of their weathered counterparts, which is in agreement with findings by other authors (Hatzinger and Alexander 1995; Carmichael *et al.* 1997).

A decrease in the extractable amounts of the added deuterated PAHs might have been due to mineralisation as well as to binding (ageing) processes. The speed with which PAHs are adsorbed to inaccessible sites has been found to depend on the soil type and organic matter content as well as the PAH-size and hydrophobicity (Hatzinger and Alexander 1995; Chung and Alexander 1998; Macleod and Semple 2000). For silty clays Chung and Alexander (1998) found a recovery rate of ^{14}C-Phenanthrene of 75 to 83% after 20 days decreasing to 64 to 75% after 60 days and 62 to 68% after 120 days. In the present study recovery rates of freshly added Acenaphthene-D10 were 10.4 to 17.8% after 21 days, decreasing to 1.8 to 4.7% after 42 days with no further significant changes. Recovery rates of Benz[a]anthracene-D12 declined to 31.1 to 82.6% after 21 days, decreasing to 11.8 to 31.2% by day 42 with no further significant changes until the end of the experiment (day 91).

Even though ageing processes might have played a role in the removal of extractable deuterated PAHs, it seems unlikely that it was a major factor. Recovery rates in this study were not only lower than those found by other authors for similar soils, but the fresh contamination was also added to an already contaminated soil. This was expected to alter the adsorption and desorption processes as binding sites may have already been occupied by native contaminants. Additionally, the microbial community was pre-adapted to PAH components and was therefore suspected to be able to mineralise the added contaminants.

Carmichael *et al.* (1997) found that for freshly added contaminants the rate of mineralisation was significantly slower than the rate of desorption, meaning that biodegradation was not limited by PAH availability, whereas for native/weathered PAHs desorption was the slower process and therefore limiting to mineralisation. Similarly to those findings, the results of this study indicate that factors influencing degradation rates were differently weighted depending on contact time of the PAH with the soil as well as PAH size. The chosen regression analysis, for example, was based on an exponential equation equal to pseudo-first-order kinetics. The good fits obtained with the regression models used indicate the possibility that PAH removal is based on processes similar to those determining PAH removal at a constant temperature. It is therefore possible that the applied temperature changes have not played a major role in those cases.

7.5.1.2. Temperature effects

Temperature has an influence on all aspects of the co-composting process (Figure 2.6) including bioavailability. Bioavailability is considered one of the main factors influencing biodegradation of contaminants in soil (Pignatello and Xing 1996; Carmichael and Pfaender 1997; Semple *et al.* 2001). Desorption kinetics are widely accepted to be the rate-limiting factor for degradation (Pignatello and Xing 1996; Carmichael *et al.* 1997; Semple *et al.* 2001) and are even proposed to be more important than the intrinsic microbial activity (Bosma *et al.* 1997). With increasing contact time (ageing) with soil PAHs exhibit decreasing extractability and bioavailability to soil organisms (Brusseau *et al.* 1991; Northcott and Jones 2001).

The results suggest that temperature played a major role in bioavailability and therefore biodegradation of weathered PAHs. This was also found in a study by Iqbal *et al.* (2007) investigating temperature effects on bioremediation of south Louisiana soils contaminated with PAHs and phenols, where the authors noted increased bioavailability and desorption for elevated temperatures. Freshly added PAHs on the other hand, have not

been subjected to ageing processes and their biodegradability should therefore be less dependent on temperature changes and other factors influencing their availability, as results of this study indicate.

7.5.2. Treatment effects

The results of this study showed no differences between the different treatments for the removal of the freshly added PAHs. In contrast to this, less removal of both native PAHs Acenaphthene and Benz[a]anthracene was found with the TC treatment than with the fungus/manure amended treatments. The similarity between the TC treatment and the other treatments in the removal of the freshly added PAHs suggests that the indigenous soil microbial community is more important for PAH degradation than added organisms if PAHs are readily available. On the other hand, *P. ostreatus* addition seemed profitable for removal of weathered and therefore less available PAHs. With PAH removal mainly occurring during the first 3 experimental weeks and treatments T-d21 and T-d35 showing higher removal rates than T-d01, chicken manure addition did not seem to be as important as the amendment with *P. ostreatus*.

7.6. CONCLUSIONS

This Chapter assessed the influence of "ageing" on PAH availability and removal from contaminated soil (Thesis aim 4). The major findings of this Chapter are summarised as follows:

- Degradation rates were 2.6 to 3.5 times higher for Acenaphthene-D10 than Acenaphthene and 1.3 to 2.4 times higher for Benz[a]anthracene-D12 than Benz[a]anthracene.
- Higher degradation rates of freshly added deuterated PAHs than weathered PAHs emphasised the importance of bioavailability and binding/release mechanisms during PAH bioremediation.

- Acenaphthene-D10 concentrations declined quicker than Benz[a]anthracene-D12 concentrations and degradation rates were 2 to 3 times higher for Acenaphthene-D10, indicating an influence of PAH size on degradation rates.

- If PAHs are relatively available (e.g. fresh contamination), the indigenous microbial community seems most capable of PAH degradation.

- If PAHs are not readily available (e.g. aged contamination), then bioaugmentation of the soil with *P. ostreatus* may enhance PAH removal.

Following those results the hypothesis that ageing influences the removal of PAHs from soil due to different removal-rate limiting processes, and the hypothesis that the effectiveness of different treatments changes with ageing of the PAHs both have to be accepted.

The results of this study lead to the recommendation that in case of a fresh contamination oxygen introduction is sufficient to remove PAHs from soil. As this study has been performed using already contaminated soil future experiments should investigate the effect of the pre-adapted microbial community on PAH degradation and possible differences in PAH removal from soils both with a history of contamination and contaminated pristine soils.

Chapter 8

Synopsis and outlook

8.1. ABSTRACT

This Chapter summarises and compares the findings obtained from the different experiments with the aim of drawing conclusions and subsequently providing an outlook for future research directions. Comparing the three performed experiments, one major difference between the performed experiments was the applied temperature profile. In the "manure" experiment (Chapter 4) the applied temperatures were increased more rapidly than in the "fungi" and "fungi plus manure" experiments (Chapters 5 and 6 respectively), where the applied temperature ramping profiles varied only slightly. The influences of the temperature profiles of the different experiments were investigated by comparing the temperature control treatments (TC) of each trial. Secondly, the addition of deuterated PAHs revealed the influence of ageing on PAH removal and indicated that different processes were determining the extent of PAH removal, depending on the bioavailability of the PAHs. Thirdly, the most promising treatments from the different experiments are summarised and compared. Due to the treatment regime, the amount of fungal inoculum added to the soil was higher in the "fungi" than the "fungi plus manure" experiment. A comparison of the treatments containing *P. ostreatus* as fungal inoculum revealed an improvement in PAH removal if *P. ostreatus* was added as the only amendment (treatment P.ost - Chapter 5), probably caused by inoculum size. Overall the treatment with *P. ostreatus* was identified as the most successful treatment out of the ones tested.

8.2. INTRODUCTION

With increasing awareness of the damaging effects of environmental contamination together with the environmental law becoming more stringent, it is becoming more imperative to reduce contamination levels in soil. A review of the literature (Chapter 2) established that different methods to remediate contaminated soil have been tested and developed. However, it is also evident that even if a successful method has been described in the laboratory, economic and logistical reasons often prevent it from being successfully implemented at contaminated sites.

Co-composting has been shown to have the potential to remediate soil contaminated with organic pollutants. Even though several studies on PAH removal by co-composting contaminated soil have been carried out, results are difficult or even impossible to compare. Not only do PAH extraction methods vary, but also large variation in the characteristics of contaminated sites including soil type, age of contamination, co-contamination, and the fact that several studies of remediation methods sometimes report seemingly contradicting results, mean that there is a need to develop a more general way to recommend remediation processes. However, generalising effects of different composting methods on PAH removal remains difficult due to the wide range of different factors not only influencing the behaviour of PAHs in soil, but also affecting the ability of the present organisms to bio-remediate those PAHs. Unfortunately, the relative importance of these various factors remains poorly understood.

In laboratory experiments several authors showed general advantages and limitations of organisms of bacterial and fungal origin (Bumpus 1989; Leahy and Colwell 1990; Cerniglia 1997). Other studies identified problems with inoculation and especially the survival of organisms added to the contaminated soil (Cases and de Lorenzo 2005). Even though both groups of organisms show different advantages and should complement each other in PAH degradation, in this study a combination of both groups within a complex inoculum matrix did not prove to be the most successful treatment under the given co-composting conditions.

8.3. INFLUENCE OF DIFFERENT TEMPERATURE REGIMES ON PAH REMOVAL

Comparison of the different experiments showed that the temperature control (TC) treatments behaved differently to each other in terms of PAH removal. The only variable that was substantially changed from the pre-treatment conditions (introducing oxygen by turning and mixing of the soil in windrows) was the applied temperature. It was therefore expected that this change in temperature had a greater influence on PAH removal than time.

In the following the TC treatments are labelled as:

- TC1 = TC treatment from the experiment on the effects of different types of manure
- TC2 = TC treatment from the experiment on the effects of different fungal inocula
- TC3 = TC treatment from the experiment on the effects of a combination of *P. ostreatus* as the fungal inoculum and chicken manure

The applied temperature profiles are given in Table 8.1

Table 8.1: Applied temperature profiles of the three experiments.

Week	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8+9**	**10+11**
Experiment 1 – Manure addition									
Temperature (°C)	20	35	50	65	35	-	-	-	-
Experiment 2 – Fungi addition									
Temperature (°C)	20	25	30	35	50	65	35	-	-
Experiment 3 – Combination of *P. ostreatus* and chicken manure									
Temperature (°C)	22	30	35	40	50	65	35	35	25

Temperatures are given in °C (±0.5); the first week spanned only 4 days for experiment 1 and 6 days for experiment 2 all other weeks spanned 7 days.

A significantly different behaviour of the TC treatments depending on temperature steps was detected for all grouped PAHs except the LMW PAHs ($p < 0.05$) (Figure 8.1). A combined influence of treatment and temperature was mainly caused by TC3 during the lower temperature phases at the beginning of the experiment. The results of the temperature control treatment from the combined fungi/manure experiment (TC3) were very variable, with standard deviations up to half the amount of the mean value, while values of replicate vessels within TC1 and TC2 were much more similar to each other. No correlation between PAH concentrations and percentages remaining with any other analytes could be found within the separate experiments. However, within all TC treatments all single PAH concentrations showed a modest positive correlation (r=0.49 to 0.64) with the total C content. No other correlations were found.

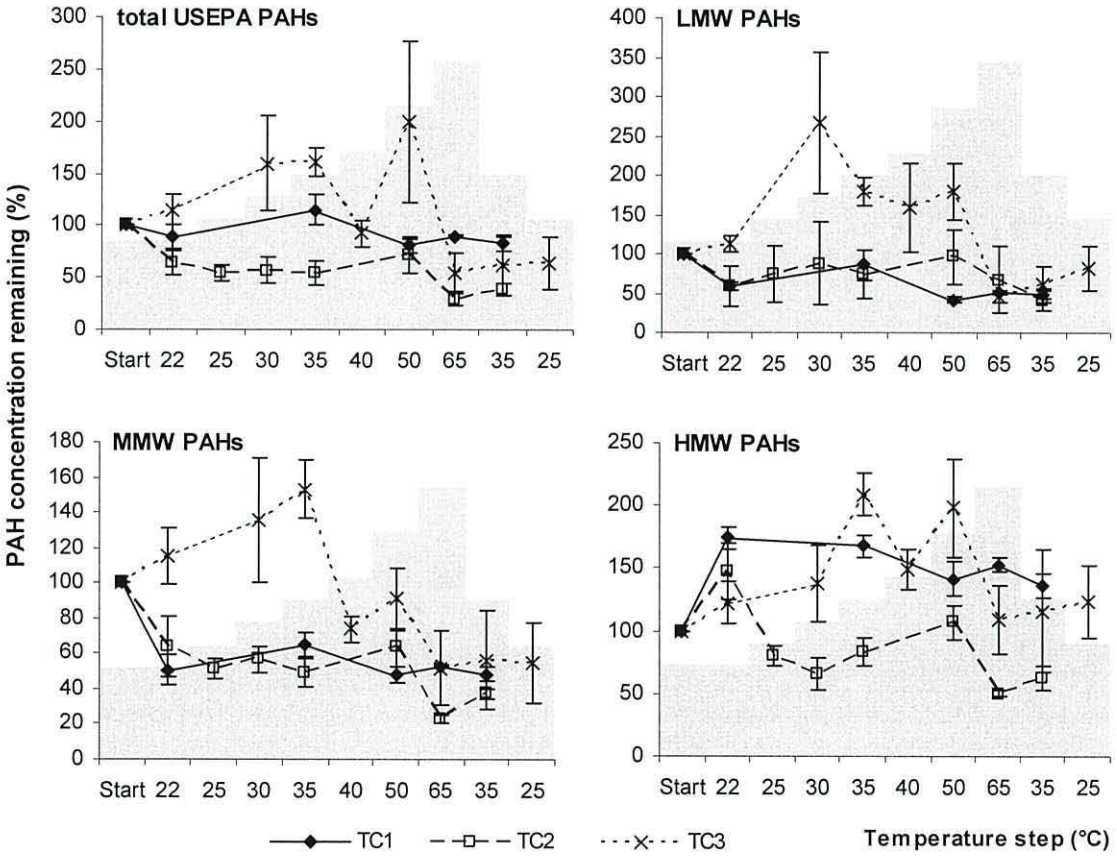

Figure 8.1: Mean PAH concentrations remaining in the temperature control (TC) treatments found at the end of the implied temperature steps in % of the initial concentrations with the different experiments (TC1 = TC "manure experiment" Chapter 4; TC2 = TC "fungi experiment" Chapter 5; TC3 = TC "fungi/manure experiment" Chapter 6). The light grey background additionally represents the applied temperatures as given on the x-axis during the experiment. Error bars represent mean ±S.D.; n=3

All TC treatments showed an increase in extractable PAH amounts during one of the highest temperature steps (50 or 65°C). TC1 showed decreases in PAH concentrations during the 50°C temperature step which was applied during week 3. Subsequently, concentrations decreased during week 4 at 65°C. TC2 and TC3 on the other hand showed the exact opposite behaviour. An effect of time has therefore to be assumed.

None of the TC treatments was particularly associated with certain microbial communities as determined by PLFA analysis. Therefore a suspected influence of the slower temperature increases during the fungi and fungi/manure experiments (TC2 and TC3) on the microbial communities and their abilities to adapt could not be shown.

8.4. THE EFFECT OF AGEING ON PAH BIOAVAILABILITY

Adding deuterated PAHs to the aged contaminated soil showed the effect of ageing on the ability of the applied treatments to remove PAHs from the mixtures. The results emphasised the importance of temperature on increasing the availability of weathered PAHs, while the effects on the freshly added PAHs were minimal.

Differences in the removal behaviour of different PAHs depending on their molecular weight were also found. These findings support the previous assumption that studies undertaken on different kinds of contamination, e.g. fresh/aged or low/high MW, cannot be compared without further investigations. Not only does the soil type influence the outcome of a treatment, but the time for which a soil has been contaminated is also influential. While treatments can prove beneficial when tested with freshly contaminated soil, they might have little impact on the removal of weathered contaminants.

8.5. SUMMARY AND COMPARISON OF THE DIFFERENT EXPERIMENTS CONCERNING PAH REMOVAL

In this thesis manures from different animals and two fungal inocula were tested to compare their effectiveness in removing aged PAHs from a clay soil that had already lost 70% of its initial total PAH concentration (mainly smaller PAHs) during irrigation and aeration pre-treatment. The properties of the different additives under changing temperatures were investigated, with the greatest emphasis on observing the effects of these additives on the removal of larger (≥ 4-ring) PAHs.

8.5.1. Manure amendment

With the manure experiment essentially no difference in final PAH concentrations could be detected between treatments. This suggests that neither the added nutrients nor micro-organisms within the manures contributed substantially to PAH removal. The seemingly better performance of the chicken manure in HMW PAH degradation is mainly based on relative removal of PAHs (% remaining). Total extractable HMW PAH concentrations were not significantly different between any of the treatments after day 4.

Differences were found in microbial community composition and development between the treatments. Much of the observed differences in treatment behaviours were caused by the chicken manure treatment. However, those differences did not make a difference to the final PAH removal. This leads to the assumption that the indigenous soil microbial community is more important for bioremediation than the one introduced with the manure. However, temperature seemed to have a large influence on PAH bioavailability and subsequently on the potential of degradation of the contaminants. Therefore manure addition is recommended if artificial temperature increases are to be avoided. An additional advantage of mixing the soil with manure is the improvement of soil quality particularly in terms of its revegetation and rhizoremediation potential (Zhang and Fang 2007).

When looking at manures to improve the quality of contaminated soil it should be taken into consideration that some types of manure might release intrinsic soil contaminants when added to the soil, as found with the cattle manure treatment. The added manures may also contain contaminants in the form of human pathogenic organisms (e.g. *Salmonella* sp., *E. coli* O157, *Campylobacter* sp.) endocrine disrupting chemicals (e.g. oestrogens) and inorganic nutrients that can pose a threat to water quality (e.g. NO_3^-, PO_4^{3-}). However, the impact of these is typically very low if managed correctly.

8.5.2. Fungi addition

The experiment investigating the addition of fungi-inoculated sawdust showed an advantage of *P. ostreatus* addition over *T. versicolor* alone or a mix of both fungi. The *P. ostreatus* amended treatment reduced PAH amounts by a greater percentage than the other two treatments as well as reducing concentrations to lower absolute levels. The most significant reduction in PAHs occurred during the first week of the experiment when numbers of total fungal biomass (measured as PLFA) were highest.

8.5.3. Combination of fungi and manure addition

Treatments T-d21 and T-d35 gave the highest PAH removal rates in the combined fungus and manure addition experiment during the early stages of the experiment when only the *P. ostreatus* inoculum had been added to the soil. An additional amendment with manure at the beginning of the experiment (T-d01) resulted in an increase in PAH concentrations after an initial decrease, delaying the PAH concentrations from reaching their final levels similar to those found in treatments T-d21 and T-d35. Manure addition at day 21 (T-d21) did not induce a substantial further decrease in PAH concentrations, while concentrations decreased during the temperature step following manure addition at day 35 (T-d35). However, the final removal of PAHs (% remaining) was not significantly different between the fungus/manure amended treatments.

8.5.4. Overall comparison of the most effective PAH removal treatments

Comparing the PAH degradation ability of the most promising treatment methods (Table 8.2), i.e. the addition of chicken manure (chicken manure treatment - Chapter 4), *P. ostreatus* inoculated sawdust (P.ost - Chapter 5), and the addition of *P. ostreatus* inoculated sawdust followed by chicken manure addition at day 21 during the treatment (T-d21 – Chapter 6), the treatment with *P. ostreatus* without manure addition resulted in the highest removal rates for all grouped PAHs. Furthermore, with less than half the absolute amounts of PAHs remaining, contamination levels were lowest with the P.ost treatment in all cases.

Table 8.2: Comparison of mean PAH concentrations (µg/g) and ratio of PAH removal (%) ±S.D. at the end of the experiments in the most promising treatments. n=3

		LMW	MMW	HMW	total USEPA
Chicken manure	µg/g ash	65 ±14	556 ±42	133 ±8	754 ±61
	% remaining	*59 ±7*	*78 ±3*	*83 ±3*	*75 ±4*
P.ost	µg/g ash	39 ±7	88 ±27	20 ±3	147 ±27
	% remaining	*69 ±8*	*9 ±2*	*17 ±2*	*13 ±2*
T-d21	µg/g ash	54 ±27	230 ±126	53 ±25	338 ±176
	% remaining	*53 ±20*	*34 ±10*	*60 ±13*	*39 ±11*

Values are means (n=3) of PAH concentration in µg per g ash ±S.D. and mean removal in % ± S.D. of the concentration at the beginning of the experiments. Chicken manure = Chicken manure treatment (Chapter 4), P.ost = *P. ostreatus* amended treatment (Chapter 5), T-d21 = *P. ostreatus* amended treatment with chicken manure addition at day 21 (Chapter 6)

These results suggest that addition of *P. ostreatus* is the most effective method to remediate an aged PAH contaminated soil. Most degradation of PAHs appeared during the early period of the experiment. In treatment T-d21, *P. ostreatus* was the only inoculant at the beginning of the experiment prior to manure addition (day 21); however, degradation rates were not as high as with the P.ost treatment. The different fungi amended treatments are compared below.

As far as could be detected by PLFA analysis in this study, microbial communities developed in similar ways during the different experiments when the same inoculum was added (chicken manure; *P. ostreatus*). However, inocula such as manures can differ greatly in their composition, which depends not only on the animal and its digestive system, but also on the specific diet. In this study manure types were chosen to give the greatest possible variation in microbial, as well as physico-chemical, composition. However, to gain a more generalised understanding of the effects on PAH removal attributable to the type of manure, future work should include a range of the same manure types from different sources (e.g. chicken manure from different farms with different housing conditions or feeds, as well as ages of the animals).

It had been intended to perform additional experiments with different manure sources and on a larger scale, as well as conducting additional analysis, but this was constrained by time and funding. In particular, other micro- and molecular-biological methods such as PCR-DGGE followed by cloning and sequencing of prominent DNA and RNA-bands would provide a more detailed insight into microbial community development than can be gained by PLFA. This would help to identify the survival and activity rates of introduced organisms, especially the introduced fungi.

8.5.5. Comparison of the fungi-amended treatments

8.5.5.1. PAH removal

The P.ost treatment, as well as the T-d21 and T-d35 treatments up to week 3 and 5, respectively, only consisted of soil mixed with *P. ostreatus* inoculated sawdust. Removal patterns should have therefore been the same within those treatments during the first 3 and 5 weeks, respectively. However, the P.ost treatment showed higher degradation rates and reduced the PAHs present to lower levels (Figure 8.2).

The greatest reductions in PAH concentrations were detected during the early stages of the process while the introduced fungi were still present in larger amounts and a greater fraction of freely available PAHs was present. The initial phase of higher reduction-rates covers the first three weeks which is a time-span of faster degradation that has also been found by other authors (Admon, Green et al. 2001; Antizar-Ladislao, Lopez-Real et al. 2005).

One difference between the experiments conducted here was that, due to environmental conditions, the applied temperatures were 2°C higher in the fungi-manure experiment (treatments T-d21 and T-d35) during the first week of the experiment and 5°C higher during weeks 2 to 4. However, the largest reduction of PAH concentrations was found in the P.ost treatment during the first week of the experiment, whilst the 2°C lower temperature was not considered to have been the significant factor in the detected PAH removal.

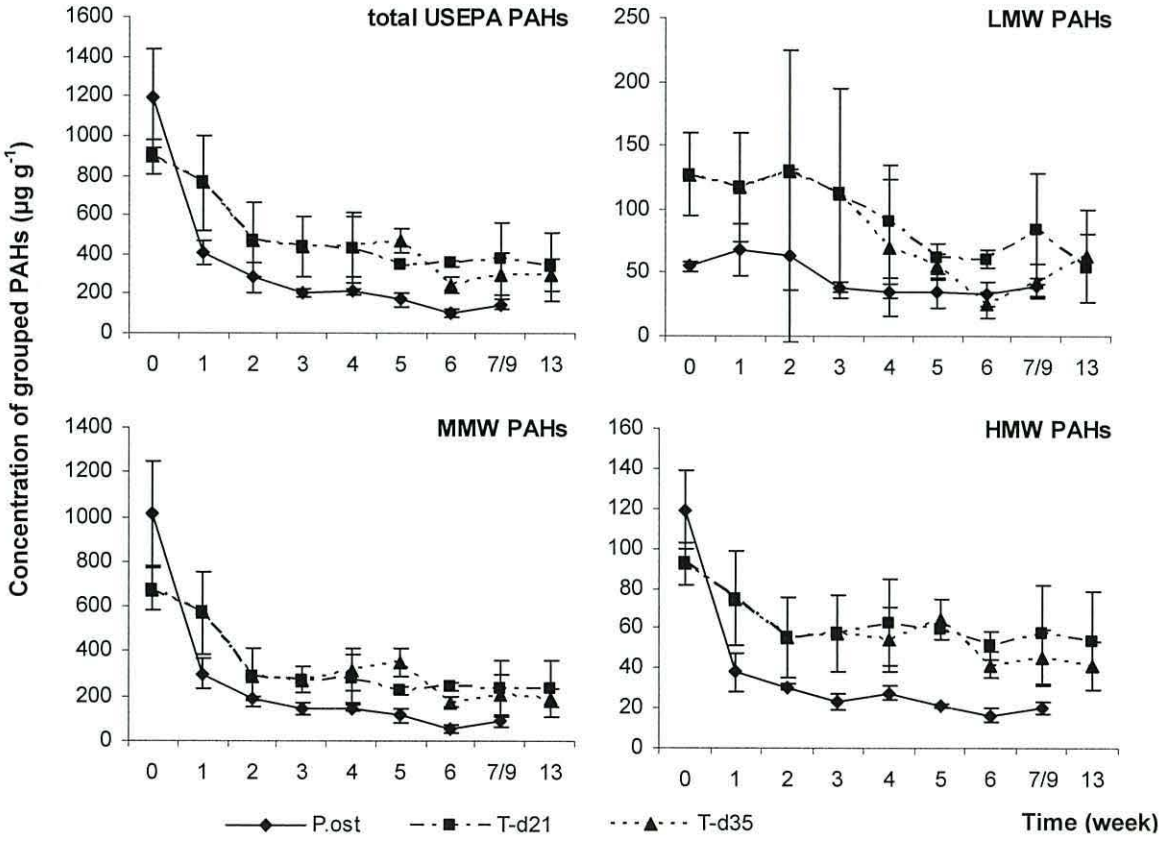

Figure 8.2: Mean concentrations of grouped PAHs in µg per g ash measured in the treatment mixtures during the two experiments involving the addition of fungi-inoculated sawdust over 7 (P.ost) and 13 (T-d21, T-d35) weeks. Week 7/9 corresponds to week 7 for the P.ost treatment and week 9 for the T-d21 and T-d35 treatments. Error bars represent mean ±S.D.; n=3 for P.ost, n=6 for T-d21/T-d35 weeks 0-3, n=3 for T-d21 weeks 4-13, n=3 for T-d35 weeks 4-13.

8.5.5.2. Inoculum size

The better performance of the P.ost treatment compared to treatments T-d21 and T-d35 can be attributed to the higher initial amount of inoculated sawdust used. During the experiment investigating the suitability of fungal additives, the soil was mixed with the fungal inoculum in a ratio of 1:1. This ratio of soil to amendment of 1:1, was also used for the following experiment. For this experiment, however, the amendment consisted of the fungal inoculum as well as chicken manure. The final ratio of soil:fungal inoculum:chicken manure was to be 25:10:15, the final ratio of soil to fungal inoculum was therefore decreased to 1:0.4. Consequently, the *P. ostreatus* inoculum was 2.5 times larger in the P.ost treatment compared to treatments T-d01, T-d21, and T-d35. This higher amount of fungal inoculum is most likely responsible for the increased degradation of PAHs.

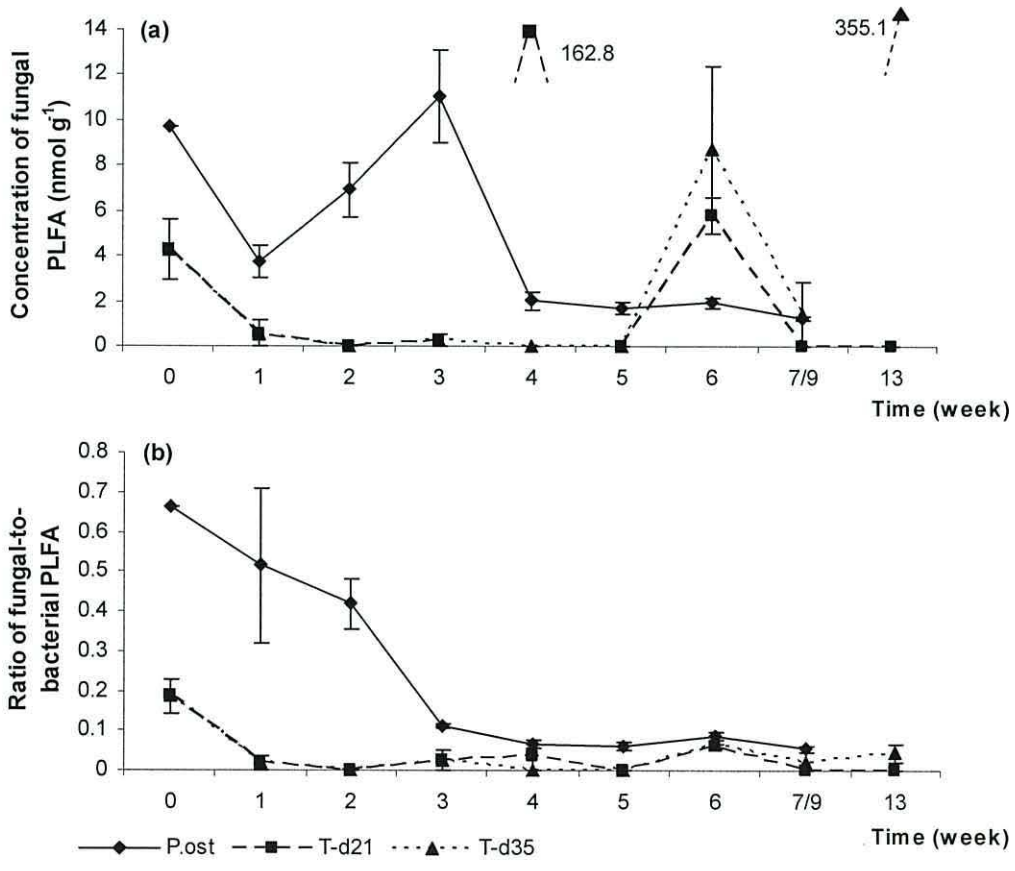

Figure 8.3: Mean total fungal PLFA amounts in nmol per g dry sample (a) and mean ratio of fungal-to-bacterial PLFAs (b) of the fungal-inoculated treatments at the end of each temperature step/week of the 7 (P.ost) and 13 (T-d21, T-d35) week experiments. Week 7/9 corresponds to week 7 for the P.ost treatment and week 9 for the T-d21 and T-d35 treatments. Error bars represent mean ±S.D.; n=3 for P.ost, n=6 for T-d21/T-d35 weeks 0-3, n=3 for T-d21 weeks 4-13, n=3 for T-d35 weeks 4-13.

PLFA analysis revealed higher amounts of fungi-indicating PLFA with the P.ost treatment, as well as a higher fungi-to-bacteria indicating PLFA ratio (Figure 8.3). The pattern of total fungal PLFA and fungal-to-bacterial PLFA was similar until manure was added to the T-d21 (day 21, week 3) and T-d35 (day 35, week 5) treatments.

The reported quantities of amendment required for optimum pollutant transformation vary widely in different studies. Investigating PAH degradation by *Mycobacterium* sp. Heitkamp and Cerniglia (1989) found that doubling the inoculum size only increased pyrene degradation slightly, while Mackova *et al.* (1997) found that PCB transformation was stimulated with increasing size of inoculum. In another study, it was found that 5% (v/v) of biomass of *Bacillus brevis* inoculum provided best degradation of phenol, compared to lower concentrations, while increasing the inoculum size only had marginal effects on the removal of phenol (Arutchelvan, Kanakasabai et al. 2006). Other authors reported that olive oil mill wastewater degradation by *T. versicolor* and *Funalia trogii* was also inoculum size dependent (Yesilada, Sik et al. 1998). The optimal inoculum size in different studies was probably dependent on soil type, the fungal inoculum used, amendment type and the age of the contamination, but it was found that generally significant quantities of amendment were required such as a corn cob to soil ratio of 4:1 (Singleton 2001). In this study an influence of the amount of the fungal additive was indicated as described above.

8.6. CONCLUSIONS AND OUTLOOK

The overall aim of this study was to find a treatment process that could remove weathered PAHs from soil; this was tested with two specific hypotheses, namely that:

- The addition of a combination of both fungal and bacterial inocula to contaminated soil enhances PAH removal.

- The time-point at which fungal and bacterial inocula are added determines the effectiveness of the micro-organisms and therefore the success of the remediation process.

The results of the experiments carried out in this thesis mean that the first hypothesis should be rejected. However, the soil had not been sterilised before the experiment meaning that an influence of the indigenous microbial community cannot be excluded. The second hypothesis was accepted because of the finding that manure addition at the beginning of the treatment inhibited quicker PAH removal and was therefore considered unfavourable, while addition at a later stage might not enhance further PAH removal, but may prove beneficial for soil quality.

The conditions under which the experiments of this study were started (i.e. high clay content of the soil; decades of ageing of the contamination in the soil; pre-treatment during which large amounts of PAHs were already lost, leaving only the more difficult to degrade and less available components) were considered difficult and unfavourable for PAH removal. However, the overall results of this study showed that the addition of fungi and especially *P. ostreatus* has the potential to further remove PAHs from this soil. It must be stated, however, that the PAH removal remained incomplete, even after extended time periods. It is therefore likely that bioremediation is not the most suitable treatment method if a complete removal of an aged PAH contamination is required and that other treatment options (e.g. incineration) could be more cost and time effective. Logistically, alternative remediation options may be more suitable for small amounts of contaminated soil where rapid removal is required. Considering the current focus on the carbon footprint of environmental operations it would be useful to undertake a life cycle analysis (LCA) of the different remediation options, whilst at the same time performing a risk assessment of the contaminated site. In such a way, an economic-based-remediation-effectiveness versus risk-curve could be obtained. This may provide an additional decision support tool for environmental practitioners.

Investigating the effects of different manure types revealed substantial increases in PAH concentrations at the beginning of the process. Those increases might pose a risk to the work force and the environment during remediation processes. This should be further investigated as in most studies samples were taken at larger intervals, wherefore increases in PAH concentrations and toxicity levels might have been missed.

Whilst valuable findings were obtained from this study there were a number of limitations which would benefit from further study, particularly with regard to temperature developments on a large scale and influences of co-contaminants. The effects of mixing the pre-treated soil with *P. ostreatus* inoculated sawdust therefore need to be investigated on a large scale without artificial temperature increases. It would also be useful to establish the effects of the inoculum on other co-contaminants, particularly as organic pollutants frequently occur in a complex cocktail of organic and inorganic pollutants. However, treating PAH contaminated soil by excavating and turning followed by *P. ostreatus* addition, after the easily degradable PAHs have been removed by the indigenous microbial community, can provide an easy to implement and less expensive remediation method.

APPENDICES

Appendix 1: Table listing studies on co-composting of PAH contaminated soil 221

Appendix 2: Tables listing PAHs and bacteria and fungi having been found to degrade those PAHs, as well as detected PAH-metabolites 233

Appendix 3: Work presented at relevant scientific seminars and conferences 239

APPENDIX 1

Table A1: List of studies on co-composting of PAH contaminated soil as published in Loick *et al.* (in press); The "Treatment"-column describes the study and experimental setup as far as they were given; the "Findings"-column summarises the main findings; the "Ref."-column refers to the literature reference.

Treatment	Findings	Ref.
Soil type: subsurface materials on the periphery of the plume at an abandoned creosote waste site **Contamination:** creosote **Amendment:** Inorganic and organic nutrient amendments **Conditions:** different pH and temperature ranges **Time:**	no increase in the extend of mineralisation of aromatic compounds by inorganic and organic nutrient amendments and changes in pH and temperature	(Thomas *et al.* 1989)
Soil type: soil from former asphalt plant in Deventer, The Netherlands **Contamination:** volatile organics, phenols, PAHs up to 10 m depth PAHs 24 g per kg soil (of this 8.4 g naphthalene per kg soil) **Amendment:** **Conditions:** groundwater circulation and treatment, extracted groundwater treated in 3 biological reactors on site and in laboratory **Time:** 146 days	46 % of the initial PAH concentration remained in the soil after prolonged flushing (146 days up to 675 times flushed) 95 % of PAHs in the groundwater could be removal from the groundwater on site with upflow aerated columns 99-100 % were removed in the laboratory when a rotating biological reactor run in parallel with an upflow aerated column	(Van der Hock *et al.* 1989) **
Soil type: 30 500 m^3 fill and clay soil from gasworks in Blackburn, UK **Contamination:** coal tar (including PAHs with total PAH concentration of ~22 g per kg soil), phenols **Amendment:** inoculated in layers with microbes from site, nutrients, surfactants (spray application) **Conditions:** layered, homogenised, treatment bed, moisture added, bed rotovation, ambient temperature **Time:**	decrease of total PAH to 148 mg per kg soil	(Bewley *et al.* 1989) **
Soil type: municipal sludge **Contamination:** radiolabelled phenanthrene (1.3-1.6 mg per kg dry sludge) **Amendment:** benchtop laboratory compost apparatus simulating aerated-pile composting conditions **Conditions:** **Time:** 10-20 days	10-11% of phenanthrene degraded 15-17% of not extractable phenanthrene metabolites remained in the compost bound to organic matter or incorporated by micro-organisms	(Racke and Frink 1989) *
Soil type: sandy loam, un-acclimatised **Contamination:** wood-preserving and petroleum-refining wastes added to soil (total PAH 490-6646 mg per kg soil) **Amendment:** **Conditions:** batch reactors, soil columns **Time:** 354 days	generally greater decrease of LMW PAHs than HMW, no detectable degradation of 5-ring PAHs; decrease in HMW correlated with oil and grease contents of the wastes and therefore attributed primarily to co-oxidation processes	(Aprill *et al.* 1990) **
Soil type: contaminated soil from wood-impregnation and coking plants **Contamination:** PAHs (total PAH ~6000 mg per kg soil of wood impregnation plant); ~145 mg per kg soil of coking plant) **Amendment:** 100ml circulating mineral salts medium **Conditions:** tickling soil columns, 100 g raschig rings, 150 g soil, ambient temp, pH 7.2 **Time:** 8 weeks	generally greater percentage in degradation of LMW PAHs than HMW; no significant degradation of any PAHs in soil from the coking plant, which contained a higher proportion of HMW	(Weissenfels *et al.* 1990) **

Soil type: sandy soil **Contamination:** gasoline, lubricating oil (lower ring PAHs), crude oil (highest concentration of HMW PAHs) **Amendment:** nutrient addition (fertiliser, KNO_3, NH_4NO_3 NaH_2PO_4, urea) **Conditions:** batch cultures (soil slurry), 20°C, no pH control **Time:**	bioremediation by in situ organisms would appear to be most effective for low and medium distillate hydrocarbons, such as lubricating oil, which consists of lower ring PAHs, volatile aromatics, and aliphatic compounds, although provision of an adequate oxygen supply and nutrients is important to enhance degradation	(Morgan and Watkinson 1990) **
Soil type: loam sand clay **Contamination:** fuel products, low to high distillate fuels added to soil **Amendment:** fertilisers (NH_2NO_3, K_2HPO_4) **Conditions:** treatability study, soil columns, weekly tilling, 50 % moisture, pH 7.5-7.6 (adjusted with lime), 17-37 °C **Time:** 4 weeks	biodegradation played a relatively minor role in reduction of lower-distillate fuels compared with vaporisation and was ineffective in removal of the heavier fuels; bioremediation of soil contaminated with medium distillate fuels was effective	(Song *et al.* 1990) **
Soil type: 68% sand, 16% silt, 22% org matter, 16% clay **Contamination:** jet fuel, heating oil, diesel oil (all are medium distillate fuels), 2.3 ml per cm^2 applied to soil (hydrocarbon content 50-70 mg per g soil) **Amendment:** fertiliser-urea 10 mg per cm^2, superphosphate 4.3 mg per cm^2, C:N (200:1), C:P (1000:1) **Conditions:** treatability study, outdoor lysimeters, watering, weekly tilling to 15 cm, pH 7.4 (adjusted with lime), 16-25 °C **Time:** 20 weeks	≥90 % reduction of hydrocarbons in soil for all fuels (to <5 mg per g soil) treatment was effective in increasing the rate of biodegradation	(Wang and Bartha 1990; Wang *et al.* 1990) **
Soil type: soil from disused refinery site in Hanover (Germany) **Contamination:** crude and mineral oil (total PAH concentration 155 mg per kg soil) **Amendment:** surfactants, microbial inoculations, nutrients (mineral salt medium, fertiliser) **Conditions:** homogenised soil bed on gravel base, lined with high density polyethylene, leachate collection, heated polytunnel (45 m x 7 m x 0.7 m), 15 % moisture, 20-25 °C, pH 6.6-7.4 (adjusted with $Ca(OH)_2$) **Time:** 2 years	~82 % reduction of total PAHs (to 28 mg per kg soil) after 10 months found by limited PAH analysis; general decrease in hydrocarbons and variability	(Bewley *et al.* 1990) **
Soil type: soil slurry, contaminated soil from wood impregnation and coking-plant **Contamination:** PAHs **Amendment:** 400 g soil in 4 litre mineral salt medium **Conditions:** airlift bioreactor, pH 7.2, 30 °C surfactant TritonX, air passed over C trap **Time:** 4 weeks (8 weeks for in-situ treatment)	average of 80 % reduction for 2-, 3-, and some 4-ring PAHs in soil from wood-impregnation (more LMW) after 4 weeks and 50% after 8 weeks for in-situ treatment no significant degradation of PAHs in coking plant soil (more HMW) except for some two and three ring compounds, even after inoculation with micro-organisms known to degrade PAHs	(Weissenfels *et al.* 1990) **
Soil type: soil slurry **Contamination:** oil **Amendment:** nutrients **Conditions:** bioreactor (25 m x 3.5 m diameter), warm-air blower, sprinkler, 30-35 °C **Time:**	Oil degrading microflora was established within 3 days Majority of biodegradation occurred in first 10 days; final petroleum oil concentrations ranged from 50-350 mg kg^{-1}; most hazardous components were removed	(Van den Munckhof and Veul 1990) **
Soil type: soil slurry **Contamination:** Oil with PAHs **Amendment:** **Conditions:** rotating drum with axial baffles-bioreactor, warm-air blower, nutrients added, 20°C, no inoculation **Time:**	Proposing soil quality standard for biodegraded soils	(Annokkee 1990) **

Soil type: fill material underlain by clay (2-15 m) from Blekholmstorget, Stockholm **Contamination:** creosote (total creosote from <10 to >32000 mg per kg soil, with an average of 4329 mg per kg soil, phenols, oil hydrocarbons and metals also detected **Amendment:** 35 % H_2O_2 (100 mg per l), nutrients (K_2HPO_4), bacterial inoculation, surfactants (Ethylan BCP&CD916, Lankro (0.5 % w:w)) **Conditions:** groundwater recirculation and treatment, 15000 m³ sheet-pile contaminant mineral salts medium, DO 8.5 ml per l, ambient temperature, **Time:** 2.5 years	overall degradation was significant. after 4 months reduction of concentration of PAHs with 3 and more rings considerably <60 %	(Ellis *et al.* 1991) **
Soil type: sandy subsurface and surface soil **Contamination:** creosote **Amendment:** nutrient solution 50 mg per 3 kg soil tiling **Conditions:** treatability study, landfarming chamber, pH 7.1, moisture 8-12% 23°C, no inoculation **Time:** 12 weeks	in surface soil: enhanced LMW PAH degradation >50 % after 12 weeks, loss of HMW PAHS considerably lower in subsurface soil: PAH concentrations remained high although actual carbon turnover was greater than in surface soils. Once creosote phenolics were extensively degraded (8-12 weeks) there was a significant increase in activity towards 2- and 3-ring PAHs. However, still little loss of HMW PAHs; nutrient addition appeared to have a positive effect on the extent of biodegradation of HMW PAHs but no significant effect on that of LMW PAHs; general rate of biodegradation for the contaminant material: phenolics> heterocyclics>LMW PAHs>HMW PAHs> pentachlorophenol	(Mueller *et al.* 1991) **
Soil type: 3500 m³ Blekholmstorget, Stockholm **Contamination:** creosote (10-30 g per kg soil; including PAHs with total PAH concentration 1024.4 mg per kg soil) **Amendment:** mineral salt medium, nutrients, microbial inoculations 10^6 cell/g soil each treatment season, surfactants 0.5 % w/w Ethylan BCP&CD916, Lankro **Conditions:** treatment bed (80 m x 60 m x 0.75 m), concrete base, bunded gravel underlayer, leachate collection, rotovation (2 x weekly), 20 % moisture **Time:**	68.4 % reduction of total PAH (to 324.1 mg per kg soil); HMW PAHs degraded less than LMW PAHs considerable increase in PAHs with ≥3 rings (except fluoranthene and pyrene) was apparent by using on-site treatment when compared with in-situ degradation on the same site	(Ellis *et al.* 1991) **
soil type: sandy, surface and subsoil **Contamination:** creosote (high concentrations of PAHs) **Amendment:** 150 mg N fertiliser per kg soil **Conditions:** treatability study, 1.5 litre bioreactors batch mode, continuous mixing, pH 7.1, DO 90 % agitation/airflow, 10% moisture **Time:** 30 days	in surface soil: after 14 days: 2-ring PAHs (except naphthalene) lost rapidly, 60 % 3-ring PAHs and 40 % 4-ring PAHs degraded, higher ring PAHs and PCPs not extensively degraded; generally no PAH degradation after day 14 High concentrations of HMW PAHs (HMW PAHs composed 90% of the total PAH abiotic loss into the sludge) and PCPs were found in reactor sludge in subsurface soil: after 5 days: 90 % 2-ring PAHs, 70 % 3-ring PAHs, and 60 % 4-ring PAHs degraded removal of HMW PAHS was significant but high concentrations were found in reactor sludge (this abiotic loss consisted of: 22 % fluoranthene, 36 % pyrene, 29 % Benzo(b)fluorene, 29 % chrysene) extensive biodegradation of all PAHs; degradation-rate more rapid, similar pattern to surface soil rate of degradation for surface and subsurface soil was: Phenolics>heterocyclics>PAHs> pentachlorophenol(PCP)	(Mueller *et al.* 1991) **

Soil type: **Contamination:** pyrene (13 mg per kg soil) **Amendment:** composted sewage sludge (from 0 to 50%) **Conditions:** laboratory scale in-vessel (40g mixtures) composting; 40% moisture, aerated, in waterbath temp. from 20 °C raised by 5 °C per day until 60 °C **Time:** 21 days	pyrene could be degraded but the rate and extend were not fully described	(Adenuga *et al.* 1992) *
Soil type: **Contamination:** PAH contaminated sewage sludge applied to different soils **Amendment:** **Conditions:** glass microcosms **Time:**	different behaviour of PAHs in each soil, probably due to different soil characteristics and history of PAH exposure half-lives for phenanthrene 83-193 days and for Benzo(g,h,i)perylene 282-535 days lowest half-life values for most PAH compounds in spiked soil suggesting a higher susceptibility of spiked PAHs to both abiotic and biological degradation PAH compounds with less than four benzene rings are susceptible to abiotic loss processes; losses by these mechanisms were insignificant for compounds with four or more benzene rings.	(Wild and Jones 1993)
Soil type: **Contamination:** 2- to 4-ring PAHs (100 mg per kg soil) and other semi-volatile compounds <10 mg per kg soil **Amendment:** composting with leaves **Conditions:** windrows (~19 m³) **Time:** 150 days	Complete breakdown after 150 days with most loss during the first 63 days. Amendment ratio did not affect the extent of degradation of PAHs during the study it was observed that temperature, moisture and C/N were deficient for optimal composting conditions	(Crawford *et al.* 1993) *
soil type: **Contamination:** pyrene **Amendment:** soil and soil-mature compost mixtures **Conditions:** **Time:** 100 days	>80 % pyrene degradation (significantly enhanced) after 20 days when mature compost was added (5% removal without compost addition)	(Mahro and Kästner 1993) *
Soil type: **Contamination:** creosote (including 2- to 4-ring PAHs) **Amendment:** municipal solid wastes and fertiliser **Conditions:** laboratory scale in vessel composting (2 kg mixtures), 45 °C, 80 % compost, 5 % fresh organic matter, 5 % fresh organic matter mixed with fertiliser (N:P:K 4:10:1), 8 % contaminated soil, 2 % fertiliser, 0.2 % creosotel to avoid toxicity effects **Time:** 15 days	81.63 % (Benzo[a]anthracene) to 98.63 % (fluorene) PAH removal <10 % volatilisation except Acenaphthylene (54%)	(Civilini 1994) *
Soil type: Ah horizon of a para-brown soil from an uncontaminated, rural area near Hamburg, Germany **Contamination:** [^{14}C]Anthracene, [^{14}C]Hexadecane **Amendment:** mature compost (1:4) compost dry wt.: soil dry wt.) **Conditions:** Co-composting contaminated soil with mature compost **Time:** 103 days	23% of [^{14}C] Anthracene transformed into $^{14}CO_2$ and 42% was irreversibly fixed to the soil matrix. In the un-supplemented control reactor more than 88 % of the original [^{14}C]Anthracene could be recovered 21% of the labelled carbon of [^{14}C] Hexadecane could not be extracted. The compost could stimulate the depletion of hydrocarbons by either mineralization or the formation of not extractable bound residues (humification)	(Kästner *et al.* 1995)

Soil type: silt loam **Contamination**: spiked with Benzo[a]pyrene (150 mg per kg soil) **Amendment:** laboratory scale (125 ml) in-vessel composting with and without *Phanerochaete chrysosporium*; soil amended with corncobs (soil:corncobs 2:1) **Conditions:** periodically aerated **Time:** 95 days	no difference between inoculated (62.8% removal) and un-inoculated (65.6% removal) systems although initial removal rates were faster in the inoculated incubations. During poison tests with 4 % $HgCl_2$, Benzo[a]pyrene removal was observed suggesting irreversible adsorption to compost material A substantial concentration of *P. chrysosporium* was found in both (inoculated and un-inoculated) systems after 95 days suggesting that amending soil with suitable fungal substrates may be sufficient	(McFarland and Qiu 1995) *
Soil type: **Contamination:** Benzo(a)pyrene (BaP) **Amendment:** inoculation with *Phanerochaete chrysosporium* **Conditions:** **Time:** 95 days	fungi increased the formation of bound residue in the first 30 days from 0.73 mg per kg soil per day to 1.58 mg per kg soil per day despite this after the 95 days fungal inoculation was found to be ineffective with 62 % (inoculated), 65 % (un-inoculated) and 49 % (poisoned compost) BaP-removal no loss through volatilisation and mineralisation, nearly all of the BaP removal was attributed to bound residue formation	(McFarland and Qiu 1995) *
soil type: **Contamination:** [^{14}C]anthracene **Amendment:** mature compost **Conditions:** 3 l compost reactors, temp 21± 2°C, continuously aerated with humidified air, 60% moisture **Time:** 103 days	23 % of [^{14}C]anthracene was mineralised to $^{14}CO_2$ and 42 % was irreversibly bound to the soil-compost matrix In soil-only incubations ~88 % [^{14}C]anthracene was recoverable by solvent extraction with the formation of bound residues less significant	(Kästner *et al.* 1995) *
Soil type: Ah horizon of a para brown soil from an uncontaminated, rural area near Hamburg, Germany **Contamination:** naphthalene (500 mg per kg soil), phenanthrene, anthracene, fluoranthene, pyrene (each 100mg per kg soil) **Amendment:** CaO, compost (3:1, soil:compost), **Conditions:** incubating 200 g mixture in 1.5l vessels, 60 % moisture, 25 °C **Time:** 98 days	The presence of the solid organic matrix of the compost seemed to be essential for enhanced degradation neither the fertilising substances of the compost, nor the shift in pH, nor the micro-organisms in the compost were responsible.	(Kästner and Mahro 1996)
Soil type: non contaminated sterilised and non-sterilised agricultural top soil **Contamination:** spiked with [^{14}C]pyrene **Amendment:** fungi *Kuehneromyces mutabilis* and *Agrocybe aegerita* **Conditions:** soil mixed with sawdust, 70 % moisture **Time:** 63 days	5.1 % [^{14}C]pyrene degraded by *K. mutabilis*, 1.5% degraded by *A. aegerita*, when soil was sterilised and inoculated 27.3 % [^{14}C]pyrene degradation by indigenous micro-organisms in non sterilised and non inoculated soil 47.7 % [^{14}C]pyrene degradation by indigenous micro-organisms and *K. mutabilis* in non sterilised and inoculated soil 38.5% [^{14}C]pyrene degradation by indigenous micro-organisms and *A. aegerita* in non sterilised and inoculated soil	(Sack and Fritsche 1996)
Soil type: spiked Ah horizon of a para brown soil at a non-contaminated rural area **Contamination:** naphthalene (500 mg per kg soil), phenanthrene (100 mg per kg soil), anthracene (100 mg per kg soil), Fluoranthene(100 mg per kg soil), pyrene(100 mg per kg soil) **Amendment:** soil and soil-mature compost (3:1) incubations **Conditions:** 25°C, 60% moisture, laboratory scale **Time:** 100 days	complete degradation of naphthalene, phenanthrene, and anthracene after 20 days complete degradation of fluorene and pyrene after 35 days (with showing a lag phase of 10 days) presence of the compost enhanced the removal of the PAHs and the presence of the organic matrix of the compost was essential for enhanced degradation	(Kästner and Mahro 1996) *

Soil type: precontaminated and uncontaminated soil **Contamination:** total PAH 0.082 to 1571 mg per kg soil (pre-contaminated soil) ^{14}C-PAHs were added to a final concentration of: [^{14}C]-Phenanthrene 500 ng per g soil, [^{14}C]-Pyrene 100 ng per g soil, [^{14}C]-Benz[a]anthracene 214 ng per g soil, [^{14}C]-Chrysene 237 ng per g soil, [^{14}C]-Benz[a]pyrene (BaP) 136 ng per g soil **Amendment:** **Conditions:** 1g soil mixed with 10 ml H_2O and incubate in the dark at 20 °C **Time:** 2 months	[^{14}C]PAHs (except BaP) readily mineralised in pre-contaminated soils with 30-60 % phenanthrene, 10-55 % pyrene, 5-40 % Benz[a]anthracene, 10-50 % chrysene, 2-9 % BaP <5 % [^{14}C]PAHs mineralised in uncontaminated soils <10% loss by metabolite production and cellular incorporation no relation between microbial-community size and the fate of [^{14}C]PAHs negative correlation between the fraction of silt and clay to the extend of [^{14}C]PAH mineralisation and positively correlated to the amount of [^{14}C]PAHs remaining in the soil after extraction	(Carmichael and Pfaender 1997)
Soil type: **Contamination:** coal tar, [^{14}C]-Phenanthrene, [^{14}C]-Pyrene, [^{14}C]-Benzo[b,j,k]fluoranthene, [^{14}C]-Benzo[a]pyrene **Amendment:** phenanthrene-utilising bacteria, non-ionic surfactants (alcohol ethoxylate and glycoside surfactants) **Conditions:** **Time:**	Inoculation with phenanthrene-utilizing bacteria stimulated the mineralization of phenanthrene. This effect was most notable in soil with a low indigenous potential for PAH degradation, and a large inoculum was required to establish phenanthrene mineralisation in the soil. Addition of alcohol ethoxylate and glycoside surfactants enhanced the mineralisation of phenanthrene and pyrene and other PAHs such as Benzo[b,j,k]fluoranthene and Benzo[a]pyrene which were resistant to degradation without surfactants. The surfactant-related enhanced degradation was less notable when a rapidly degradable glycoside surfactant was used suggesting that surfactants that are mineralised at moderate rates may be more applicable for increasing the availability of PAHs in soil.	(Madsen and Kristensen 1997)
Soil type: spiked Ah/Al horizon **Contamination:** PAHs/N-PAHs (28-181 mg per kg soil) **Amendment:** **Conditions:** 415 g soil-compost mixture in a 1 litre bioreactor; 50 % moisture **Time:** 180 days	in un-amended soils only 2- to 3-ring PAHs were degraded over 105 days. in soil-compost mixtures 2-ring PAHs were depleted after a lag phase of 8 days within the following 49 days and 3-ring PAHs were eliminated to <3 % during 105 days; out of the larger PAHs only fluoranthene and pyrene were almost completely transformed within 105 days; the residual concentration of Benzo[a]anthracene, chrysene and Benzo[a]pyrene decreased to 2, 3 and 27 % within 180 days. similar losses in poisoned and un-amended soil suggest predominant abiotic losses within the un-amended soil	(Wischmann and Steinhart 1997) *
Soil type: soil from oil-lake beds in Kuwait; high salinity **Contamination:** total petroleum hydrocarbon (40 g per kg soil for landfarming; 35 g per kg soil for windrows; 14 g per kg soil for bioventing piles **Amendment:** contaminated soil supplemented with inorganic fertiliser, compost and woodchips **Conditions:** landfarming, windrow composting, and static bioventing piles, constant water and nutrient supply **Time:** 12 months	82.5 % reduction (landfarming) 74.2 % reduction (windrow) 64.2 % reduction (static pile)	(Balba *et al.* 1998)

Soil type: **Contamination:** oil sludge (16.8 g per kg dry matter) and petroleum residues (78.5 g per kg dry matter) from a refinery (both including USEPA and other PAHs with naphthalene being the dominant PAH (50 %)) **Amendment:** horse manure **Conditions:** co-composting in 208 l composting bins, 4 treatments: 1.8 % oil sludge, 2.1 % petroleum residues, 7.1 % petroleum residues, 7.0 % paraffin oil, aerated, temp. increasing from 15 °C to 30 °C during days 12-17, then decreasing to 22-25 °C **Time:** 135 days	78–93 % removal of the majority of PAHs with concentrations close or below the detection limit of 0.1mg per kg dry matter, except for pyrene, chrysene, and Dibenz[a,h]anthracene which were only partly removed (10 %) to 0.2-0.8 mg per kg dry matter	(Kirchmann and Ewnetu 1998) *
Soil type: simulated municipal solid waste **Contamination:** spiked with a mixture of 3- and 4-ring PAHs **Amendment:** **Conditions:** laboratory scale, batch-type, in-vessel composter; 50-60 % moisture during active composting and 30 % during curing; aerated and/or stirred; 50 °C during active composting; nutrients (0.8 % ammonia nitrogen, 2.3 % nitrate, 32.9 % urea) during active composting **Time:** 30 days active composting + 30 days compost curing	removal of anthracene, phenanthrene, and pyrene mainly through biotic processes fluorene lost with abiotic processes accounting for 75% Benz[a]anthracene was resistant to biodegradation throughout both phases, but 40-50% was lost abiotically most biodegradation occurred during active composting	(Joyce *et al.* 1998) *
Soil type: weathered-hydrocarbon contaminated soil from Imperial Oil, Sarnia, ON, and uncontaminated soil **Contamination:** mineral oil and grease including aliphatic-, polar-, and aromatic hydrocarbons **Amendment:** alfalfa and maple-leaves adding either 4.2 % $CaCO_3$ and PO_4^{3-} **Conditions:** 1 litre jars, aerated, 50-60 % moisture, temperature profile; altering C/N ratio (17-49 mol/mol), addition, temperature, length of thermophilic phase **Time:** 30 days	increased degradation with: - low C:N ratio (~17-18) - 23°C, than 5 days at 50°, but - maintaining 50°C for 30 days much better than 50°C for 6 days and 23°C for the remaining time - adding $CaCO_3$ or PO_4, but adding both together had no effect - increasing the leaves/alfalfa amendment	(Beaudin *et al.* 1999)
Soil type: from abandoned wood preservation site in southern Norway **Contamination:** creosote (including 16 USEPA PAHs) **Amendment:** fertiliser pre-treated and not pre-treated, straw inoculated with *Pleurotus ostreatus* **Conditions:** soil was mixed and layered with straw inoculated with *Pleurotus ostreatus* at 8 °C or 22 °C **Time:** 2 months	*P. ostreatus* had an overall positive effect on the degradation of aged creosote contaminated soil enhanced degradation by increased temperature and fertilizer pre-treatment which had a good effect on the microbial community at low temperatures even without addition of fungi	(Eggen and Sveum 1999)
Soil type: real contaminated soil and spiked sterile soil **Contamination:** PAH **Amendment:** fungal (*Bjerkandera* sp.) pre-colonised hemp stem wood **Conditions:** **Time:** 90 days	up to 70% PAH removal in real aged soil in real soil the *Bjerkandera* sp. inoculated soil showed lowest residual PAH concentrations compared with not inoculated soil after 56 days in spiked soil very broad optimal growth ranges for fungi and high residual concentrations after *Bjerkandera* sp. treatment which could be partly overcome by miscible solvent or surfactant addition the limited bioavailability of PAHs seems to be the most important bottleneck for implementation of a white-rot-fungus-based remediation technique for polluted soils	(Grotenhuis *et al.* 1999)

Soil type: **Contamination:** soil spiked with [^{14}C]Naphthalene, [^{14}C]Phenanthrene, [^{14}C]Pyrene, and contaminated soil (naphthalene 24 mg per kg soil, phenanthrene 1.3 mg per kg soil, and other PAHs) **Amendment:** mixed microbial culture isolated from PAH contaminated soil **Conditions:** aerobic aqueous systems, 25 or 30 ml reaction vessel **Time:**	results showed that in a mixture of compounds the rates of more degradable compounds will be inhibited and the rates of the more recalcitrant compounds will be enhanced	(Guha *et al.* 1999)
Soil type: Ah horizon of a Luvisol (organic C content, 1%) from an uncontaminated, rural area near Hamburg, Germany **Contamination:** [^{14}C]anthracene **Amendment:** compost (4:1, soil:compost) **Conditions:** 2 kg soil in continuously aerated 3 l soil bioreactors, 60 % moisture **Time:** 176 days	Addition of compost increased metabolism and decreased residue formation Differences as to where (what position) [^{14}C]anthracene is labelled were found	(Kästner *et al.* 1999)
Soil type: contaminated sewage sludge **Contamination:** PAHs **Amendment:** ligneous waste **Conditions:** co-composting **Time:** 90 days.	31 % decrease of total PAHs during the first 20 days followed by an 8 % increase up to day 90	(Lazzari *et al.* 1999)
Soil type: soil from Reilly Tar and Chemical Company Superfund Site St. Louis Park, MN **Contamination:** creosote with 19 PAHs (total PAHs 1606-4445 mg per kg soil) **Amendment:** 10 in vessel bench scale compost units **Conditions:** 30% (w/w) corn cobs; std nutrients or modified OECD nutrients (C:N:P=100:5:1), cow manure, activated sludge, autoclaved sludge; 30-35% moisture, continuous vertical airflow; temperatures increased to 41-53°C during the first 15 days and subsequently decreased to ambient temperature **time:** 12 weeks	PAHs degraded to 888-1556mg/kg 87% average decrease of 2-3 ring PAHs 61% average decrease of 4-ring PAHs no reduction of 5-6 ring PAHs plateau concentrations appeared after week 8 with no significant differences in the final concentrations for all treatments removal rates during first 4 weeks correlated with starting concentrations but not with reactor biomass concentration	(Potter *et al.* 1999) *
Soil type: artificially contaminated pine wood and, and really PAH contaminated pine wood **Contamination:** spiked wood: phenanthrene and pyrene (1000 mg per kg wood each); contaminated wood: total PAH 5485 mg per kg wood **Amendment:** mixing the contaminated wood with decomposed wood (50 g per kg contaminated wood) and 26 l pig manure **Conditions:** pilot scale percolator system, **Time:** 61 days	93 % of phenanthrene degraded with the artificially contaminated wood 90% of pyrene degraded with the artificially contaminated wood slower degradation of PAHs in really polluted wood resulting in 86% phenanthrene and 32% pyrene degradation fastest degradation by compost addition most intensive CO_2 evolution with hydrocarbon polluted soil as additive	(Löser *et al.* 1999) *
Soil type: **Contamination:** [^{14}C]anthracene (100 mg per kg soil) **Amendment:** pure soil and soil-compost mixture (4:1) **Conditions:** bioreactor in laboratory scale continuously aerated, 60% moisture **Time:** 176 days	complete transformation of [^{14}C]anthracene less formation of bound residues (20.7 %) and a higher mineralisation (67.2 %) in the compost mixture than in pure soil (43.8 % mineralised, 45.4 % transformed into bound residues)	(Kästner *et al.* 1999) *

Soil type: silty soil **Contamination:** TPH 40 g per kg soil and PAH 630 mg per kg soil **Amendment:** 640 g soil + 250 g maple leaves + 750 g alfalfa + 80 g $CaCO_3$ **Conditions:** moisture content 50%, incubated at 55°C, aerated continuously or intermittently **Time:** 35 days active composting + 90 days maturing at ambient temp; after that mixing the resulting compost with more PAH contaminated soil (1:4) and compost for 100 days	>50 % mineralisation of pyrene by day 15 <3 % mineralisation of pyrene by day 15 in unamended 0.7 % maximum total mineralisation in abiotic controls enhanced pyrene mineralisation by addition of humic acid to the soil compost mixture inhibited pyrene mineralisation with fulvic acid addition	(Haderlein *et al.* 1999; Haderlein *et al.* 2001) *
Soil type: silty clay **Contamination:** tar residues, total PAH 4.3-6915 mg per kg soil of this 180-300 mg naphthalene per kg soil, 70-230 mg phenanthrene per kg soil, 58-71 mg Benzo(a)pyrene per kg soil **Amendment:** green tree waste (*Eucalyptus* sp. leaf and stem waste):manure:soil (3:1:16) **Conditions:** 130m^3 windrow, regular mixed, 60-80 % moisture, max temp (42°C) reached after 35 days **Time:** 244 days	complete removal of LMW PAHs 90 % removal of MMW PAHs 70% HMW PAHs; ~50 % of each of the most resistant PAHs (Indeno[1,2,3-cd]pyrene, Benzo[g,h,i]perylene) was lost with 120mg/kg the total PAH concentration was lower than with land treatment no volatilisation	(Guerin 2000) *
Soil type: **Contamination:** diesel fuel **Amendment:** two carbon-to-nitrogen ratios by adding ammonium sulfate or urea. **Conditions:** laboratory scale sealed bioreactors, 25 °C, **Time:**	highest hydrocarbon degradation rates for the ammonium sulfate (20:1 at 0.032 per day; 40:1 at 0.019 per day) and urea treatments (20:1 at 0.025 per day; 40:1at 0.011 per day) A degradation rate correlation as a function of nitrate and ammonia concentrations was developed, suggesting the occurrence of nitrate inhibition at elevated nitrate concentrations	(Brook *et al.* 2001)
Soil type: from manufactured gas plant site commissioned in 1838 at Clitheroe, Lancashire, UK **Contamination:** coal-tar (including 16 USEPA PAHs) **Amendment:** fungi (*Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Coriolus versicolor*, and Wye isolate #7) inoculated wheat-straw (single species and any combination) in a ratio of 5:2 (soil:fungi) **Conditions:** 50 % moisture, 22 °C **Time:** 32 days	greatest PAH losses in biotic control small or negligible differences in microcosms inoculated with one or more fungi results suggest that the use of the autochthonous micro-flora, with no introduction of foreign micro-organisms, offers the greatest potential for PAH degradation	(Canet *et al.* 2001)
Soil type: spiked loamy sand **Contamination:** petroleum hydrocarbons (5000 mg HC per kg soil) by mixing diesel fuel and motor oil (1:1, v:v) **Amendment:** steer manure and inorganic fertiliser [$(NH_4)2SO_4$] **Conditions:** **Time:** 41 days	32 % reduction in hydrocarbon-concentrations in the control treatment ~ 54 % reduction in hydrocarbon-concentrations with the $(NH_4)2SO_4$ fertilizer-treatment up to 81 % reduction in hydrocarbon-concentrations with 20 % manure amendment	(Wellman *et al.* 2001)
Soil type: from a former tar-contaminated site **Contamination:** 11 individual three- to six-ring unsubstituted aromatic hydrocarbons (PAH) **Amendment:** **Conditions:** compost pile, aerated **Time:** 42 days active composting + 100 days maturation	42-68 % removal of 3-4 ring PAHs 35-57 % removal of higher-molar mass PAHs no further decrease of PAHs in additional 100 d of compost maturation in open-air field	(Cajthaml *et al.* 2002)
Soil type: **Contamination:** phenanthrene, anthracene, pyrene (100 mg per kg soil each) **Amendment:** pig manure at three different ratios (12.5 %, 25 % and 50 % w/w dry weight basis) **Conditions:** co-composting **Time:**	90 % removal of PAHs 25 % pig manure application rate showed the most efficient removal of 3-ring PAHs (phenanthrene and anthracene) no significant difference in pyrene removal for the treatments with 25 or 50 % pig manure	(Wong *et al.* 2002)

Soil type: soil from a sawmill area **Contamination:** creosote (23.6 g PAH per kg fresh weight soil) about 78% were small PAHs; also highly contaminated with arsenic, chromium, and copper **Amendment:** *Mycobacterium sp.*, nutrients, spruce bark chips **Conditions:** windrows (5m³), some of the soil was pre-treated with 50% hydrogen peroxide **Time:** 163 days	almost 90 % reduction of 2- and some 3-ring PAHs PAH reduction in all windrows PAH concentrations in inoculated piles temporarily increased (after 4 months) but decrease to the same amount than the control pile (~80 %) after 5 months inoculation did not speed up the process markedly pre-treating soil with hydrogen peroxide achieved similar removal rates (96 % small PAHs and 57 % medium and large PAHs) in shorter periods of time	(Ahtiainen *et al.* 2002) *
soil type: wood preservation site **Contamination:** creosote (total PAH 6473 mg per kg soil) **Amendment:** inoculation with spent mushroom substrate **Conditions:** **Time:** 12 weeks + 3 weeks	50 % (anthracene and acenaphthene) to 87 % (phenanthrene, fluorene) reduction of 3-ring PAHs after 12 weeks 87 % (anthracene) and 97-99 % (fluorene, phenanthrene, acenaphthene) PAH-removal after additional re-inoculation and 3 weeks incubation 43 % (fluoranthene) and 34 % (pyrene) reduction of 4-ring PAHs after 12 weeks 59 % (fluoranthene) and 51 % (pyrene) after additional re-inoculation and 3 weeks incubation	(Eggen and SaSek 2002)
Soil type: former tar-producing plant **Contamination:** 16USEPA PAHs (total conc. 2832 mg per kg soil) **Amendment:** adding mushroom compost (consisting of wheat straw, chicken manure, and gypsum) in mid phase; thermally insulated composting chamber (~1000kg volume) **Conditions:** 57:53 soil:compost; 64% moisture **Time:** 42 days in composting chamber + 100 days in windrow	42-68 % removal of 3- to 4-ring PAHs after 42 days 35-57 % removal of 5- and 6- PAHs after 42 days no further decrease after additional 100 days	(Cajthaml *et al.* 2002) *
Soil type: two contaminated soils **Contamination:** PAHs **Amendment:** *Pleurotus ostreatus* in the form of homogenised refuse from the commercial production of fungi **Conditions:** concrete cylinders (height, 50 cm; diameter, 60 cm), treatments (control, soil mixed with autoclaved sawdust medium, and soil mixed with P. ostreatus refuse) **Time:** 9 weeks	78 % reduction of 3-ring PAHs 41 % reduction of 4-ring PAHs 4 % reduction of 5- and 6-ring PAHs	(Hestbjerg *et al.* 2003)
Soil type: spiked garden soil (sandy loam soil) **Contamination:** naphthalene, phenanthrene, Benzo[a]pyrene and Benzo[g,h,i]perylene (200 mg per kg soil) **Amendment:** spent mushroom compost of *Pleurotus pulmonarius* (mixture of wheat straw, dried blood, horse manure, ground chalk) (19:1 soil:compost) **Conditions:** 60% moisture 4 to 80°C at 200 rpm continuous shaking **Time:** 2 days	82 % (naphthalene) to 59 % (phenanthrene) PAH removal due to biodegradation and sorption when treating 100 mg PAH per litre at room temp with 1% spent mushroom compost highest sorption removal with phenanthrene (46%) increase in PAH removal with temperature; complete removal of three PAHs (not phenanthrene) at 50°C; complete removal of all 4 PAHs at 80°C with 5% mushroom compost	(Lau *et al.* 2003) *
Soil type: interior thermophilic and exterior mesophilic zone of yard waste compost between 3 and 6 months old **Contamination:** phenanthrene (100 mg per kg soil) **Amendment:** **Conditions:** incubated in the dark at 22±2 or 60±2 °C in biometers **Time:** 90 days	dominant effect due to heterogeneity of compost waste from thermophilic zone: - 1-2 % mineralisation when incubated at 60°C - 17% mineralisation when incubated at 22°C waste from mesophilic zone: negligible - mineralisation when incubated at 60°C - 8% mineralisation when incubated at 22°C	(Carlstrom and Tuovinen 2003) *

Soil type: manufactured-gas plant soil **Contamination:** 12 USEPA PAHs (total conc. 610 mg per kg dry soil) **Amendment:** adding mushroom compost (consisting of wheat straw, chicken manure, and gypsum) in mid phase; thermally insulated composting chamber (~1000kg volume) **Conditions:** 64:36 soil:compost; 64% moisture **Time:** 54 days in composting chamber + 100 days in windrow	20-60 % of individual PAHs were degraded until day 54 37-80 % of individual PAHs were degraded until day 154 no volatilisation temp increased to 70 up to day 12 then it progressively decreased	(ŠaŠek *et al.* 2003) *
Soil type: soil from manufactured-gas-plant-area **Contamination:** petroleum hydrocarbons **Amendment:** inoculating soil with white rot fungi *Irpex lacteus, Pleurotus ostreatus,* and bacterium *Pseudomonas putida*. **Conditions:** **Time:** 10 weeks	up to 66 % decrease in phenanthrene, anthracene, fluoranthene and pyrene concentrations	(ŠaŠek *et al.* 2003) *
Soil type: mispah form (FAO:lithosol) from creosote treatment plant **Contamination:** total PAHs >3g per kg soil **Amendment:** sawdust with poultry manure **Conditions:** soil:sawdust:poultry manure (2:2:1), 350 kg as static pile **Time:** 19 months	removal below the remediation target (1 mg per kg soil) of all PAHs except chrysene after 11 months removal of chrysene below 1 mg per kg soil after 17 months after 19 months only traces were left (0.1-0.5 mg per kg)	(Atagana 2004)
Soil type: lagooning sewage sludge **Contamination:** PAHs (total 0.24 mg per kg sludge) **Amendment:** straw (8.25:1, sludge:straw) **Conditions:** co-composting pile, turned every 25 days **Time:** 180 days	75 % decrease until day 30 ~50 % increase until day 60 slight decrease until day 90 88 % further decrease until day 180 Each single PAH showing similar behaviour to the total PAH profile Decrease during stabilisation (more notable for larger PAHs (≥4-rings) maximum desorption after day 60 for naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, Benzo[a]anthracene, chrysene	(Amir *et al.* 2005)
Soil type: manufactured gas plant site commissioned in 1838 at Clitheroe, Lancashire, UK **Contamination:** 16 USEPA PAHs (total PAHs 100.3 mg per kg dry soil) **Amendment:** silver-sand, green-waste (3:3:10 soil:silver-sand:green-waste) **Conditions:** incubated at different temperatures (38°C, 55°C, 70°C) **Time:** 98 days	80.9 % highest PAH-removal after 111d at 38°C generally more removal of PAHs with fewer aromatic rings	(Antizar-Ladislao *et al.* 2005)
Soil type: manufactured gas plant site commissioned in 1838 at Clitheroe, Lancashire, UK **Contamination:** 16 USEPA PAHs (total PAHs 100.3 mg per kg dry soil) **Amendment:** silver-sand, green-waste ((3:3:10); (35:35:10); (40:40:10), (45:45:10) soil:silver-sand:green-waste) **Conditions:** incubated at different temperatures (38°C, 55°C, 70°C) **Time:** 98 days	75.1 % highest PAH-removal after 8 weeks at 38°C with a mixing ratio of (0.4:0.4:1)	(Antizar-Ladislao *et al.* 2005)
Soil type: **Contamination:** soot waste (total PAHs ~200 mg per kg soil) **Amendment:** sewage sludge and yard waste (high alkalinity pH 12.8) **Conditions:** co-composting in closed tank with forced aeration **Time:** 60 days in closed tank followed by 70 days with natural aeration	68 % PAH removal significant self-drop in waste pH and increase in mass temperature after 30 days, progressive drop in the PAH concentration following this degradation more effective on lower molecular weight PAHs (2-4 rings).	(Moretto *et al.* 2005)

Soil type: **Contamination:** creosote **Amendment:** nutrients (final C:N:P ratio of 300:10:1 adding one-third in the beginning, one at day 45 and one at day 90) and either a bio-surfactant (added in the beginning and at day 130), a microbial consortium (added at days 14, 26, 57, 120, 165), or iron octate (added in the beginning and at day 145) **Conditions:** **Time:** 200 days	higher degradation values without nutrient addition moisture content (40 % optimal) and aeration are key factors no differences in PAH or TPH degradation with Bio-surfactant addition, bioaugmentation, or ferric octate addition compared to nutrient treatment	(Vinas *et al.* 2005)
Soil type: manufactured gas plant site commissioned in 1838 at Clitheroe, Lancashire, UK **Contamination:** 16 USEPA PAHs (total PAHs 100.3 mg per kg dry soil) **Amendment:** silver-sand, green-waste ((3:3:10); (35:35:10); (40:40:10), (45:45:10) soil:silver-sand:green-waste) **Conditions:** incubating at different temperatures (38°C, 55°C, 70°C), and with different moisture contents (40%, 60%, 80%) **Time:** 98 days	82.0 % highest PAH-removal rate at 38°C with a mixing ratio of (0.35:0.35:1) and a moisture content of 60 % more removal of small PAHs than large except for 70 °C there the other way round	(Antizar-Ladislao *et al.* 2006)

* reviewed by Antizar-Ladislao *et al.* (2004)
** reviewed by Wilson and Jones (1993)

APPENDIX 2

Table A2: List of PAHs and bacteria that have been found to degrade those PAHs, as well as metabolites detected during the investigated degradation-pathways; as published in Loick *et al.* (in press); The "Organism"-column gives the PAH and lists the organisms having been found to be able to degrade this PAH; the "Detected Metabolites"-column lists the found metabolites; the "Reference"-column refers to the literature reference

Organism	Detected Metabolites	Reference
Naphthalene		
Acinetobacter calcoaceticus	1,2-dihydroxynaphthalene	(Sutherland *et*
Alcaligenes denitrificans	1-naphthol	*al.* 1995;
Bacillus cereus	2,3-dihydrocoumarin (chroman-2-one)	Hamann *et al.*
Bacillus thermoleovorans	2,3-dihydroxynaphthalene	1999;
Comamonas testosteroni	2-carboxycinnamic acid	Annweiler *et*
Corynebacterium renale	2-hydroxybenzoic acid (salicylic acid)	*al.* 2000; Li *et*
Cyclotrophicus sp.	2-hydroxychromene-2-carboxylic acid	*al.* 2000;
Hyphomicrobium sp.	2-hydroxycinnamic acid	Samanta *et al.*
Methylococcus capsulatus	2-naphthol	2002; Mrozik
Moraxella sp.	3-(2-carboxyphenyl)-2-propenoic acid (2-carboxycinnamic	*et al.* 2003)
Mycobacterium sp.	acid)	
Nocardia sp.	3-(2-hydroxyphenyl)-propanoic acid	
Oscillatoria sp.	4-(2-hydroxyphenyl)-2-oxo-but-3-enoic acid	
Pseudomonas sp.	benzene-1,2-diol (catechol)	
Pseudomonas acidovorans	benzoic acid	
Pseudomonas cepacia	catechol	
Pseudomonas fluorescens	*cis*-1,2-dihydro-1,2-dihydroxynaphthalene	
Pseudomonas putida	*cis*-1,2-naphthalenedihydrodiol	
Pseudomonas	*cis*-2-hydroxybenzalpyruvate	
saccharophila	coumarin (chromen-2-one)	
Pseudomonas stutzeri	gentisate	
Pseudomonas testosteroni	gentisic acid	
Pseudomonas vesicularis	naphthalene1,2-oxide	
Rhodococcus sp.	phthalic acid	
Sphingomonas	pyruvate	
paucimobilis	salicylaldehyde	
Streptomyces sp.	salicylate	
Streptomyces griseus	salicylic acid	
Vibrio sp.	*trans*-1,2-dihydrodiol	
	trans-o-hydroxybenzylindenepyruvic acid	
Acenaphthylene		
Beijerinckia sp.	1,2-acenaphthenedione	(Grifoll *et al.*
Pseudomonas sp.	1,2-dihydroxyacenaphthylene (possibly)	1995;
Pseudomonas aeruginosa	1,8-naphthalenedicarboxylic acid	Sutherland *et*
Sphingomonas sp.	acenaphthoquinone	*al.* 1995;
	cis-1,2-acenaphthylenedihydrodiol	Pinyakong *et*
	cis-acenaphthene-1,2-diol	*al.* 2004)
Acenaphthene		
Alcaligenes eutrophus	1,2-acenaphthenedione	(Grifoll *et al.*
Alcaligenes paradoxus	1,2-dihydroxyacenaphthylene (possibly)	1995;
Beijerinckia sp.	1,8-naphthalenedicarboxylic acid	Sutherland *et*
Cycloclasticus sp.	1-acenaphthenol	*al.* 1995;
Coriolopsis gallica	1-acenaphthenone	Pickard *et al.*
Pseudomonas sp.	3-hydroxyphthalic acid	1999;
Pseudomonas aeruginosa	7,8-diketonaphthyl-1-acetic acid	Pinyakong *et*
Sphingomonas sp.	acenaphtoquinone	*al.* 2004)
	cis-1,2-acenaphthenedihydrodiol	
	trans-acenaphthene diols	
Fluorene		
Arthrobacter sp	1,10-dihydro-1,10-dihydroxyfluorene-9-one	(Boldrin *et al.*
Brevibacterium sp.	1,1a-dihydroxy-1-hydrofluoren-9-one	1993; Trenz *et*
Cycloclasticus sp.	1,1-dihydroxy-1-hydro-9-fluorenone	*al.* 1994; Grifoll
Mycobacterium sp.	1-hydroxy-9-fluorenone	*et al.* 1995;
Mycobacterium	1-indanone	Bogan *et al.*
austroafricanum	2-formyl-1-indanone	2003; Mrozik

Pseudomonas sp.	2-hydroxy-4-pentenoate	*et al.* 2003)
Pseudomonas aeruginosa	2-hydroxy-6-(2-carboxyphenyl)-6-oxo-2,4-hexadienoic	
Pseudomonas cepacia	acid	
Pseudomonas	2-indanone	
saccharophila	2-oxo-4-pentenoate	
Rhodococcus sp.	3-(2-hydroxyphenyl)propionate	
Staphylococcus auriculans	3,4-dihydrocoumarin	
	3,4-dihydroxyfluorene	
	3-hydroxy-1-indanone	
	3-isochromanone	
	4-hydroxy-9-fluorenone	
	8-hydroxy-3,4-benzocoumarin	
	9-fluorenol	
	9-fluorenone	
	9-hydroxyfluorene	
	cis-1,1a-dihydroxy-1-hydrofluoren-9-one	
	cis-3,4-dihydrodiol	
	formyl-indanone	
	phthalic acid	
	substituted biphenyl	
	tricarboxylic acid cycle	
Phenanthrene		
Aeromonas sp.	1,2-dihydroxynaphthalene-> mineralised via naphthalene	(Grifoll *et al.*
Agmenellum	pathway	1995;
quadruplicatum	1-hydroxy-2-naphthaldehyde	Sutherland *et*
Alcaligenes denitrificans	1-hydroxy-2-naphthoic acid	*al.* 1995;
Alcaligenes faecalis	1-methoxyphenanthrene	Geiselbrecht *et*
Arthrobacter	1-naphthol	*al.* 1998;
polychromogenes	2-carboxybenzaldehyde	Hamann *et al.*
Bacillus sp.	2-carboxycinnamic acid	1999; Pickard
Beijerinckia sp.	2-formylbenzoic acid	*et al.* 1999;
Brevibacterium sp.	2-hydroxy-1-naphthaldehyde	Samanta *et al.*
Comamonas testosteroni	2-hydroxy-1-naphthoic acid	2002; Bogan *et*
Coriolopsis gallica	2-hydroxy-3-naphthoic acid	*al.* 2003;
Cycloclasticus sp.	3,4-dihydroxy-3,4-dihydronaphthalene	Mrozik *et al.*
Flavobacterium sp.	3,4-dihydroxyphenanthrene	2003; Jacques
Micrococcus sp.	3,4-phenanthrenediol	*et al.* 2005;
Mycobacterium sp.	4, 5-dihydroxyphthalic acid	Keum *et al.*
Nocardia sp.	4-hydroxy-1-tetralone	2006)
Pseudomonas sp.	5,6-benzocoumarin	
Pseudomonas aeruginosa	7,8-benzocoumarin	
Pseudomonas cepacia	catechol	
Pseudomonas citronellolis	*cis*-1,2-dihydroxy-1,2-dihydrophenanthrene	
Pseudomonas fluorescens	*cis*-1,2-phenanthrenedihydrodiol	
Pseudomonas putida	*cis*-3,4-dihydroxy-3,4-dihydrophenanthrene	
Rhodococcus sp.	*cis*-3,4-phenanthrenedihydrodiol	
Sinorhizobium sp.	*cis*-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid	
Sphingomanas sp.	*cis*-4-(2-hydroxynaph-1-yl)-2-oxobut-3-enoic acid	
Sphingomonas	*cis*-o-hydroxybenzylidene-pyruvic acid	
paucimobilis	coumarin salicylic acid	
Streptomyces sp.	gentisic acid	
Streptomyces flavovirens	naphthalene-1,2-dicarboxylic acid	
Vibrio sp.	naphthalene-1,2-diol	
Xanthomonas maltophila	*o*-phthalic acid	
Mycobacterium sp.	phthalic acid	
Mycobacterium flavescens	protocatechuate	
Mycobacterium	protochatechuic acid	
austroafricanum	pyruvic acid	
	salicylic acid	
	trans-3,4-phenanthrenedihydrodiol	
	trans-9,10-phenanthrenedihydrodiol	
	tricarboxylic acid cycle	
Anthracene		
Beijerinckia sp	1,2-dihydroanthracene	(Grifoll *et al.*
Comamonas testosteroni	1,2-dihydroxyanthracene	1995;
Coriolopsis gallica	1-methoxy-2-hydroxyanthracene	Sutherland *et*
Cycloclasticus sp.	2,3-dihydroxynaphthalene	*al.* 1995; Goyal
Flavobacterium sp.	2-hydroxy-3-naphthaldehyde,	and Zylstra

Mycobacterium sp.	2-hydroxy-3-naphthoic acid	1996; Kästner
Nocardia sp.	2-hydroxynaphthoic acid	*et al.* 1998;
Pseudomonas sp.	3-(2-carboxyvinyl)naphthalene-2-carboxylic acid	Hamann *et al.*
Pseudomonas aeruginosa	4-[3-hydroxy(2-naphthyl)-2-oxobut-3-enoic acid	1999; Pickard
Pseudomonas cepacia	6,7-benzocoumarin	*et al.* 1999;
Pseudomonas fluorescens	9,10-anthracenedihydrodiol	Dean-Ross *et*
Pseudomonas citronellolis	9,10-anthraquinone	*al.* 2001;
Sphingomonas sp.	9,10-dihydroxyanthracene	Mrozik *et al.*
Sphingomonas	catechol	2003; Jacques
paucimobilis	cis-1,2-dihydrodiol	*et al.* 2005)
Rhodococcus sp.	cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid	
Xanthomonas maltophila	salicylic acid	
	simple aliphatic compounds	
Fluoranthene		
Alcaligenes sp.	1-acenaphthenone	(Mueller *et al.*
Alcaligenes denitrificans	2-carboxybenzaldehyde	1990;
Cycloclasticus sp.	3-hydroxymethyl-3,4-dihydrobenzocoumarin	Sutherland *et*
Gordonia sp.	3-hydroxymethyl-4,5-benzocoumarin	*al.* 1995;
Mycobacterium sp.	4-hydroxybenzochromene-6-one-7-carboxylic acid,	Harayama
Mycobacterium	7-acenaphthenone	1997; Šepic *et*
austroafricanum	7-hydroxyacenaphthylene	*al.* 1998;
Mycobacterium flavescens	8-hydroxy-7-methoxyfluoranthene	Hamann *et al.*
Pasteurella sp.	9-carboxymethylene-fluorene-1-carboxylic acid,	1999; Samanta
Pseudomonas sp	9-fluorenone	*et al.* 2002;
Pseudomonas putida	9-fluorenone-1-carboxylic acid	Mrozik *et al.*
Rhodococcus sp.	9-hydroxy-1-fluorene-carboxylic acid	2003)
Sphingomonas	9-hydroxyfluorene	
paucimobilis	acenaphthene-7-one	
	adipic acid	
	benzene-1,2,3-tricarboxylic acid	
	benzoic acid	
	cis-1,9-dihydroxy-1-hydro-fluorene-9-one-8-carboxylic acid	
	cis-2,3-fluoranthene dihydrodiol,	
	fluorene-9-one	
	phenylacetic acid	
	phthalic acid	
Pyrene		
Coriolopsis gallica	1,2- and 4,5-dihydroxypyrene	(Heitkamp *et*
Cycloclasticus sp.	2-hydroxy-2-(phenanthren-5-one-4-enyl)acetic acid	*al.* 1988;
Gordonia sp.	4,5-dihydroxy-4,5-dihydropyrene	Sutherland *et*
Mycobacterium sp.	4,5-phenanthrenedioic acid	*al.* 1995; Sack
Mycobacterium	4-hydroxyperinaphthenone	and Fritsche
austroafricanum	4-phenanthroic acid	1996; Kästner
Mycobacterium flavescens	6-6'-dihydroxy-2,2'-biphenyl dicarboxylic acid	*et al.* 1998;
Mycobacterium gilvum	cinnamic acid	Pickard *et al.*
Pseudomonas aeruginosa	*cis*-2-hydroxy-3-(perinaphthenone-9-yl)propenic acid	1999; Samanta
Pseudomonas citronellolis	*cis*-4,5-dixydroxy-4,5-dihydropyrene	*et al.* 2002;
Pseudomonas	*cis*-4,5-dihydro-4,5-dihydroxypyrene	Bogan *et al.*
saccharophila	*cis*-4,5-pyrenedihydrodiol	2003; Mrozik
Rhodococcus sp.	4,5-pyrenedione	*et al.* 2003;
Sphingomonas	*cis*-dihydrodiol	Jacques *et al.*
paucimobilis	phenanthrene 4,5-dicarboxylic acid	2005)
Sphingomonas yanoikuyae	phenanthrene 4-carboxylic acid	
	phthalic acid	
	pyrenol	
	trans-4,5-dihydroxy-4,5-dihydropyrene	
	trans-4,5-pyrenedihydrodiol	
	trans-dihydrodiol	
Benz[a]anthracene		
Beijerinckia sp.	1- hydroxy-2-carboxyanthracene	(Gibson *et al.*
Mycobacterium sp.	or the corresponding phenanthrenes	1975;
	1-hydroxy-2-anthranoic acid	Sutherland *et*
	2-hydroxy-3-phenanthroic acid	*al.* 1995;
	3-hydroxy-2-phenanthroic acid	Mrozik *et al.*
	5,6-benz[a]anthracenedihydrodiol	2003)
	cis-1,2-benz[a]anthracenedihydrodiol	
	cis-1,2-dihydroxy-1,2-dihydrobenz[a]anthracene	

	cis-10,11-benz[a]anthracenedihydrodiol *cis*-8,9-benz[a]anthracenedihydrodiol	
Chrysene		
Gordonia sp. *Mycobacterium sp.* *Rhodococcus sp.* *Sphingomonas paucimobilis*		(Sutherland *et al.* 1995; Harayama 1997; Samanta *et al.* 2002)
Benzo[b]fluoranthene		
Sphingomonas paucimobilis		(Samanta *et al.* 2002)
Benzo[a]pyrene		
Beijerinckia sp. *Coriolopsis gallica* *Mycobacterium sp.* *Mycobacterium austroafricanum* *Pseudomonas sp.* *Pseudomonas cepacia* *Selenastrum capricomutum* *Sphingomonas paucimobilis*	4,5-chrysene-dicarboxylic acid 4,5-dihydroxy[a]pyrene 7,8-dihydro-pyrene-7-carboxylic acid 7,8-dihydro-pyrene-8-carboxylic acid *cis*-11,12-benzo[a]pyrenedihydrodiol *cis*-4-(7-hydroxypyren-8-yl)-2-oxobut-3-enoic acid *cis*-4-(hydroxypyren-7-yl)-2-oxobut-3-enoic acid *cis*-4,5-benzo[a]pyrenedihydrodiol *cis*-7,8-benzo[a]pyrenedihydrodiol *cis*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene *cis*-9,10-benzo[a]pyrenedihydrodiol *cis*-9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene	(Gibson *et al.* 1975; Sutherland *et al.* 1995; Juhasz *et al.* 1996; Harayama 1997; Pickard *et al.* 1999; Bogan *et al.* 2003; Mrozik *et al.* 2003)
Dibenz[a,h]anthracene		
Pseudomonas cepacia *Sphingomonas paucimobilis*		(Juhasz *et al.* 1996)

Table A3: List of PAHs and fungi that have been found to degrade those PAHs, as well as metabolites detected during the investigated degradation-pathways; as published in Loick *et al.* (in press); The "Organism"-column gives the PAH and lists the organisms having been found to be able to degrade this PAH; the "Detected Metabolites"-column lists the found metabolites; the "Reference"-column refers to the literature reference.

Organism	Detected Metabolites	Reference
Naphthalene		
many fungi	1-naphthol 2-naphthol 4-hydroxy-1-tetralone glucuronide sulphate conjugates *trans*-1,2-naphthalenedihydrodiol	(Sutherland *et al.* 1995)
Acenaphthene		
Coriolopsis gallica *Cunninghamella elegans*	1,2-acenaphthenedione 1,2-acenaphthenedioneol 1,5-dihydroxyacenaphthene 1-acenaphthenol 1-acenaphthenone 6-hydroxyacenaphthenone *cis*-1,2-dihydroxyacenaphthene *trans*-1,2-dihydroxyacenaphthene	(Sutherland *et al.* 1995; Pickard *et al.* 1999)
Fluorene		
Cunninghamella elegans *Laetiporus sulphureus* *Penicillium janthinellum* *Phanerochaete chrysosporium* *Pleurotus ostreatus* *Trametes versicolor*	2-hydroxy-9-fluorenone 9-fluorenol 9-fluorenone	(Sutherland *et al.* 1995; Cerniglia 1997; Boonchan *et al.* 2000)

Phenanthrene		
Aspergillus niger	1-methoxyphenanthrene	(Sutherland *et*
Coriolopsis gallica	1-phenanthrol	*al.* 1995;
Cunninghamella elegans	2,2'-diphenic acid	Cerniglia 1997;
Flammulina velutipes	2-phenanthrol	Pickard *et al.*
Laetiporus sulphureus	3-phenanthrol	1999;
Marasmiellus sp.	4-phenanthrol	Boonchan *et*
Naematoloma frowardii	9-phenanthrol	*al.* 2000)
Penicillium sp.	glucoside conjugate	
Penicillium janthinellum	phenanthrene 9,10-quinone	
Phanerochaete	*trans*-1,2-phenanthrenedihydrodiol	
chrysosporium	*trans*-3,4-phenanthrenedihydrodiol	
Phanerochaete laevis	*trans*-9,10-dihydroxy-9,10-dihydrophenanthrene	
Pleurotus ostreatus	*trans*-9,10-phenanthrenedihydrodiol	
Syncephalastrum		
racemosum		
Trametes versicolor		
Trichosporon penicillatum		
Anthracene		
Bjerkandera sp.	1-anthrol	(Field *et al.*
Bjerkandera adusta	1-anthryl sulfate	1992;
Coriolopsis gallica	9,10-anthraquinone	Sutherland *et*
Cunninghamella elegans	anthraquinone	*al.* 1995;
Naematoloma frowardii	glucuronide	Bezalel *et al.*
Penicillium sp.	phthalate	1996; Cerniglia
Phanerochaete	phthalic acid	1997; Kästner
chrysosporium	sulfate	*et al.* 1998;
Phanerochaete laevis	trans-1,2-anthracenedihydrodiol	Pickard *et al.*
Pleurotus ostreatus	xyloside conjugates	1999)
Pleurotus sajor-caju		
Ramaria sp.		
Rhizoctonia solani		
Trametes sp.		
Trametes versicolor		
Fluoranthene		
Cunninghamella elegans	8-hydroxyfluoranthene trans-2,3-dihydrodiol	(Sutherland *et*
Penicillium sp.	9-hydroxyfluoranthene trans-2,3-dihydrodiol	*al.* 1995;
Penicillium janthinellum	glucoside conjugates	Cerniglia 1997;
Laetiporus sulphureus	*trans*-2,3-fluoranthenedihydrodiol	Boonchan *et*
Naematoloma frowardii		*al.* 2000)
Trametes versicolor		
Pleurotus ostreatus		
Pyrene		
Aspergillus niger	1,6-dihydroxypyrene	(Lambert *et al.*
Agrocybe aegerita	1,6-pyrenequinone	1994; Lange *et*
Candida parapsilopsis	1,6-quinone	*al.* 1994;
Coriolopsis gallica	1,8-dihydroxypyrene	Sutherland *et*
Crinipellis maxima	1,8-pyrenequinone	*al.* 1995;
Crinipellis perniciosa	1,8-quinone	Bezalel *et al.*
Crinipellis stipitaria	1-hydroxy-8-pyrenyl sulfate	1996; Cerniglia
Crinipellis zonata	1-hydroxypyrene	1997; Pickard
Cunninghamella elegans	1-pyrenol	*et al.* 1999;
Fusarium oxysporum	1-pyrenylsulfate	Boonchan *et*
Kuehneromyces mutabilis	6-hydroxy-1-pyrenyl sulfate	*al.* 2000)
Marasmiellus ramealis	glucoside conjugates	
Marasmius rotula	*trans*-4,5-dihydro-4,5-dihydroxypyrene	
Mucor sp.	*trans*-4,5-pyrenedihydrodiol	
Naematoloma frowardii		
Penicilium sp.		
Penicillium janczewskii		
Penicillium janthinellum		
Phanerochaete		
chrysosporium		
Pleurotus ostreatus		
Syncephalastrum		
racemosum		
Trichoderma harzianum		

Benz[a]anthracene		
Candida krusei	glucuronide conjugates	(Sutherland *et al.* 1995; Cerniglia 1997; Boonchan *et al.* 2000)
Cunninghamella elegans	phenolic derivates	
Penicillium janthinellum	sulfate conjugates	
Phanerochaete chrysosporium	tetrahydroxy derivates	
	trans-10,11-benz[a]anthracenedihydrodiol	
Phanerochaete laevis	*trans*-3,4-benz[a]anthracenedihydrodiol	
Pleurotus ostreatus	*trans*-8,9-benz[a]anthracenedihydrodiol	
Rhodotorula minuta		
Syncephalastrum racemosum		
Trametes versicolor		
Chrysene		
Cunninghamella elegans	2-chrysenyl sulfate	(Cerniglia 1997)
Penicillium janthinellum	2-hydroxy-8-chrysenylsulfate	
Syncephalastrum racemosum	*trans*-1,2-chrysenedihydrodiol	
Benzo[a]pyrene		
Aspergillus ochraceus	1,6-quinone	(Haemmerli *et al.* 1986; Sanglard *et al.* 1986; Field *et al.* 1992; Sutherland *et al.* 1995; Cerniglia 1997; Grotenhuis *et al.* 1999; Boonchan *et al.* 2000)
Bjerkandera sp	3,6-quinone	
Bjerkandera adusta	3-hydroxybenzo[a]pyrene	
Candida maltosa	6,12-quinone	
Candida tropicalis	6-hydroxybenzo[a]pyrene	
Chrysosporium pannorum	7,8-dihydrodiol-9-10-epoxide	
Coriolopsis gallica	7β,8α,9α,10β-tetrahydrobenzo[a]pyrene	
Cunninghamella elegans	7β,8α,9α,10β-tetrahydroxy-7,8,9,10tetrahydrobenzo[a]pyrene	
Mortierella verrucosa	9-hydroxybenzo[a]pyrene	
Naematoloma frowardii	benzo[a]pyrene (+)-7,8-diol-9,10-epoxide-2 (highly carcinogenic – with C.elegans trace amounts detected (Sutherland *et al.* 1995))	
Neurospora crassa	glucuronide	
Penicillium janczewskii	sulfate conjugates	
Penicillium janthinellum	*trans*-4,5-benzo[a]pyrenedihydrodiol	
Phanerochaete chrysosporium	*trans*-7,8-benzo[a]pyrenedihydrodiol	
Phanerochaete laevis	*trans*-9,10-benzo[a]pyrenedihydrodiol	
Pleurotus ostreatus	various conjugates	
Ramaria sp.		
Saccharomyces cerevisiae		
Syncephalastrum racemosum		
Trametes sp.		
Trametes versicolor		
Trichoderma viride		
Benzo[e]pyrene		
Cunninghamella elegans	10-hydroxy-3-benzo[e]pyrenyl sulfate	(Cerniglia 1997)
	3-benzo[e]pyrenyl sulfate	
	benzo[e]pyrene-3-0-β-glucopyranoside	

"Joint SAFS/SBS PhD Winter Conference 13/12/2005"

University of Wales, Bangor, Wales

Poster presentation

Title: Degradation of Poly-Aromatic-Hydrocarbons by Composting

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Abstract

Persistent organic chemicals and in particular poly aromatic hydrocarbons (PAHs) are of environmental concern, especially when it comes to remediation of polluted soils. Bioremediation by biodegradation has been shown to be most effective at degrading PAHs. With this method PAHs are mineralised by micro-organisms and not "locked up". Although "locking up" might reduce the overall risk, it leaves the long-term fate of those contaminants uncertain. To achieve good mineralization rates of PAHs by micro-organisms composting conditions must be optimal. Micro-organisms may use PAHs directly as carbon sources or degrade PAHs co-metabolically or by unspecified oxidative metabolism. In this study aerobic thermophilic composting processes are being investigated and established. These incorporate specific inocula and novel accelerants to optimise PAH degradation. The processes are being investigated using small-scale compost vessels and characterised using chemical, physical and microbiological methods. Nutrient concentrations and physical conditions like the temperature profile are being manipulated and the impact on the microbial community successions and PAH degradation rates are being characterised and determined.

Interdependencies of the microbial communities, physical and chemical conditions, and PAH degradation rates will be revealed by developing a model which can then predict the development and succession of a PAH degrading process by composting. This will make it possible to find optimal conditions for a "PAH-degradation composting process" under certain given conditions like PAH concentrations.

Degradation of Poly-Aromatic Hydrocarbons by Composting

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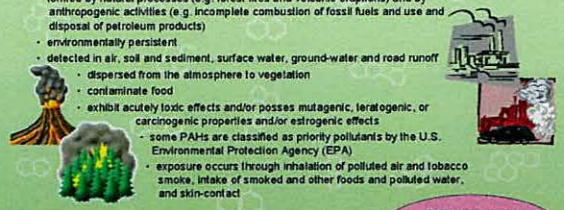
PAHs - sources and characteristics

- organic chemicals consisting of multiple interconnected 6-carbon (benzene) rings
- formed by natural processes (e.g. forest fires and volcanic eruptions) and by anthropogenic activities (e.g. incomplete combustion of fossil fuels and use and disposal of petroleum products)
- environmentally persistent
- detected in air, soil and sediment, surface water, ground-water and road runoff
  - dispersed from the atmosphere to vegetation
  - contaminate food
  - exhibit acutely toxic effects and/or posses mutagenic, teratogenic, or carcinogenic properties and/or estrogenic effects
    - some PAHs are classified as priority pollutants by the U.S. Environmental Protection Agency (EPA)
    - exposure occurs through inhalation of polluted air and tobacco smoke, intake of smoked and other foods and polluted water, and skin-contact



PAHs are major environmental pollutants and are distributed worldwide!

Introduction

Environmental pollution and contaminations due to polycyclic aromatic hydrocarbons (PAHs) in particular are of environmental concern, especially when it comes to remediation of polluted areas. Several studies have investigated composting as a bioremediation process for hydrocarbon contaminated soil. Most investigations have focused on operational considerations rather than on biological mechanisms that underpin bioremediation and composting technologies. Most effective bioremediation and biodegradation of PAHs is achieved when PAHs are mineralised by micro-organisms and not "locked-up" through sorption onto organic matter where the PAHs will not be available for degradation. Even though "locking-up" PAHs reduces the overall risk, the long-term fate of the "locked up" organic contaminants remains uncertain. To attain a good mineralization rate composting conditions must be optimal for PAH-degrading micro-organisms. Such organisms are either able to grow on PAHs using them as their carbon source or degrade PAHs co-metabolically or by unspecified oxidation metabolism.

In this study, aerobic thermophilic composting processes including the use of inocula and novel accelerants to degrade persistent materials such as hydrocarbons are investigated and established. By setting small scale compost experiments imitating parts of a natural composting process the processes will be characterised using chemical, physical, and microbiological methods. Doing this, the microbial community successions and their dependence on the feedstock material will be investigated concluding in an attempt to develop a model to predict microbial community developments, and degradation rates and estimate optimal conditions for best degradation rates depending on the given surrounding conditions.

Composting - characteristics and potential

Composting is a process by which organic materials are biodegraded by micro-organisms, resulting in the production of organic and/or inorganic by-products and energy in the form of heat.

- a typical composting process consists of four phases:
  - mesophilic - thermophilic - cooling down – maturation
- involve wide diversity of organisms, both microbial and invertebrate
- mainly relies on micro-organisms degrading and transforming the feedstock
- Various micro-organisms in the compost utilize the available carbon sources in different ways and therefore have the potential to biodegrade organic contaminants as well
  - contaminants can be degraded directly by using them as their carbon source or indirectly by co-metabolic processes using exoenzymes or unspecified oxidation metabolism

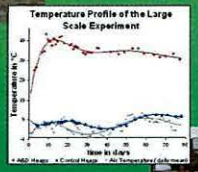
Because of the micro-organisms, composting processes have the potential to degrade persistent materials!

Large Scale Experiment

Set by A&D Biotech to validate their new accelerant

- two heaps consisting of hydrocarbon contaminated soil, sawdust, chicken manure, and accelerant
- two 'control' heaps consisting of hydrocarbon contaminated soil and mature greenwaste-compost

The composting processes taking place were characterised and compared using physical, chemical and biological methods.

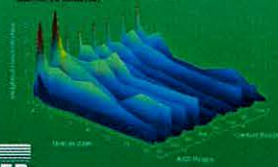


Results

The experimental setup was not optimal, even so:

- good insight in the temperature profile of the composting process (see graph)
- higher hydrocarbon degradation rate for the 'compost + accelerant' heaps (data not shown)
- surrounding conditions [e.g. the ambient air temperature] have a great influence on the composting process.
- results will be used for establishing the conditions for the small scale experiments.

3D plot of PAH Concentrations throughout the Large Scale Experiment for all sample points, normalised to Anthracene Concentration



The outside conditions have a great effect on the composting process!

Small Scale Experiments

As it was shown by the large scale experiment, the composting process was influenced by several factors and to investigate the influences of each factor more controlled conditions are needed.

The small scale experiments will be carried out in aerated 5 litre vessels placed in a water-bath for a controlled outside temperature.

Controlled conditions

- temperature
- moisture content
- aeration
- mixing rate
- particle size

+

Different treatment methods

- temperature: set at different temperatures and changed over time
- nutrients: cattle-, sheep-, chicken-manure; fertilizer; accelerants
- organisms: inocula (e.g. fungus *Pleurotus ostreatus*)

Physical Analysis
- Temperature
- Aeration

Chemical Analysis
- Nutrients (N, C, P)
- pH
- Redox
- Conductivity

Biological Analysis
- PLFA
- PCR-DGGE
- Sequencing
- Culturing

Results

Combining the results of the different methods will

- give holistic view of the composting process
- show how different factors are interrelated
- which factors influence microbial communities are
- give an idea on the optimal conditions for hydrocarbon degradation

Combining and relating different data will give a holistic view of the degradation process!

Conclusions

By relating the chemical and physical conditions in a composting process to the changes in the microbial communities and the hydrocarbon degradation rate the optimal conditions for the organisms present can be defined and the organisms /communities responsible for the degradation of hydrocarbons can be identified.

Identifying organisms/communities that can be used to degrade specified material and knowing the optimal conditions will give the opportunity to develop models that can predict the development of microbial communities for the composting of a specific waste type, give advice on how to set conditions to effectively compost different materials and which materials to add or organisms to use when inoculating material like contaminated sites.

IGER

BANGOR

"1st Network Conference on Persistent Organic Pollutants: Human Exposure and Impacts 29-30/03/2006"

University of Birmingham, Birmingham, England

Poster presentation

Title: Volatilisation of PAHs using different treatments with manure

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Abstract

Persistent organic chemicals and in particular poly-aromatic hydrocarbons (PAHs) are of environmental concern, especially when it comes to remediation of polluted soils. Bioremediation has been shown to be most effective at degrading PAHs. With this approach PAHs are mineralised by micro-organisms and not "locked up in the soil". Locking-up may reduce the overall risk but leaves the long-term fate of those contaminants uncertain. To achieve good mineralization rates of PAHs composting conditions must be optimal for the micro-organisms that use PAHs as a carbon source or degrade PAHs co-metabolically or by unspecified oxidative metabolism. In this study the use of different fungi under different physical and chemical conditions is investigated using small-scale compost vessels to assess PAH degradation. Species included are the basidiomycetes *Pleurotus ostreatus* and *Trametes versicolor*. The processes are characterised by measuring chemical and physical conditions and changes and by microbiological analysis. Nutrient concentrations and sources like chicken manure, cattle manure, and fertilizer, and physical conditions like the temperature profile, are manipulated and the impact on the microbial community successions and PAH degradation rates are characterised and determined using phosphor lipid fatty acid analysis (PLFA). Interdependencies of the microbial communities, physical and chemical conditions, and PAH degradation rates reveal the timescale in which the degradation rate of PAHs is highest and principal component analysis shows microbial groups associated with the given conditions and PAH degradation rates.

Volatilisation of PAHs using different treatments with manure

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Introduction

Persistent organic chemicals and in particular poly-aromatic hydrocarbons (PAHs) are of environmental concern, especially when it comes to remediation of polluted soils. Bioremediation has been shown to be most effective at degrading PAHs. With this approach PAHs are mineralised by micro-organisms and not bound to the soil. Binding these compounds to the soil may reduce the overall risk but leaves the long-term fate of those contaminants uncertain. To achieve good mineralization rates of PAHs composting conditions must be optimal for the micro-organisms that use PAHs as a carbon source or degrade PAHs co-metabolically or by unspecified oxidative metabolism.

In this study the use of additives under different physical and chemical conditions is investigated using small-scale composting vessels to assess PAH degradation.

During the composting process PAHs volatilise. This poster presents the results of PAH volatilisation that occurred during the treatment of PAH-contaminated soil with manure.

Methods

Three replicates for three treatments and a control were monitored in 12 x 5 litre glass vessels placed in a water-bath (fig. 1). Temperatures were increased from 20°C up to 65°C on a weekly basis and decreased to 35°C for the final week (fig. 7). By heating the vessel from the outside a natural composting process was reproduced and the temperature effect on PAH volatilisation and the succession of microbial communities are investigated. To sustain aerobic conditions the compost mixtures were aerated at 500ml*min-1. In order to maintain the moisture content of the compost the air was moisturised by bubbling through water of the same temperature as the water-bath. To ensure a homogeneous mixture and homogeneous sampling the compost was thoroughly mixed each time a sample was taken.

Gas samples of 500ml were taken from the headspace of the composting vessels and PAHs were adsorbed onto appropriate thermal-desorption-tubes. The PAHs are thermally desorbed and cryogenically trapped before subsequent identification and quantification by gas chromatography mass spectrometry (GC-MS).

Results and Discussion

The treatments with cattle and horse manure showed very similar volatilisation rates with minor differences to those from the control treatment. Volatilisation rates were highest with the chicken manure treatment (fig. 2).

The 2-ring PAH Naphthalene showed highest volatilisation rates at 50°C for the horse- and cattle manure treatment and at 35°C for the chicken manure and at 65°C for the control treatment. The possible influence of a higher microbial load and a higher nitrogen (ammonium) content on the higher volatilisation rates in chicken manure (fig. 3) will be investigated.

The 3-ring PAHs (Acenaphthalene, Acenaphthene, Fluorene, Phenanthrene, and Anthracene) showed the highest volatilisation rates at a temperature of 50°C with highest rates for the chicken manure treatment (figs. 3 and 4).

The lower molecular weight 4-ring PAHs (Fluoranthene and Pyrene; MW = 202) showed highest volatilisation rates at 50°C with the control and chicken manure treatment showing the highest rates (fig. 5).

The high molecular weight 4-ring PAHs (Benz(a)anthracene, and Chrysene; MW = 228) showed highest volatilisation rates at a temperature of 65°C. Again, for these compounds the rates were higher for the chicken manure treatment (fig. 6).

Microbial activity was investigated by measuring the CO_2 concentrations in the compost headspace. Highest concentrations were measured at 50°C (day 18) indicating a higher microbial activity at this temperature (fig. 7).

Conclusions

Across treatments the volatilisation of naphthalene relative to its abundance in the soil was generally higher than for the heavier PAH compounds. This agrees with other studies where it was found that volatilisation was likely to be important for naphthalene but not for higher ringed compounds (Park *et al.* 1990; Wild *et al.* 1990).

As expected, the results of this experiment show that volatilisation rates were higher for compounds with a lower molecular weight (i.e. in order of decreasing observed volatilisation rate: Naphthalene - Acenaphthene - Acenaphthalene – Fluorene - Phenanthrene – Anthracene - Pyrene - Fluoranthene - Chrysene - Benz(a)anthracene). Volatilisation rates increase until day 18 (50°C) for all compounds except naphthalene in chicken manure treatment (maximum at 35°C). From day 18 (50°C) volatilisation rates decrease (or stay unchanged) for all compounds except the heavy weight PAHs Chrysene and Benz(a)anthracene, volatilisation rates continued to increase and reached their highest levels on day 25 at 65°C.

Future Work

Ongoing and future work will assess PAH degradation under different composting conditions and with fungal additives such as the basidiomycetes. Microbial community succession will be investigated using phospholipid fatty acid analysis (PLFA) and polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE). Interdependencies of the microbial communities, physical and chemical conditions, PAH volatilisation- and degradation rates will reveal the fate of the PAHs.

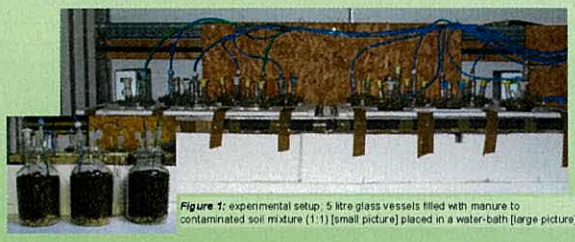

Figure 1: experimental setup: 5 litre glass vessels filled with manure to contaminated soil mixture (1:1) [small picture] placed in a water-bath [large picture]

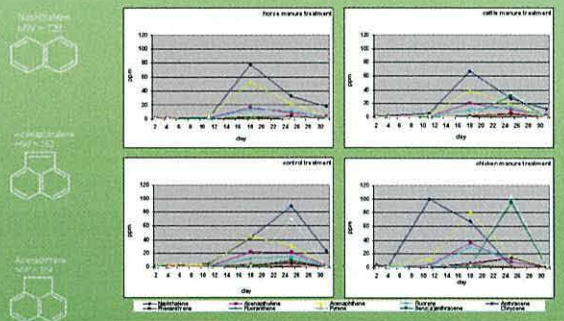

Figure 2: PAH emission rate (relative to concentrations in the compost mixture) for the different treatments

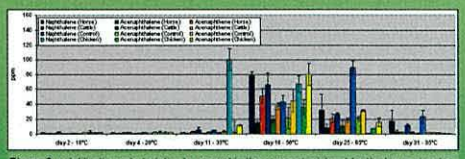

Figure 3: volatilisation rate (relative to amount in the compost-mixture) for the low molecular weight 2-3 ring PAHs Naphthalene, Acenaphthalene, and Acenaphthene for the different treatments

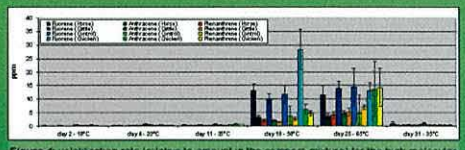

Figure 4: volatilisation rate (relative to amount in the compost-mixture) for the high molecular weight 3 ring PAHs Fluorene, Anthracene, and Phenanthrene for the different treatments

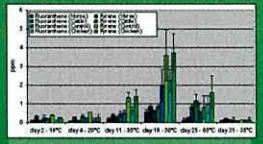

Figure 5: volatilisation rate (relative to amount in the compost-mixture) for the lower molecular weight 4 ring PAHs Fluoranthene and Pyrene for the different treatments

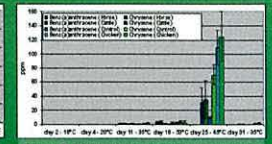

Figure 6: volatilisation rate (relative to amount in the compost-mixture) for the high molecular weight 4 ring PAHs Benz(a)anthracene and Chrysene for the different treatments

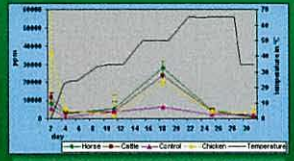

Figure 7: CO_2 emission rate for the different treatments. On the second axis the temperature development over the experimental period is shown

References:

Park, K.S., Sims, R.C., Dupont, R.R., Doucette, W.J., and Matthews, J.E. (1990) Fate of PAH compounds in two soil types: Influence of volatilization, abiotic loss and biological activity. *Environmental Toxicology and Chemistry* **9**: 187-195

Wild, S.R., Waterhouse, K.S., McGrath, S.P., and Jones, K.C. (1990) Organic contaminants in an agricultural soil with a known history of sewage sludge amendments: polynuclear aromatic hydrocarbons. *Environ. Sci. Technol.* **24**: 1706-1711

"12th Ramiran International Conference 11-13/09/2006"

Aahus, Denmark

Poster presentation

Published in: DIAS Report no. 123, August 2006, 12th Ramiran International ConferenceTechnology for Recycling of Manureand Organic Residues in a Whole Farm Perspective. Vol. 2, p. 137-139.

Optimising degradation of PAHs in soils using different composting approaches

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[2]*Institute of Grassland and Environmental Research, North Wyke Research Station, Okehampton, EX20 2AB, UK;*

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Introduction and aim of study

The aim of this study was to investigate and optimise the usage of manure to bio-remediate hydrocarbon contaminated soil. A small scale composting experiment was carried out and the effect of different types of manure on the degradation of hydrocarbons in oil-contaminated soil were analysed.

Persistent organic chemicals and in particular poly-aromatic hydrocarbons (PAHs) are of environmental concern, especially when remediating polluted soils e.g. oil spills. Bioremediation by biodegradation has been shown to be most effective at degrading PAHs. Composting is a bioremediation method whereby PAHs are mineralised by micro-organisms and not just immobilised. Although immobilisation might reduce the overall risk of dispersing PAHs, it leaves the long-term fate of PAH contaminants uncertain.

To achieve good PAH mineralisation rates by micro-organisms, composting conditions such as pH, temperature and especially the nutrient content and availability of the substrate must be optimal. Manure is readily available and rich in nutrients as well as micro-organisms. In this study different aerobic thermophilic composting processes were investigated by adding different manure types to optimise PAH degradation.

Methods

To investigate the decay process, small-scale compost vessels were filled with a mixture of contaminated soil, sawdust, and a manure type as shown in Table 1.

To imitate the natural composting process and to estimate the optimum conditions for PAH degradation the temperature profile was simulated to reflect that naturally

occurring during composting. The temperature was gradually increased from room temperature (19°C) to 25°C (day 4), 35°C (day 11), 50°C (day 18), 65°C (day 25), and decreased again to 35°C (day 32). To maintain aerobic conditions, air at the desired temperature was blown through the bottom of each vessel. The air was pre-humidified to prevent the compost-mixture drying out.

Table 1. Description of the different treatments used in the PAH bioremediation trial.

Treatment	Description
A	Horse manure mixed with contaminated soil and sawdust
B	Cattle manure mixed with contaminated soil and sawdust
C	Chicken manure mixed with contaminated soil and sawdust
D	Control treatment: Contaminated soil mixed with sawdust

The composting process was characterised using a range of chemical, physical and microbiological methods (e.g. pH, redox-potential, electrical conductivity, and CO_2 and NH_3 emissions). The impact of time and treatment on microbial community structure was also examined using PLFA (phospholipid fatty acid) analysis. PAH degradation rates were determined using GC-MS (gas chromatography-mass spectrometry). To assess the metabolic decomposition rate and any loss of PAHs through volatilisation, gaseous emissions from the headspace of the composting vessels were analysed using GC-MS.

Results and Discussion

The results showed significant differences in compost quality, PAH degradation and microbial community development between the various treatments and with the duration of composting. Those differences and their significance were confirmed statistically by principal component analysis (PCA). Extractable PAH concentrations decreased for all of the four treatments. In agreement with previous studies (Antizar-Ladislao *et al.* 2005), our results showed that greatest PAH degradation rates generally occurred when the temperature was *ca.* 35°C (both at the start and end of the composting process). Overall, there was a 50% decrease for single PAHs at each of these start and end stages.

The bacterial community structure, as observed by PLFA analysis, showed the largest changes from day 4 to 18 for the horse manure treatment (A), from day 11 to 25 for the cattle manure treatment (B), and from day 4 to 11 for the chicken manure treatment (C). The control treatment (D) showed no significant changes over the whole process.

The volatilisation rates of PAHs were highest with the chicken manure treatment, whereas the treatments with cattle and horse manure showed very similar volatilisation rates with only minor differences to those observed in the control treatment. In this study, the volatilisation of naphthalene relative to its abundance in the contaminated soil was generally higher than for the heavier PAH compounds across all the treatments. This was in agreement with other studies, where it was found that volatilisation was likely to be important for naphthalene but not for higher ringed compounds (Park *et al.* 1990; Wild *et al.* 1990).

Conclusions

By excluding the influence of climatic factors and conducting the experiments under controlled monitoring conditions this study has revealed the fate of the PAHs during the co-composting of animal wastes and contaminated soil. It has also demonstrated the importance of microbial community structure on the mineralisation of PAHs. Overall, PAH concentrations were decreased by up to 50% within all composting treatments compared to a decrease of around 20% within the control treatment. Initial results, with reductions of up to 65% for naphthalene, indicate that the chicken manure was the most successful amendment. Principal component analysis revealed interdependencies of the microbial communities and the effects of physical and chemical conditions on PAH degradation. In conclusion, manure is effective for the bio-remediation of hydrocarbon contaminated soil by co-composting.

References

Antizar-Ladislao, B., Lopez-Real, J., and Beck, A.J. (2005) Laboratory studies of the remediation of polycyclic aromatic hydrocarbon contaminated soil by in-vessel composting. Waste Management 25: 281-289.

Park, K.S., Sims, R.C., Dupont, R.R., Doucette, W.J., and Matthews, J.E. (1990) Fate of PAH compounds in two soil types. Influence of volatilization, abiotic loss and biological activity. Environmental Toxicology and Chemistry 9: 187-195.

Wild, S.R., Waterhouse, K.S., McGrath, S.P., and Jones, K.C. (1990) Organic contaminants in an agricultural soil with a known history of sewage sludge amendments: polynuclear aromatic hydrocarbons. Environmental Science and Technology 24: 1706-1711.

Optimising degradation of PAHs in soils using different composting approaches

The suitability of manure to biodegrade contaminated soil

Nadine Loick[1]; Phil J. Hobbs[2]; Mike D. Hale[1]; Davey L. Jones[1]

[1] School of the Environment and Natural Resources, University of Wales, Bangor;
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contact: nadine.loick@bbsrc.ac.uk

Background

The aim of this study was to investigate and optimise the usage of manure to bio-remediate hydrocarbon contaminated soil.
Being persistent organic chemicals poly-aromatic hydrocarbons (PAHs) are of environmental concern, especially when remediating polluted soils e.g. oil spills. Bioremediation by biodegradation has been shown to be most effective at degrading PAHs. Composting is a bioremediation method whereby PAHs are mineralised by micro-organisms. To achieve good PAH mineralisation rates by micro-organisms, composting conditions such as pH, temperature and especially the nutrient content and availability of the substrate must be optimal. Manure is readily available and rich in nutrients as well as micro-organisms.

In this study different aerobic thermophilic composting processes were investigated by adding different manure types to weathered PAH contaminated soil to investigate their effects on and optimise PAH degradation.

Methodology

To investigate the decay process, small-scale compost vessels were filled with a mixture of contaminated soil, sawdust, and a manure type. The experiment was carried out in 5 litre glass vessel placed in a water-bath (fig. 1). Temperatures were changed weekly reproducing a natural composting process. To sustain aerobic conditions the compost mixtures were aerated at 500ml*min-1 with moisturised air and the compost was thoroughly mixed each time before a sample was taken.

The temperature effect on PAH degradation and volatilisation and the succession of microbial communities are investigated by extraction of PAHs from samples, analysing gas samples from the headspace and extracting phospholipid fatty acids (PLFAs).

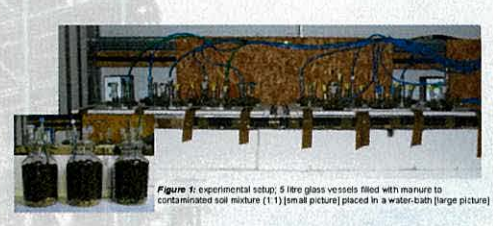

Figure 1: experimental setup; 5 litre glass vessels filled with manure to contaminated soil mixture (1:1) [small picture] placed in a water-bath [large picture]

Results

PAH amounts in soil (fig. 2):

- generally decrease over the course of the experiment, even though for single PAHs increases were detected until day 25.
- chicken manure showed with 23% the highest degradation of total extractable PAHs, followed by the horse manure treatment (21%) and the control treatment (19%).
- the cattle manure treatment showed an increase in total extractable PAHs of 5%.

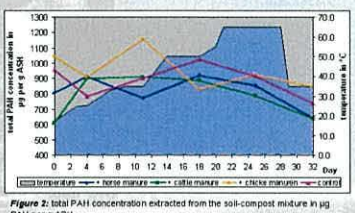

Figure 2: total PAH concentration extracted from the soil-compost mixture in µg PAH per g ASH

Gaseous emissions of PAHs (fig 3):

- highest emission rates at days 18 and 25 where temperatures were increased to 50 and 65°C.
- total PAHs of less than 60ppm of the amount in the soil at the sampling point were evaporated
- chicken manure treatment showed highest emission rates for nearly all measured PAHs, followed by the control treatment.
- horse- and cattle manure treatment show similar evaporation rates with the ones for the horse manure being generally lower than the cattle manure.

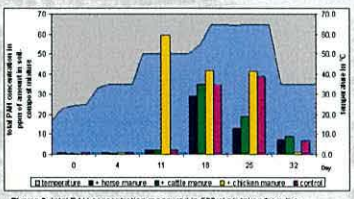

Figure 3: total PAH concentration measured in 500ml air taken from the headspace of the composting vessels. The measured amounts were related to the amount of extractable PAHs in the respective vessel.

Phospholipid fatty acids (PLFA) extractions (fig 4):

- highest amounts of PLFAs were measured for the horse- and chicken manure treatment.
- for the manure treatments PLFA amounts decreased over the course of the experiment, whereas with the control treatment the total PLFA content is increasing by a factor of 1.7
- the community structure for the manure treatments generally shows a decrease of gram⁻-bacteria and fungi
- gram⁺-bacteria stay at about the same percentage over the whole experiment for the control, cattle- and chicken manure treatment, but increase for the horse manure treatment
- for the manure treatments PLFAs indicative for Actinomycetes, which are mainly thermophilic bacteria, show an increase over the whole experiment with the main increase from day 18 to 25, when temperatures were increased from 50 to 65°C.
- in contrast to the manure treatments the control treatment did not show changes until day 18 when gram⁺-bacteria, gram⁻-bacteria, and Actinomycetes indicating PLFAs decrease and fungal PLFAs increase.

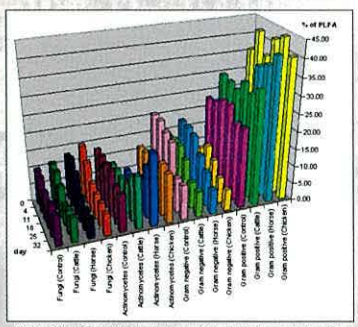

Figure 4: Microbial community changes as measured by PLFA extractions. PLFAs are grouped into the four groups (Fungi, Actinomycetes, gram⁻, and gram⁺-bacteria) and expressed as their percentage of all measured PLFAs.

Conclusions

By excluding the influence of climatic factors and conducting the experiments under controlled monitoring conditions this study has revealed the fate of the PAHs during the co-composting of animal wastes and contaminated soil. It has also given a first insight into the microbial community structure which development over the whole composting process and its importance on the mineralisation of PAHs will be investigated further. Considering the amounts of extractable PAHs and the evaporation of PAHs initial results indicate that the horse- and chicken manure were the most successful amendments. Statistical analysis showed significant differences between the different treatments. In conclusion, manure is effective for the bio-remediation of hydrocarbon contaminated soil by co-composting.

The funding of this PhD by the European Social Fund is gratefully acknowledged

"SENR/SBS PhD Conference 18/06/2007"

University of Wales, Bangor, Wales

Oral presentation

Composting hydrocarbon contaminated soil: Bioremediation of poly-aromatic hydrocarbon (PAH) contaminated soil by composting

N. Loick[1], M.D.C. Hale[1], P.J. Hobbs[2], D.L. Jones[1]

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Abstract

Persistent organic chemicals and in particular poly aromatic hydrocarbons (PAHs) are of environmental concern, especially when it comes to remediation of polluted soils. PAH contaminated sites are still a big problem world wide as such point sources represent the most significant releases of PAHs into the environment. The aim of this study is to investigate and optimise the usage of manure and fungal inocula to bio-remediate hydrocarbon contaminated soil using a composting process. The composting process mainly relies on micro-organisms degrading and transforming the feedstock. With most microbial inocula the problem occurs that the micro-organisms are not able to survive under natural conditions, as they face resistance to colonisation effects and a niche specificity which is not optimal and might make them vulnerable to predators. In this study manure was used as a source of micro-organisms (mainly bacteria) in their natural habitat decreasing the risk of competition and vulnerability towards predators and increasing the viability of those organisms. Bacteria and fungi possess different PAH-degradation pathways and abilities. Therefore two different white rot fungi applied as inoculated sawdust have also been investigated. The experiment was performed in small-scale compost vessel. Results show greater decomposition of specific PAHs in the chicken manure over cattle- and horse manure and the fungus *Pleurotus ostreatus* shows a greater decomposition of PAHs over *Trametes versicolor* or a mix of both fungal inocula. A further experiment is investigating the effects of a combined application of both types of additives at different stages of the composting process.

"ISPAC – 21st International Symposium for Polycyclic Aromatic Compounds 05-10/08/2007"

Trondheim, Norway

Oral presentation

Bioremediation of poly-aromatic hydrocarbon (PAH) contaminated soil by composting

Nadine Loick[a], Mike D. Hale[a], Davey L. Jones[a] and Phil J. Hobbs[b]

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Abstract

PAH contaminated sites are still a big problem world wide as such point sources represent the most significant releases of PAHs into the envoironment. The aim of this study was to investigate and optimise the usage of manure and fungal inocula to bio-remediate hydrocarbon contaminated soil using a composting process. The composting process mainly relies on micro-organisms degrading and transforming the feedstock. During co-composting of PAH contaminated soil the contaminant can be degraded directly by being used as carbon source, or indirectly through unspecified oxidation metabolism or co-metabolic processes using exo-enzymes. Most microbial inocula bear the problem that the micro-organisms are not able to survive under natural conditions, as they face resistance to colonisation effects and a niche specificity which is not optimal and might make them vulnerable to predators. In this study manure was used as a source of micro-organisms (mainly bacteria) in their natural habitat. This decreases the risk of competition and vulnerability towards predators and therefore increases the viability of those organisms. Bacteria and fungi possess different PAH-degradation pathways and abilities. Therefore two different white rot fungi applied as inoculated sawdust have been investigated. The experiment was performed in small-scale compost vessel placed in a water-bath simulating a temperature profile as would appear during a large scale composting process. The compost mixture was aerated with moisturised air to maintain aerobic conditions and prevent the mixture from drying out. The composting process was characterised using a range of chemical, physical and microbiological methods (e.g. pH, electrical conductivity, and CO_2 and NH_3 emissions). The impact of time and treatment on the microbial community structure was examined using PLFA (phospholipid fatty acid) analysis. PAH degradation rates were determined using GC-MS (gas chromatography-mass spectrometry). To assess the metabolic decomposition rate and any loss of PAHs through volatilisation, gaseous emissions from the headspace of the composting vessels were analysed using GC-MS.

"CSI - Young Researcher's Meeting: Contaminants in the Environment 24/09/2007"

York, England

Poster presentation

Bioremediation of poly-aromatic hydrocarbon (PAH) contaminated soil using manure

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Abstract

PAH contaminated sites are still a big problem world wide and point sources like this represent the most significant releases of PAHs into the environment. This study investigates the usage of manure to bio-remediate hydrocarbon contaminated soil using a composting process. The composting process mainly relies on micro-organisms degrading and transforming the feedstock. During co-composting of PAH contaminated soil the contaminants can be degraded directly by being used as carbon source, or indirectly through unspecified oxidation metabolism or co-metabolic processes using exo-enzymes. Most microbial inocula have the problem that the micro-organisms are not able to survive under natural conditions, facing resistance to colonisation effects and a niche specificity which is not optimal and might make them vulnerable to predators. In this study manure was used as a source of micro-organisms in their natural habitat. This decreases the risk of competition and vulnerability towards predators and therefore increases the viability of those organisms. The experiment was performed in small-scale compost vessel placed in a water-bath set at temperatures as would appear during a large scale composting process. The compost mixture was aerated with moisturised air to maintain aerobic conditions and prevent the mixture from drying out. The process was characterised using a range of chemical and physical methods and the impact of temperature/time and treatment on the microbial community structure was examined using PLFA (phospholipid fatty acid) analysis. PAH degradation rates as well as any loss of PAHs through volatilisation were determined using gas chromatography-mass spectrometry. Results show a slight advantage of the chicken manure treatment over cattle- and horse manure with biggest differences between the treatments in the early stages of the experiment. Over the course of the experiment results indicate that the different treatments become more alike towards the end of the experiment.

Bioremediation of poly aromatic hydrocarbon (PAH) contaminated soil using manure

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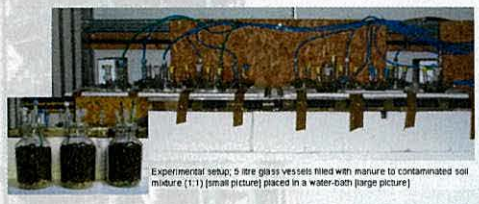
Background

PAHs are persistent organic chemicals consisting of multiple interconnected 6-carbon rings. Those compounds are formed by natural processes such as forest fires and volcanic eruptions, and anthropogenic activities such as the incomplete combustion of fossil fuels and use and disposal of petroleum products. Exhibiting acutely toxic effects and/or possessing mutagenic, teratogenic, or carcinogenic properties some are classified as priority pollutants by the USEPA. PAH contaminated sites are still a big problem world wide and such point sources represent the most significant releases of PAHs into the environment. This study investigated the usage of different manure-types to bio-remediate hydrocarbon contaminated soil using a composting process. The composting process mainly relies on micro-organisms degrading and transforming the feedstock. Most microbial inocula however have the problem that the micro-organisms are not able to survive under natural conditions. In this study manure was used as a source of micro-organisms in their natural habitat, which decreases the risk of competition and vulnerability towards predators and therefore increases the viability of those introduced organisms. At the same time the manure supplies nutrients for the indigenous micro-organisms, enhancing their growth conditions.

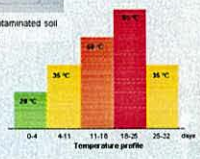
Methodology & Results

Soil (silty clay, pH 8.17) from a former refinery site had been piled in windrows and turned regularly for 2 months. The still heavily contaminated (1800 - 2000 mg kg^{-1}) soil was then used for this experiment.

- 4 x 3 glass vessel filled with soil/manure mixture (v:v)
  - **Horse manure + Soil + Sawdust (2:1:1)**
  - **Cattle manure + Soil + Sawdust (2:1:1)**
  - **Chicken manure + Soil + Sawdust (2:1:1)**
  - **Soil + Sawdust (1:1)**
- forcefully aerated at 200 ml*min^{-1} with moisturised air
- placed in a water-bath whose temperature was changed weekly
- sampling after each temperature step and thorough homogenisation of the mixture



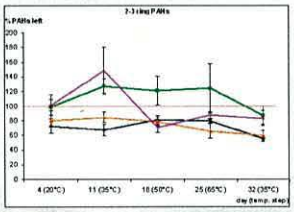
Experimental setup; 5 litre glass vessels filled with manure to contaminated soil mixture (1:1) [small picture] placed in a water-bath [large picture]

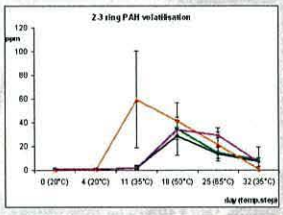


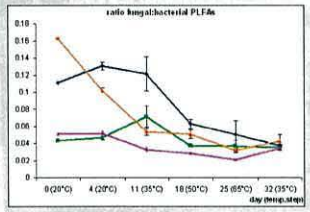
PAH concentration in the soil
ISO/DIS 18287 method
[amounts related to ash content]

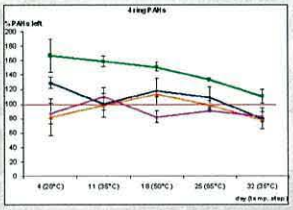
PAH volatilisation
thermo-desorption tubes
[500ml headspace-air]

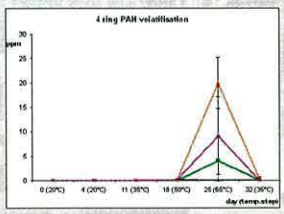
microbial community profile
phospholipid-fatty-acid (PLFA) extraction

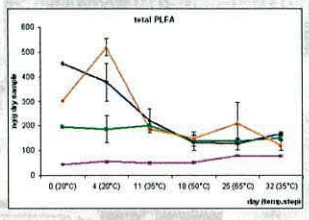


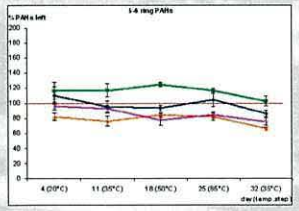


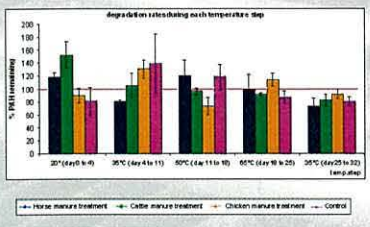












Conclusions

- Due to de- and adsorption effects as well as degradation extractable PAH amounts increase as well as decrease during the course of the experiment.
- Highest degradation rates were found for 4-ring PAHs; lower molecular weight PAHs have probably been degraded during the 2 months of windrow treatment; the higher molecular weight PAHs are usually more difficult do degrade and show more stable ratios in the soil.
- Losses through volatilisation stayed below 100 ppm at all times and represented an insignificant loss pathway; however results showed an increase of volatilisation with decreasing PAH size as well as with increasing temperature.
- Higher amounts of PLFAs (corresponding to the total amount of organisms in the sample) were detected when manure was added. While the treatments differ in the beginning of the experiment they become more alike towards the end of the treatment period with no significant differences from day 11 on.

In conclusion, as to PAH degradation no significant differences could be found between the amendments with the different types of manure, but a slightly better performance was detected with the chicken manure amendment. Considering that the process without manure amendment would not reach the implied temperatures this experiment indicates that manure addition is effective for the bio-remediation of hydrocarbon contaminated soil by co-composting. However, a large scale experiment will be needed to investigate the effects under natural conditions

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