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Biogeochemical characterisation of extreme environments

A Thesis presented for the Degree of Doctor of Philosophy

By

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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Abstract

There is currently a considerable interest in characterising extreme environments, since they offer the opportunity to envision practical applications and to understand microbial diversity as an adaptive response that reflects environmental diversity. It is now well recognized that microorganisms thrive in extreme conditions such as contaminated soils/sediments and the pressurised depth of the Earth. Morphological, physiological, biochemical and genetic adaptations to extreme environments by these microorganisms have generated immense interest amongst scientists who continuously discover new occurrences and modes of microbial life on Earth.

In this thesis, biogeochemical processes are investigated in two different extreme environments. (*i*) The deep biosphere, with a focus on shale gas basin and coal-bed methane (CBM). These environments are currently gaining momentum across the scientific community for the production of gaseous fuel. (*i*) Coal tar-contaminated soil and concentrated organic-phase coal tar, which was studied for bioremediation purposes.

The core of this thesis consists of three articles dedicated to combination of different molecular and chromatographic methods of experimentation, analysis and interpretation. These include molecular tools such as DNA extraction techniques, PCR, 454-pyrosequencing and culturing-based approaches. The chemical experiments were metabolomic and isotopic chromatographic analyses.

This study presented an extensive review of the biogeochemistry of unconventional gas systems, which provide an improved level of information of such environments. A robust culture-independent methodology was developed for the characterisation of microbial life in extreme environments, which was applied to describe, for the first time, the presence of bacteria in concentrated organic-phase coal tar. The deep sequencing methods were then used in combination with multidimensional

compound specific isotope analysis (CSIA) to investigate community structure. The combined approach of deep sequencing methods with multidimensional CSIA was confirmed by statistics. Thus, high-throughput 16S rRNA gene sequencing and multidimentional CSIA, can be applied to investigate microbial community structure in extreme environments.

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Poster, presentations and publications

Peer-reviewed publications

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Presentations

Colosimo F., Boothman C., Lloyd J.R, Thomas R., Lord R. and Kalin R.M. Bacterial community structure and multidimensional isotope signature of PAHs in coal tar. *Organic Geochemistry Society 2015.* 02nd and 3rd July 2015, University of Glasgow. Glasgow, UK.

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Colosimo F., Thomas R., Lord R. and Kalin R.M. Biogeochemistry of shale gas. *TotalDecom 2014*. 8th-9th April 2014; Glasgow Hilton Hotel. Glasgow, UK.

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Abbreviations

ACE	Acenaphthene
ACY	Acenaphthylene
ANT	Anthracene
BaA	Benz[a]anthracene
BaF	Benzo[b]fluoranthene
BaP	Benzo[a]pyrene
BGS	British Geological Survey
BkF	Benzo[k]fluoranthene
Blast	Basic Local Alignment Search Tool
BP	Benzo[g,h,i]perylene
CHR	Chrysene
CSIA	Compound specific isotope analysis
CWG	Carburetted water gas
DBA	Dibenz[a,h]anthracene
DNA	Deoxyribonucleic acid
DNAPL	Dense non-aqueous phase liquid
FLT	Fluoranthene
FLU	Fluorene
FMGP	former manufactured gas plant
GC-IRMS	Gas chromatography - isotope ratio mass spectrometry
GC-C-IRMS	Gas chromatography - combustion - isotope ratio mass spectrometry
GC-MS	Gas chromatography – mass spectrometry
GC-TC-IRMS	Gas chromatography – thermal conversion - isotope ratio mass spectrometry
HMW	High molecular weight
IP	Indeno[1,2,3-cd]pyrene
LMW	Light molecular weight

MAH	Mono aromatic hydrocarbon
MGP	Manufactured gas plant
N	Naphthalene
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
NRB	Nitrate reducing bacteria
OM	Organic matter
OTU	Operational taxonomy unit
PAH	Polycyclic aromatic hydrocarbon
PHE	Phenanthrene
PYR	Pyrene
SRB	Sulphate reducing bacteria
SSU	Small subunit
US EPA	United States Environmental Protection Agency
16S rRNA	16 ‘Svedberg’ ribosomal ribonucleic acid

Preface

This thesis is submitted in partial fulfilment of the requirement for the Ph.D. degree at the Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow. The Ph.D. project was carried out under supervision of Professor Robert Kalin, Dr Richard Lord and Dr Russell Thomas (WSP Parsons Brinckerhoff). The Ph.D. project consisted of interdisciplinary research at the chemistry-biology interface with the main focus on microbial and chemical methods for the characterisation of extreme environments. The project draws on different aspects to form the basis to describe the biogeochemistry of such environments.

Various extreme environments have been the topic of numerous studies dealing with the characterization of microbial communities and related processes. These studies have done much to enrich our understanding of microbial diversity and of biogeochemical mechanisms. There is currently a considerable interest in characterising extreme environments, since they offer us the opportunity to envision practical applications and to understand microbial diversity as an adaptive response that reflects environmental diversity. Indeed, these environments can be hot or cold, acidic or basic, wet or dry, saline, radioactive, polluted with heavy metals or hydrocarbons, or located in the deep subsurface.

In this thesis, biogeochemical processes are investigated in two different extreme environments. The first is the deep biosphere, with a focus on shale gas basin and coal-bed methane (CBM); these environments are currently gaining momentum across the scientific community for the production of gaseous fuel. The second extreme environments investigated in this thesis are coal tar-contaminated soils, which were studied for bioremediation purposes. The challenges presented by this project are met through standard experimental approaches both quantitative and qualitative in nature.

The project started with the chemical and microbial characterisation of two Carboniferous and one Jurassic shale core samples, obtained from the collection of the British Geological Survey (BGS, Keyworth, Nottingham). These preliminary experiments lead to the development of methodologies for the molecular characterisation of tight rocks. However, due to the nature of these samples, no indigenous microbial communities were found in the low porosity of the cores, demonstrating that these samples were not subjected to re-inoculum of microbial cells, that could happen through recharge of water or weathering processes. The complete absence of DNA, verified through molecular and culturing methods, demonstrate that the protocol used for the preparation of samples is effective in avoiding cross-contamination.

The absence of indigenous microbial communities in these samples and the difficulties in obtaining new fresh shale/CBM cores samples, have spurred the exploration for biodegradative microorganisms and for an analogue extreme environment to be used for comparison. The molecular tools developed in the preliminary experiments were then applied for the characterisation of a digestate sample obtained from the mesophilic Bran Sands Advanced Digestion Facility (Middleborough, UK).

To help much needed studies on bioremediation, molecular and isotopic tools for the analysis of complex substrates such as concentrated organic-phase coal tar were developed and applied for the first time. The methodologies developed for the DNA extraction represent an easy and cost-effective step toward the molecular characterisation of complex substrates such as NAPLs.

Finally, this project seeks to contribute to our understanding of the complex biogeochemical processes involved in microbial methane production and hydrocarbon biodegradation. In addition, it is expected that the new molecular tools developed throughout the duration of this project will be a significant catalyst for future studies of microbial processes in extreme environments. This is particularly significant for contaminated groundwaters, where insoluble contaminants generate a

contamination plume such as DNAPL (dense non-aqueous phase liquid) or LNAPL (light non-aqueous phase liquid). At the plume fringe, microbial life thrives thanks to the continuous exchange of electron donors and acceptors.

Chapter 1

Introduction

1.1. Background

Microorganisms played key roles in the life of human beings since the beginning of time. We have been using and exploiting microbes much before the knowledge and realization of their existence on Earth. Even before the appearance of mankind on Earth, tiny cyanobacteria (blue-green algae) began to proliferate for still unclear reasons. Like their descendants today, these cyanobacteria could turn sunlight, water, and carbon dioxide (CO_2) into carbohydrates and oxygen (O_2), allowing other forms of life to thrive. The systematic applications of microorganisms by human beings date back to the days of Louis Pasteur, when he established the relationship between chemical transformations and the existence of microbes. The active involvement of microbes in daily life is due to their versatility, diversity and fast growth. They affect almost every spheres of our life ranging from agriculture to industry and from environment to health. Due to their role in human society, microorganisms have been extensively studied by researchers.

Considering the contemporary developments towards modern biology, the elucidation of the Deoxyribonucleic acid (DNA) structure by American biologist James Watson and English physicist Francis Crick in the 1950s (thanks to the previous work of Friedrich Miescher, Phoebus Levene and Erwin Chargaff) has been a major turning point. Their groundbreaking conclusion gave birth to a new way to study life on Earth, which resulted in the advent of modern molecular biology: the ability to isolate and manipulate genes and genomes and the availability of tools to rapidly sequence DNA and proteins.

However, despite the significant developments in the molecular biology, microbial activities remain to be under limited set of conditions. Due to different restrictions on cultivation of the naturally occurring population, only fraction (1-10%) of microbial world is known, and biocatalysts from microbes are able to function only under delicate and defined laboratory set of conditions.

In the light of the above considerations, exploration of newer habitats, particularly extreme ones for environmental and biotechnological applications become quite significant. In this context, the search for microorganisms in extreme environments and the application of biological processes, organisms, or systems to manufacture products intended to improve the quality of human life is still a topical concept with many opportunities for development.

1.2. Hypotheses

In the first part of this Ph.D. project, the relationships between microbial community composition, biogeochemical conditions and microbial methane generation in unconventional gas systems were investigated and the following primary hypotheses were identified:

1. The stimulation of microbial methane generation in unconventional gas systems is complicated by several biogeochemical factors such as the bioavailability of water and organic matter, redox conditions, pore size of the formation, temperature, pH, salinity, microbial competition, etc. However, biogenic methane is observed in several shallow, low temperature shale and coal formations.
2. The current state of research regarding the stimulation of microbial methane generation in extreme environment is poorly linked with commercial activities. Managing the microbial communities in unconventional gas systems might have implications for both recovery practices and a sustainable development of unconventional resources.

3. Microbial communities in unconventional gas systems could be analysed in shale and coal core samples with culture-independent techniques.

In order to chemically and microbially characterise concentrated organic-phase coal tar, this dissertation addressed three primary hypotheses:

1. Microbial communities might be present in concentrated organic-phase coal tar.
2. If there is any microbial community in concentrated organic-phase coal tar, their structure could be analysed with culture-independent methods.
3. Microbial communities in concentrated organic-phase coal tar may slowly change the isotopic signature of hydrocarbons.

1.3. Research aims and objectives

The aim of this research is to investigate and expand experimental tools for the characterisation of extreme environments using microbial, molecular, chemical and isotopic techniques. To achieve the aims of this project and to test the above-mentioned hypotheses, the following research objectives were identified:

- To develop and verify a protocol to handle and process shale and coal core samples for microbial community analyses.
- To optimise bioinformatics tools for the analyses of high-throughput 16S rRNA gene sequencing.
- To develop analytical methodologies for the analysis of the microbial community structure in concentrated organic-phase coal tar DNAPL.
- To develop a fast, accurate and precise extraction procedure to allow analysis of concentrated organic-phase coal tars by multidimensional compound specific isotope analysis (CSIA) without the need for complex fractionation processes.
- To develop a comprehensive method for the analyses of complex organic matter from coal tar using GC-MS (gas chromatography - mass spectrometry) and

multidimensional CSIA, with focus on the 16 US priority polycyclic aromatic hydrocarbons (PAHs).

- To compare the output of high-throughput 16S rRNA gene sequencing with CSIA results with statistical methods to identify potential correlations with microbial processes.

1.4. Thesis Outline

This thesis details the development of methodologies for the microbial characterisation of extreme environments. The two environments investigated were (*i*) the deep biosphere, in particular shale gas and coal bed methane; and (*ii*) coal tar contaminated soils with a focus on concentrated organic-phase tars.

Chapter 1 present an introduction to the biogeochemistry of extreme environments, describing the metabolic diversity of the microorganisms encountered, their main microbial processes and introducing genomic techniques used to analyse them.

Chapter 2 also provides background information on coalification and coal conversion processes, and the various forms of contamination found at the site of former gasworks. Lastly, a review of biological techniques applied for enhancing hydrocarbon biodegradation is presented, with a focus on molecular and isotopic methodologies.

Chapter 3 presents an overview of the methodologies employed in this research. The specifications and parameters of instrumental techniques are provided, as well as quality control procedures. Brief site summaries are also provided for each shale and coal tar samples investigated in this study.

Chapter 4 consists of a short introduction to the first paper presented in this thesis. Paper I has been published in The International Journal of Coal Geology (Colosimo et al., 2016). Paper I is a comprehensive review on biogenic methane in shale gas and

coal bed methane: an extensive search in the literature highlighted current knowledge and gaps in the microbiology of shale gas and coal basins.

Chapter 5 is composed by a short bridge chapter that explain the rationale for the work presented in the second paper submitted during the Ph.D. project. Paper II presents deep sequencing methods for the characterisation of digestate samples obtained from a mesophilic anaerobic digester and relates the microbial population to the processes used for gas production.

Chapter 6 presents the third paper produced during the Ph.D. project, which was submitted to The Journal of Contaminant Hydrology (currently under review). Paper III describe, for the first time, the presence of bacteria in concentrated organic-phase coal tar. Furthermore, this research article developed and applied molecular and isotopic methodologies for the analysis of this complex substrate. Lastly, a statistical comparison of high-throughput 16S rRNA gene sequencing with multidimensional CSIA is presented.

Finally, ***Chapter 7*** summarises the major findings of this Thesis, its impact and highlights the recommendations for further work.

Chapter 2

Literature review

2.1. Introduction

The investigation of life processes in extreme environments has a broad spectrum of relevance, including both social and economic considerations. These exciting areas of research are at an early stage and focus on environments that have been difficult to investigate in the past. However, they are set to benefit dramatically from the new technological developments, as well as from the use of the rapidly developing tools of molecular biology and bioinformatics and are ideal targets for the consideration of species within their whole ecosystems.

Actions should be taken to move research in this direction. At the European level the scientific community currently studying extreme environments is significant and well regarded but its structure is relatively fragmented. The benefits resulting from improved coordination and information exchange within this community are clear and implementing greater networking represents a significant challenge to Europe for the future.

Various extreme soils have been the topic of numerous and fruitful studies dealing with the characterization of microbial communities and processes. These studies have done much to enrich our understanding of microbial diversity and of biogeochemical and other biological mechanisms. They allow us to grasp microbial adaptability and to envision practical applications. Reading the enclosed collection of chapters will make it clear that unifying these studies under the general theme of "extreme soils," and associating this concept with the broader notion of "extreme environments" leads to essential theoretical and practical advances.

2.1.1. Biogeochemistry of extreme environments

Microorganisms populate every habitable environment on Earth and, through their metabolic activity, affect the chemistry and physical properties of their surroundings. Nearly every environment on Earth is populated by microorganisms. Bacteria and Archaea were discovered and described in a number of extreme environments, including low and high pH, temperature, pressure and redox conditions.

So intimate is the relationship microorganisms-Earth, that to discover a part of Earth that has not been fundamentally affected by life, it may be necessary to penetrate hundreds of kilometres into the mantle. Yet, even at great depths, the physico/chemical properties of the mantle may have been modified by the slow but continuous subduction of sediments with elemental and isotopic features arising from biological activity.

Over geologic time, multicellular eukaryotic organisms such as fungi, algae, higher plants, and mammals have caused significant changes on Earth's geochemistry, but the most important geochemical "changers" by far have been unicellular microorganisms (e.g., Bacteria and Archaea). Microbes have changed Earth in a number of ways.

They have altered the chemistry of the atmosphere via oxygenic photosynthesis (ancestral phototrophic bacteria), nitrogen fixation, and carbon sequestration (Kasting et al., 2002).

Microbes have also modified the compositions of oceans, rivers, and pore fluids through control of mineral weathering rates or by inducing mineral precipitation.

Bacteria have influenced the speciation of metals and metalloids in water, soils, and sediments by releasing complex molecules and by enzymatically catalysing redox reactions; they have shaped the physical characteristics of every environment by binding sediments, precipitating ore deposits, and weathering rocks; and they have

sustained communities of higher organisms through primary production and by remineralizing organic carbon.

Most remarkably, they perform these functions in every environment, from the near surface to the depths, including even the most extreme environments (Reysenbach et al., 2002). Microbes affect the chemistry and distribution of nearly all elements in the periodic table, with the ultimate aim to influence their bioavailability.

In this work, a brief revision of recent findings that have expanded the knowledge and the versatility of microbial metabolisms in geochemistry was carried out. A focus was given to extreme environments such as the deep subsurface and heavy polluted sites.

2.1.2. Metabolic diversity

The metabolic diversity of microbial life on Earth is surprisingly vast. New discoveries are continuously made about occurrences and modes of microbial life, ranging from proteorhodopsin-based phototrophy in the open ocean (Béjá et al., 2001) to methanogenesis driven by geochemical reactions in Earth's depths (Chapelle et al., 2002).

Niches once considered to be uninhabitable (such as those with pH of 0 in extremely rich metal solutions) or coal tar polluted soils have been found to harbour active microbial communities (Edwards, et al., 2000). Chemical compounds once thought to be recalcitrant, such as kerogen, or long-chain alkanes (under anaerobic conditions) are now known to be microbial growth substrates (Petsch et al., 2001; Zengler et al., 1999). Microorganisms previously believed to be unculturable, such as anaerobic benzene oxidizers, have been brought into culture (Coates et al., 2001). Geochemical transformations such as the anaerobic oxidation of methane or ammonium have been recently attributed to the activity of syntrophic consortia of bacteria and archaea (Boetius et al., 2000; Jetten et al., 2001).

The vast extent of microbial diversity has triggered geochemical studies, useful to define and explore interesting biogeochemical problems. For decades, microbiologists and microbial ecologists have observed that microorganisms inhabit almost every thermodynamically favourable environment (Beijerink, 1888; Winogradsky et al., 1889; Kluyver, 1924).

Although clearly there are limits to life, with recent findings these limits seem ever more remote. For example, it was once believed that the minimum quantum of free energy that could be biochemically converted was -20 kJ/mol (Schink, 1997). A recent study with syntrophic microbial cultures, however, has questioned this statement (Jackson and McInerney, 2002). Free energies as low as -4.5 kJ/mol were found to support the growth of butyrate-degrading organisms in co-culture with methanogens; values lower than -20 kJ/mol were calculated for different fermenters under different conditions (including sulphate- and nitrate-reduction). These recent findings increase the number of niches where we might expect microbial activity.

The thermodynamic extent of microbial life is particularly relevant for reservoirs on Earth where energy is a limiting factor and where microbial growth is expected to be slow, such as in the deep subsurface. Clearly not only thermodynamic factors control microbial growth, including pH, temperature, pressure, salinity, radiation, toxins, and trace metals. How life responds (from the perspective of both single organisms and communities) to environmental constraints and how it changes the environment in the process is perhaps the most fundamental and fascinating topic in geomicrobiology. Although geomicrobiology can be defined broadly as the study of how microorganisms shape Earth's geochemistry, this thesis is limited to the investigation and discussion of microbial processes in terrestrial systems. Within this, the focus is given to the deep biosphere and hydrocarbon-polluted environments.

2.1.3. Activity in the environment

Beginning near the surface, sedimentary microorganisms play important roles in the cycling of several elements, including carbon, iron, and sulphur. These include heterotrophs that are sustained by organic carbon originating from primary production in surface waters, as well as chemolithotrophs who get their energy from the inorganic products of heterotrophic metabolism.

As oxygen is used in near-surface sediments, anaerobic microorganisms take over as the primary degraders of organic matter. Recently, a specific enrichment of bacteria of the family *Geobacteraceae* (anaerobic heterotrophs that can couple the oxidation of organic compounds to the reduction of insoluble Fe(III) oxides) was shown to grow by oxidizing organics with a graphite electrode as the sole electron acceptor (Bond, et al., 2002). This type of activity may hold promise for the bioremediation of organic contaminants and/or energy harvesting for instruments, as members of this family are known to represent a sizable fraction of the microbial population in diverse sedimentary environments.

In addition to the *Geobacteraceae*, many guilds couple organic matter oxidation to the reduction of inorganic compounds such as selenate, nitrate, manganese oxides, arsenate, sulphate, and carbonate for energy generation. Collectively, their metabolism is versatile, and they may help immobilize inorganic contaminants such as technetium and uranium (by catalysing mineral precipitation) (Lloyd and Lovley, 2001) or degrade organic contaminants such as benzene and chlorinated hydrocarbons through either oxidative or reductive reactions (Fennell et al., 2001). For example, microorganisms able to use (per)chlorate as a terminal electron acceptor are ubiquitous in pristine and hydrocarbon-contaminated soils and sediments. They have been shown to oxidize a variety of monoaromatic compounds (including benzene) to CO_2 under anaerobic conditions (Coates et al., 2001). Elucidation of the metabolic pathways that govern these transformations is in progress, and identification of the genes involved will soon be aided by the

completion of the genomic sequence of *Dechloromonas aromatica* strain RCB, a representative from this group.

The exciting concept that microorganisms have contributed to the formation of certain ore deposits over geologic time arise from the recognition that they can precipitate metals from solution. Though most ore deposits are thought to relate to transport and deposition by high-temperature fluids and magmas, in some cases low temperature origins may be possible. For example, it has been suggested that iron-oxidizing phototrophs may have played a role in the deposition of Banded Iron Formations over 2.5 billion years ago (Widdel et al., 1993).

New evidence has revealed that natural communities of sulphate-reducing bacteria (SRB) can generate essentially pure ZnS deposits from diluted groundwater solutions, providing support for a biogenic origin of many low-temperature metal sulphide ore deposits (Labrenz et al., 1993). In addition, recent evidence indicates that hyperthermophilic and mesophilic dissimilatory Fe(III)-reducing bacteria and Archaea can couple oxidation of hydrogen to reduction of Au(III), leading to Au(0) precipitation (Kashefi et al., 2001).

Hyperthermophilic microorganisms can couple oxidation of hydrogen or organics to the reduction of metals in hydrothermal solutions, leading to the formation of magnetite (Fe_3O_4) and uraninite (UO_2) ore deposits at 100°C. Given their potential importance to ore deposit formation, it was essential to define how to determine the distribution of metal-precipitating microorganisms. In this case, SRB were first used for these studies: Devereux, (1996) used probes that bind specifically to the 16S ribosomal RNA (rRNA) of target organisms to quantify the abundance of SRB in a sediment and to identify the predominant group in a zone characterized by mercury methylation (an activity attributed to SRB).

The 16S rRNA gene has been widely used to expand our understanding of microbial diversity in the environment (Pace, 1997). However, in microbial ecology, species identification does not always correlate with metabolic function. Accordingly, an

alternative to assessing microbial activity in the environment is to target functional genes. In the case of SRB, most of these investigations have focused on analysis of sequences for dissimilatory sulphite reductase. This approach was used to identify SRB in a hypersaline microbial mat and to evaluate their distributions relative to oxygen gradients (Minz et al., 1999).

Phylogenetic analysis of partial dissimilatory sulphite reductase gene sequences indicates that Archaeoglobus, an archaeal sulphate reducer, acquired the ability to reduce sulphate via an ancient lateral transfer from a bacterial donor (Klein et al., 2001). These studies show that it is possible to determine which species are controlling key geochemical transformations as well as to resolve details of the evolution of a pathway central to biogeochemical sulphur cycling over much of Earth's history. In addition to promoting mineral formation, microorganisms also catalyse mineral dissolution.

In aerobic environments, microbes may dissolve minerals through the excretion of various organic compounds. For example, because iron in aerobic soils is sequestered into minerals, organisms produce a class of biomolecules called siderophores that strongly bind iron and shuttle it to the cell surface, thereby increasing mineral dissolution rates (Macrellis et al., 2001). Kinetic data for the dissolution of goethite ($Fe^{+3}O(OH)$) by trihydroxamate siderophores suggest that these molecules adsorb iron via a single hydroxamate group in bidentate ligation with an Fe(III) center (Cocozza et al., 2002). A synergistic effect in the presence of an additional ligand, oxalate, was attributed to coupling of oxalate-promoted dissolution with complexation of Fe(III) by the siderophore (Cervini-Silva and Sposito, 2002). In anaerobic environments, anaerobic respiration may also promote mineral dissolution (or mineral transformation, depending on the geochemical conditions). An example of this is the reductive dissolution of Fe(III) oxides, which liberates metalloids such as arsenic that are adsorbed to the oxides (Cummings et al., 1999). Culture-independent methods are now being used to identify the genes that encode the production of organic molecules in the soil (Boetius et al., 2000) that likely participate in this type of reaction.

Deeper into Earth, caves and mines provide direct access to microbial communities kilometres beneath Earth's surface. Cave habitats may support a wide variety of species, including invertebrates that are sustained by chemoautotrophic microorganisms that oxidize hydrogen sulphide (Angert et al., 1998). A microbial role in the formation of cave deposits (such as speleothems) has been proposed based on microstructural, morphological, and isotopic data (Melim et al., 2001). However, at the present time, the role of organisms in speleothem formation is controversial (Jones 2001; Barton et al., 2001). Subsurface mine systems are populated by a diversity of archaeal, bacterial, and eucaryal species. Metal sulphide ore deposits dominated by pyrite (FeS_2) are widespread and are of great interest because they are the source of environmentally damaging metal-rich sulphuric acid solutions (acid mine drainage). In contrast to some cave systems (Sarbu et al., 1996), acid mine drainage populations are typically dominated by only a small number of species belonging to diverse phylogenetic groups. The simple community structure is probably due to the small number of electron donors and acceptors, extremely low pH, and high concentrations of toxic metals (Bond et al., 2000).

Because these communities are essentially isolated from fixed carbon and nitrogen compounds formed at Earth's surface, acid mine drainage may provide ideal environments for detailed studies of how microbial communities work. Acid mine drainage sites provide perhaps the best known example of microbially controlled mineral solubilisation. Ferric iron, the primary sulphide oxidant at low pH, is regenerated slowly in abiotic systems by reaction of ferrous iron released by pyrite dissolution with oxygen. Some bacteria and archaea are able to enzymatically oxidize iron, which provides them with energy to sustain their growth (Blake et al., 1993). The ferrous iron enzymatic oxidation rate is up to six orders of magnitude faster than the inorganic reaction (Singer and Stumm, 1970).

However, this does not mean that inorganic oxidation is completely insignificant. Evaluation of the microbial effect requires consideration of the number of active iron-oxidizing cells and the rate at which each cell oxidizes iron. Edwards et al.,

(2000) quantified these rates for individual microbial species and consortia of Fe(II)-oxidizing prokaryotes at low pH. A preliminary estimate indicated that the microbial populations in one sampled region of an acid mine drainage site account for 70% of Fe(II) released by pyrite dissolution.

Subsurface microbiology is not limited to caves and mines. Deep drilling projects in many parts of the world have revealed evidence for microbial life in rocks at great depth (Krumholz, 1998). Examples include sulphate reduction occurring between sandstone and shales that were deposited during the Cretaceous period (90 to 93 million years ago) (Krumholz et al., 1997) and methanogenesis driven by geothermal waters rich in hydrogen (Chapelle et al., 2002).

In both of these cases, microbial activity was detected at least 200 m below land surface. Even deeper environments have been sampled for signs of microbial life. Notable examples of this are the gold mines of South Africa, which represent the deepest accessible excavations in the world. Some fissure water samples from these mines are thought to originate from anaerobic, saline groundwater at a depth of 5 to 6 km, where temperatures are in the hyperthermophic range (Takai et al., 2001). Through culture-independent analysis, novel archaeal sequences have been retrieved from these samples, including a clone that is related to known hypothermophilic species of *Pyrococcus* from a marine vent system (Takai et al., 2000). One question that remains open is whether the organisms that inhabit these environments are metabolically active in situ. The recent finding that *Shewanella oneidensis* and *Escherichia coli* can survive and oxidize formate at pressures greater than 1000 mPa (corresponding to around 50 km below Earth's crust) suggests that metabolic activity may occur at depth (Sharma et al. 2002).

2.1.4. Biochemical considerations

Many of the above studies were performed using culture-independent methods, and such approaches are an example of an exciting new frontier in geomicrobiology. A topic that has inspired several studies at the molecular level is how bacteria respire minerals. Most terminal electron acceptors that bacteria use for respiration, such as oxygen, nitrate, and sulphate, are soluble. This means they can freely diffuse to the cell to receive electrons from the membrane-bound molecules of the respiratory chain. How bacteria transfer electrons to solids like hematite (α Fe_2O_3) and goethite presents a real problem. Because these minerals are effectively insoluble under environmentally relevant conditions, simple dissolution and diffusion of ferric iron to the cell cannot be the answer. Different mechanisms for electron transfer to minerals during respiration have been proposed.

The first is that bacteria solubilize the minerals by producing chelators. Although the addition of synthetic chelators has been shown to stimulate microbial electron transfer to iron minerals, to date no evidence has been found that bacteria use this mechanism in respiration (Nevin and Lovley 2000).

The second is that they use soluble shuttles, such as organic compounds with quinone moieties, to transfer electrons from the cell to the mineral (Lovley et al., 1996). These shuttles may be exogenous substances or may be produced by the organisms themselves (Newman and Kolter 2000). Recent results from genetic screens with the iron-reducing organism *S. oneidensis* strain MR-1 suggest that these shuttles may share structural and functional properties with redox active antibiotics (Hernandez and Newman 2001; Shyu et al., 2002).

The third mechanism is that bacteria directly transfer electrons from the cell surface to the mineral. A variety of biomolecules (including cytochromes, quinones, dehydrogenases, and secretory proteins) have been identified as participating in this electron transfer pathway (Richardson 2000; Beliaev et al., 2001; Magnuson et al., 2000; Di Christina et al., 2002). Of these, several are located on the outer membrane

of the cell and presumably make contact with the mineral directly (Lower et al., 2001).

Another topic that has received molecular attention is the mechanism of precipitation of manganese oxides by diverse *Bacillus* species. Dormant spores produced by these organisms enzymatically oxidize soluble Mn(II) to insoluble Mn(IV) oxides. A representative of this group, *Bacillus sp*. strain SG-1, is believed to catalyze this process by a multicopper oxidase, MnxG. Recently, phylogenetic analysis based on 16S rRNA and mnxG sequences obtained from 15 different Mn(II)-oxidizing spore formers (including SG-1) revealed extensive diversity within the genus *Bacillus*, with organisms falling into several distinct clusters and lineages (Francis and Tebo, 2002). In addition, active Mn(II)-oxidizing proteins of various sizes were recovered from the outer layers of purified dormant spores of the isolates (Francis and Tebo, 2002). Mechanistic details of important biogeochemical activities may also be identified from uncultured organisms. An outstanding example of this is the recent finding that proteorhodopsin-based phototrophy occurs globally in the marine environment. This discovery was made by expressing select DNA fragments from uncultured marine organisms in *E. coli* and observing the production of functional proteorhodopsin (Béjá et al., 2000). Biophysical techniques were then used to show that native organisms expressing proteorhodopsin are widespread (Béjá et al., 2001).

Whether these organisms can fix CO_2 is currently unknown. But given their global distribution, it seems likely that they may have a significant impact on carbon and energy flux in the oceans. Lastly, an important step forward in the mechanistic analysis of natural samples has been the combination of gene probes with molecular-level isotopic measurements. This approach has permitted the direct correlation of geochemical activities (such as anaerobic methane oxidation) with likely source organisms (methanogenic archaea and SRB), even though the latter may have never before been cultured (Boetius et al., 2000; Orphan et al., 2001).

2.1.5. Genomics

The most rapid recent advances in our understanding of non-culturable microorganisms can be attributed to the advent of genomics. The first microbial genome sequencing projects focused almost entirely on pathogenic strains, but within the past few years biogeochemically relevant microorganisms have been sequenced too.

The use of genomic sequencing has increased dramatically in the past decade, as it can be seen in the rising number of microbial genome projects that are listed on the Website of the National Center for Bioinformatics (NCBI) (www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi). However, in the majority of geologic settings, only a few isolated microbial strains have been characterized, and they do not necessarily represent the dominant organisms in a given environment. A desirable goal for future sequencing projects will be to identify the major players in any given environment and bring them into culture so that their physiology and biochemistry may be studied with the aid of genomic information. It will also be important to determine the degree to which gene content, genome organization, and gene regulation vary between strains in a given environment.

Despite the limited data set, much has already been learned from genome sequencing projects that is causing us to re-evaluate the history of life. Comparative genomics (e.g., comparisons of coding regions from different organisms for similar biomolecules) has revealed phylogenetic incongruities that span the universal tree. For example, the vast majority of gene products from the Archaea most resemble counterparts among the Bacteria and not the Eucarya, yet the rooted phylogenetic tree [based on small subunit rRNA (SSU rRNA)] clearly places the Archaea as specific relatives of the Eucarya (Doolittle, 1999). Although re-examination of the universal tree on the basis of alignments of 23 orthologous proteins conserved across 45 species from all domains has resulted in trees that support the SSU rRNA trees with respect to the separate monophyly of domains (Brown et al., 2001), differences in GC content and codon usage patterns within genomes suggest that lateral gene

transfer has been a primary evolutionary mechanism throughout Earth's history (Olsen, 2001; Woese, 1998). It is still difficult to establish which cell types emerged first and how, but as more genome sequences from evolutionarily interesting microbes (such as anaerobic protists) become available, it will simplify to use DNA as a fossil in speculating about evolution.

A significant advance will probably come by linking the emergence of particular genes to major events in Earth's history. This is a difficult task, but some intriguing work has already been done in this area (Kirschvink et al., 2000). A powerful new tool that has been made possible by whole-genome sequencing is DNA microarray technology. This involves the simultaneous monitoring of gene expression patterns for all messenger RNAs (mRNAs) of an organism. DNA microarray technology makes it possible to determine how (and how rapidly) organisms respond to changes in the geochemistry or biology of their habitats (e.g., to an increase in concentration of a toxic metal; decrease in temperature, variations in ionic strength and pH; or additions or deletions of microbial species). An example of this is a recent microarray study that was performed with *S. oneidensis* (Belieav et al., 2002). In that work, it was possible to identify the networks of genes that were up- or down-regulated depending on the type of metabolism in which the cells were engaged (e.g., growth on alternative electron acceptors, including iron).

Recent studies, where microarrays have been used to study microbial development [e.g., studies of the life cycle of the stalked bacterium *Caulobacter crecentus* (Stephens, 2001) or biofilm formation by *Pseudomonas aeruginosa* (Whiteley et al., 2001), will probably be a significant catalyst for the use of microarrays in investigating geomicrobiological problems. For example, once enzymes are identified (such as those involved in metal oxidation or reduction) it may be possible to monitor the activity of genes that encode these enzymes with organism-specific resolution in mixed communities. As more information are gathered about important gene sequences from the environment, it will be feasible to extend array technology to monitor expression patterns of genes from uncultured organisms.

2.2. Coal conversion processes

2.2.1. Background on coalification

Coalification is the process by which organic remains matures to coals of increasing rank. The biochemical stage consists in the conversion of cellulose to humic acid, and this is followed by the geochemical stage (Jones & Godefrey, 2002). Coalification is characterised by a loss of volatiles (CH_4, H_2O, CO_2 and CO) and relative increase in the carbon content with increasing rank. Kopp et al., (2000) identified a decrease in the atomic oxygen/carbon (O/C) ratio and hydrogen/carbon (H/C) ratio with increasing rank.

Coalification temperatures are thought to range from 100˚C to 170˚C for bituminous coals and from 170˚C to 250˚C for anthracites (Taylor et al, 1998). Coal is characterised by a complex cross-linked structure (Scott, 1999), with organic material typically representing 85% to 95% (wt/wt) of dry coal (Levine et al., 1982). Various inorganic materials, particularly alumina-silicates and pyrites (especially in high-sulphur coals) comprise 5% - 15% of the coal. Coal has an extensive network of pores resulting in high surface areas (> 100 m2/g for bituminous and sub-bituminous coals and lignites).

At the atomic level, bituminous coal is composed of relatively small aromatic and naphthenic rings, coupled to one another by “bridges” of aliphatic chains and hetero-atoms (i.e. nitrogen, oxygen and sulphur) (Levine et al., 1982). In addition to the covalent bridges, there are a significant number of polar groups, such as hydroxyls (-OH), that can contribute to the integrity of the coal structure via electrostatic binding. During the carbonisation process, the cross-linked coal structure is broken into its constituent parts and existing constituents are altered. Walker et al., (2007) undertook extensive studies into high volatile bituminous coals from Indiana (USA). Samples were carbonised in retorts at temperatures of between 275˚C and 425˚C and

subsequently analysed. Above 375˚C an increase in aromaticity was observed. The predominance of aromatic chemistry is apparent in the tars resultant from coal carbonisation. Typical operating temperatures of a Manufactured Gas Plant (MGP) retort range from 600˚C to > 900˚C depending on the retort design.

2.2.2. Carbonisation of coal

Coal tar is a by-product of coal carbonisation, which involves the destructive heating of coal. Coal tar was historically produced as a by-product of manufactured gas operations until approximately 1950 (Environment Agency, 2003a). The temperature of coal carbonisation is dependent on the desired product. Gentry (1928) defines low temperature carbonisation to mean the destructive distillation of coal at, or below, the cracking temperature of the hydrocarbons in to primary tar. Due to the inherent heterogeneity between coals of different ages and source, the temperature at which cracking occurs spans over a range generally accepted to be in the region of 500˚C and 800˚C.

Where town gas production was of primary importance such as Former Manufactured Gas Plants (FMGPs) early engineers found that the greatest yield was achieved at high temperatures and retorts were operated accordingly. High temperature coking is used in the metallurgical industries (Gentry, 1928). Creosote and coal tar are examples of multi-component DNAPLs (Environment Agency 2003a). The chemical components of creosote and coal tar are too numerous to warrant individual consideration and it is usual practice to consider selected key contaminants during environmental risk assessment. Determinants are selected for which there is detailed physicochemical and toxicological published literature to allow groundwater risk assessment in accordance with the Environment Agency Remedial Target Methodology (Environment Agency 2006).

2.2.3. History of manufactured gas

Manufactured gases are fuel gases derived from a solid or liquid source of organic carbon. Coal was carbonised by heating in an oxygen-depleted environment to produce coal gas, coke, tar and ammoniacal liquor (Murphy et al., 2006). The principle of manufactured gas production for lighting and heating was developed by the British engineer William Murdoch in the late 18th century. Murdoch employed the first industrial-scale manufactured gas plant to light a Boulton and Watt Soho factory in 1798 (Findlay, 1918). However, the first public gas works in Britain did not begin operation until 1813 (Thorsheim, 2002).

The manufactured gas industry expanded rapidly over the following century, especially within Europe and the United States (Thomas and Lester, 1994). By the mid 19th century, every town and city in Britain had at least one gasworks plant (Thorsheim, 2002). The manufactured gas industry evolved over almost 200 years, with many advancements in technology providing better gas quality and quantity. However, the discovery of natural gas supplies in the mid-20th century resulted in the industry's rapid decline.

A concise list of former gas works sites has never been published, but it is estimated that approximately 3000 ± 1000 FMGP sites exist within the United Kingdom alone (Thomas and Lester, 1994). These sites could prove harmful to the population and the environment, before, during or after redevelopment of the land if appropriate remediation techniques are not used (Thomas and Lester, 1994). Manufactured gas was generally used for lighting and heating, however, a number of markets also developed for the consumption of the considerable quantities of by-products generated by the manufacturing process (Thomas and Lester, 1994). During the manufacturing process, by-products, such as coal tars and ammoniacal liquors, and impurities, such as H_2S, were removed from the gas (Hatheway, 2002).

Crude coal tar is a dark-coloured DNAPL (Hatheway, 2006). When coal tars are released into the environment the more soluble components migrate into the

groundwater leaving the residual coal tar as a DNAPL and thereby forming a contamination plume. Consequently, these coal tar DNAPLs migrate down through the water table until they reach a less permeable layer, where they begin to spread laterally to form underground plumes (D'Affonseca et al., 2008). DNAPLs are composed of thousands of organic and inorganic compounds, many of which may be found in trace quantities (Birak and Miller, 2009). A large percentage of coal tar composition is made up of PAHs, including the 16 priority US-EPA pollutant PAHs. The vast majority of coal tar components are toxic, with many compounds known to have carcinogenic, teratogenic and mutagenic properties (Benhabib et al., 2006).

2.2.4. Manufacturing processes

Manufactured gas is a fuel gas derived from a source of organic carbon, such as coal or oil. In the early years of the manufactured gas industry, coal was the main carbon source utilised in United Kingdom. The process conditions, such as temperature, carbonisation method and coal composition all played a role in determining the composition of the coal gas and any by-products formed (Brown et al., 2006). The general procedure of gas manufacture was coal retort carbonisation (Fig. 2.1). Retort were sealed, cast iron cylinders in which coal was placed and heated, in order to allow volatiles to be driven off as a gas, which was collected via an exhauster pump (Hatheway, 2002). A number of retort types were employed throughout the gas manufacture era, including horizontal, inclined and vertical designs. The initial gas produced by heating coal contained a number of impurities which had to be removed prior to use for lighting or heating. Purification processes were employed to remove liquors, residual hydrocarbons, sulphur compounds and tar from the gas (Hatheway, 2002). The final gas product distributed to consumers consisted mainly of hydrogen, carbon monoxide and a small portion of hydrocarbons.

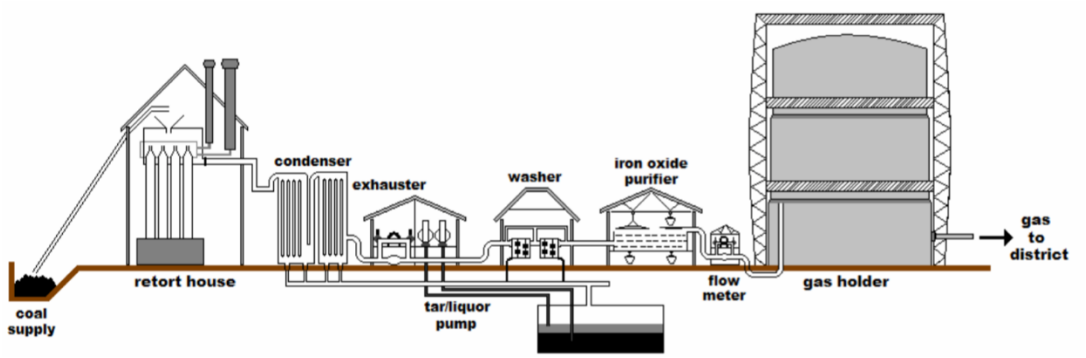

Figure 2.1. Schematic representation of the process involved in manufactured gas plant. Modified from Thomas and Lester, (1994)**.**

2.2.5. Coal tar generation

The term 'coal tar' is used to describe the thick viscous liquid by-product of coal carbonisation. Birak and Miller (2009) highlight the fact that coal tar at a contaminated site may have arisen from any one of a number of technologies employed to carbonise coal (horizontal and vertical retort, coke oven, carburetted water gas). Gas manufacture through coal carbonisation spans from the early 1800's until it demise in 1950's, although coal carbonisation for the metallurgical industry continues to the present day. Initially (c.1800) coke plants were constructed using 'bee-hive' ovens to generate coke from bituminous coal, with no consideration given to capturing the coal gas (known as 'off-gas') generated in the process. Later, some coke plant facilities were modified to allow the capture of the 'off-gas'.

The gas generated by coal carbonisation contains impurities including ammoniacal liquor, tar, hydrocarbon oils and sulphur compounds. Prior to distribution the gas required cleaning. The majority of the tar and water vapour contained in the gas at the outlet of the retort house was removed as liquor in the condensers. Most of the associated ammonia and hydrogen sulphide was dissolved in this liquor. The residual tar was removed using Livesey washers. The remaining ammonia and some hydrogen sulphide and hydrogen cyanide were removed by scrubbing the gas with water or weak ammoniacal liquor. In 1812 the first commercial gas works was

established in London to provide street lighting (Rhodes, 1945). The early engineers recognized to some extent the value of low temperature processes, but as the production of gas was of primary importance to them, they resorted to the practice which gave the greatest yield and so adopted the high temperature method exclusively (Gentry, 1928).

The development of electric lighting in the 1880's provided the incentive for the development of additional uses of coal gas, such as heating and cooking (Thomas and Lester, 1994). Gas used for these domestic purposes was named 'town gas'. For use as town gas further purification was required to reduce the hydrogen sulphide content of the gas. Gas leaving the ammonia scrubber could contain up to 20,000 ppm hydrogen sulphide, but the statutory requirements for the quality of distributed gas set a limit of 0.7 ppm. The early gas works used slaked lime (calcium hydroxide) to remove hydrogen sulphide (Thomas and Lester, 1994). Between 1850 and 1900 this technology was superseded by the use of iron oxide to remove hydrogen sulphide. The iron oxide also served to remove hydrogen cyanide from the gas.

Birak and Miller (2009) recognised three primary methods by which gas was manufactured, each of which produced a different type of tar with its own chemical fingerprint. Coal gas comprises the volatiles collected when bituminous coal is heated in sealed chambers. Before 1850, this was performed in horizontal cast iron retorts at temperatures typically of 600 - 800˚C. Post 1850, the introduction of clay retorts allowed temperatures of > 900˚C to be achieved. Post 1910, vertical retorts were re-introduced, with lower operating temperatures. An outline of retort design is presented by Gentry (1928). Carburetted Water Gas (CWG) was generated by passing steam over red-hot coke. The reaction was strongly endothermic. The resultant gas contained hydrogen and carbon monoxide known as water-gas. Oil was then sprayed into this gas to increase its calorific value, creating CWG. In locations where coal was not readily available (i.e. Eastern USA) alternative hydrocarbon sources have been employed.

In the production of oil gas, oils alone were heated and cracked to produce a mixture of mostly hydrogen and methane, along with other illuminates (Rhodes, 1966). The plants were generally situated within urban areas where the gas was used (DoE, 1995). This minimised the length of pipe work required to distribute the gas. Due to the positioning of these former coal carbonisation sites, they often represent highly desirable plots of land which remain undeveloped on account of the present contamination. Murphy et al., (2005) note that tars not sold to refiners were either landfilled or disposed of in open pits. Hamper (2006) states that historically, tars with a water content of greater than 3% was unsalable to refiners.

The investigation on coal tar chemical composition is still a complicated analysis. Tars are typically rich in aromatic compounds: isotopic ^{13}C and ^{1}H NMR analysis performed by Pindoria et al., (1997) revealed that within the tar from manufactured gas plant 94% of the carbon was in aromatic compounds and 86% of the hydrogen was in aromatic compounds. Abraham (1922), using sulfonation testing, revealed that over 90% of the mass of tar is aromatic. Birak and Miller (2009) notes that a significant portion of coal tar lies above the range of standard analytical techniques with the pitch fraction constituting up to 70% of coal tar. Morgan et al., (2008) quote compound comprising up to 210 aromatic rings identified during analysis. Brown et al., (2006) analysed eleven samples of coal tar from ten FMGPs sites across the USA. Samples were analysed for monoaromatic hydrocarbons (MAH), PAHs and alkylated aromatics. In addition, the aromatic and aliphatic fractions were quantified and their water and ash contents were studied.

Brown et al., (2006) observed that, whilst MAH and PAH concentrations varied by up to an order of magnitude or more between coal tar samples, the relative distribution of PAHs and MAHs were similar for all the eleven samples. In all instances coal tar was dominated by naphthalene, 1-methyl-naphthalene, 2-methylnaphthalene and phenanthrene. Brown et al., (2006) also observed that the 2-ring (naphthalenes), 3-ring (phenanthrenes and anthracenes) and 4 ring (flouranthenes and pyrenes) PAHs displayed an inverse relationship between the degree of alkylation and their relative concentration in coal tar (i.e. un-substituted

naphthalene > 1 carbon substituted naphthalene > 2 carbon substituted naphthalene > 3 carbon substituted naphthalene etc.). In respect to benzene, 2 carbon-substituted benzene dominated.

2.3. PAHs

PAHs are the product of incomplete combustion, i.e. the burning of fossil fuels, forest fires, coal tar, creosote, diesel and cigarette smoke. These organic chemicals are found almost everywhere and pose a risk for human health because of their potentially carcinogenic nature and bioavailability in water, soil, sediment, and air resources that humans come in contact with daily.

Because PAHs are emitted naturally through forest fires and volcanoes, microorganisms have the ability to breakdown PAHs and contaminated sites can be remediated through microbial and environmental manipulations. Previous research on remediation of PAH contaminated resources have been conducted in situ, ex situ in bioreactors, or in a lab setting with soils spiked with various levels of PAHs.
The following section provides information on:

- PAH chemistry;
- behaviour and PAH concentration ranges in different environments;
- PAH bioavailability;
- transfer, degradation and sequestration of PAHs in sediments and soils;
- biological techniques for enhancing PAH degradation;

2.3.1. Chemistry of PAHs

PAHs are products of incomplete combustion of organic material. High temperatures (i.e. coking process) create simple PAHs, low temperatures (i.e. smouldering) result

in more complex PAHs (Harvey, 1997). PAHs are composed of two or more rings of carbon and hydrogen atoms bonded in either a linear, angular or clustered way (Sims and Overcash, 1983; Harvey, 1997). In general, PAHs are considered organic pollutants that are widely distributed in the environment, are toxic, and very persistent (Cerniglia, 1992; Haeseler et al., 1999). PAHs are formed and released into the environment through both natural and anthropogenic sources. Natural sources include volcanoes and forest fires. Anthropogenic sources include (Harvey, 1997):

- combustion of fossil fuels, including motor vehicle emission and power generation;
- wood burning;
- municipal and industrial waste incineration;
- coal-gasification and coke production processes;
- coal tar, coke, asphalt, crude oil, creosote, asphalt roads, roofing tar;
- discharges from industrial plants and waste water treatment plants;
- smoke houses;
- aluminium and steel production plants;
- atmospheric contamination of leafy plants;
- cigarette smoke;
- charbroiled meat.

Anthropogenic sources are the primary source of PAHs in atmospheric pollution (Grimmer, 1983). Activities like power generation, refuse burning, and coke production provide 50% of the annual benzo[a]pyrene (BaP) emissions, which are widely used as a standard of PAHs emissions. The emissions of PAHs by vehicles are believed to be responsible for up to 35% of the total emission in highly populated urban areas of the United States (Dabestani and Ivanov, 1999). Diesel power is a source of lighter molecular weight PAH, while gasoline power is a major source of heavy molecular weight PAHs (Juhasz and Naidu, 2000). Although most PAHs enter the environment via the atmosphere, sediment and soil is the primary environmental repository (Dabestani and Ivanov, 1999; Juhasz and Naidu, 2000).

Molecular weight, structure, water solubility, and vapour pressure of each PAH compound affects the potential for transfer (i.e. volatilization, absorption, leaching and erosion) degradation (biological and chemical), and sequestration (adsorption and diffusion) (Reid et al., 2000a). Understanding the fate of each PAH compound in the natural environment based on its specific characteristics is important in determining appropriate remediation techniques.

2.3.2. Molecular weight

PAHs are classified as low molecular weight (LMW) if they have two or three fused rings or high molecular weight (HMW) if they have four or more fused rings. LMW PAHs are degraded and volatilized more rapidly than HMW PAHs (Harvey, 1997). As molecular weight increases, hydrophobicity/lipophilicity increases, water solubility decreases, vapour pressure decreases, and the compound will have a more recalcitrant structure. High molecular weight PAHs persist in the environment because of low volatility, resistance to leaching, and recalcitrant nature (Wild and Jones, 1995). Molecular weights of the 16 US EPA PAHs are provided in Table 1.

2.3.3. Structure

PAHs are also classified into two groups based on ring structure: alternant and non-alternant.

Alternant PAHs such as anthracene (ANT), phenanthrene (PHE), and chrysene (CHR) are derived from benzene by fusion of additional six-membered benzoid rings, and contain fewer than eight benzoid rings (Harvey, 1997).

Non-alternant PAHs may contain rings with fewer than six carbon atoms in addition to six membered rings. This group is extremely broad in structure, which increases PAHs diversity and properties (Harvey, 1997).

Examples of four-, five-, and six-membered rings are fluorine and fluoranthene (FLT) (Harvey, 1997; Dabestani and Ivanov, 1999). CHR consists of four fused, six carbon benzene rings. FLT, on the other hand, contains naphthalene (N) and a benzene unit connected by a five-membered ring (in the center of the structure) and is indicative of lower temperature and less efficient combustion. Information on number of rings for select PAH compounds are provided in Table 2.1.

Table 2.1. US EPA's 16 priority pollutant PAHs and selected properties (adapted from Lundstedt, (2003) and Bojes and Pope, (2007).

PAHs	**Abbreviation**	**N° of rings**	**Molecular weight (g/mole)**	**Solubility in water (mg/L)**	**Vapor pressure (Pa)**	**Log K_{ow}**
Naphthalene	N	2	128.17	31	11.866	3.37
Acenaphthene	ACE	3	154.21	3.8	0.500	3.92
Acenaphthylene	ACY	3	152.2	16.1	3.866	4.00
Anthracene	ANT	3	178.23	0.045	3.40×10^{-3}	4.54
Phenanthrene	PHE	3	178.23	1.1	9.07×10^{-2}	4.57
Fluorene	FLU	3	166.22	1.9	0.432	4.18
Fluoranthene	FLT	4	202.26	0.26	1.08×10^{-3}	5.22
Benz[a]anthracene*	BaA	4	228.29	0.011	2.05×10^{-5}	5.91
Chrysene*	CHR	4	228.29	0.0015	1.04×10^{-6}	5.91
Pyrene	PYR	4	202.26	0.132	5.67×10^{-4}	5.18
Benzo[a]pyrene*	BaP	5	252.32	0.0038	6.52×10^{-7}	5.91
Benzo[b]fluoranthene*	BaF	5	252.32	0.0015	1.07×10^{-5}	5.80
Benzo[k]fluoranthene*	BkF	5	252.32	0.0008	1.28×10^{-8}	6.00
Dibenz[a,h]anthracene*	DBA	6	278.35	0.0005	2.80×10^{-9}	6.75
Benzo[g,h,i]perylene*	BP	6	276.34	0.00026	1.33×10^{-8}	6.50
Indeno[1,2,3-cd]pyrene*	IP	6	276.34	0.062	1.87×10^{-8}	6.50

*US EPA PAHs classified as possible human carcinogens.

2.3.4. Solubility

Solubility of PAHs in water is dependent upon temperature, pH, ionic strength (concentration of soluble salts), and other organic chemicals (i.e. dissolved organic

carbon) (Pierzynski et al., 2000). Solubility is estimated by chemical structure and octanol-water partition coefficients (Pierzynski et al., 2000).

Chemical structure

In general, as the number of benzene rings in a PAH compound increases, solubility decreases (Wilson and Jones, 1993). Symmetry, planarity, and the presence of substituents affect PAH solubility in organic solvents. Solubility has been found to increase in linearly-fused PAH as the number of rings increase because the bonds become weaker (olifinic) in character, but has not observed in angularly-fused PAHs (Harvey, 1997). Planar PAHs are less soluble and biologically less toxic (Dabestani and Ivanov, 1999). Substituted PAHs are those in which a functional group in the compound has been replaced with another functional group. For example, in a methyl-substituted PAHs, one of the functional groups has been replaced by a univalent compound with the general formula CH_3^-. Alternant PAHs that are planar and symmetrical require a relatively high energy of solubilisation because of their ability to fit closely in a lattice. Thus, they tend to be less soluble (Harvey, 1997). As the compounds deviate from planarity or symmetry they tend to be more soluble in organic solvents. Methyl and polar substitution may also increase the solubility of PAHs in certain solvents (Harvey, 1997). Most by-products of PAH biological and chemical degradation tend to be more polar and have higher solubility in the environment than the parent compounds.

Octanol-water partition coefficients

There is a substantial amount of data on the relationship between aqueous solubility and octanol-water partition coefficients (K_{ow}) for the partitioning of PAH between water and organic matter in soils (Mackay and Callcott, 1998). There is an inverse relationship between K_{ow} and solubility which is determined with the following equation (Eq 2.1):

(Eq. 2.1) K_{ow} = OC in octanol (mg/L) / OC in water (mg/L)

Where OC is the amount of organic chemical in octanol. The octanol-water coefficient is often expressed as the log K_{ow}. Naphthalene has a log K_{ow} of 3.37, while the K_{ow} of IP is 6.50. In this case, N is more soluble than IP. This is also in agreement with the influence of chemical structure on solubility. Solubility and K_{ow} of select PAH are provided in Table 2.1.

2.3.5. Vapour pressure

Vapour pressure defines the point at which PAHs in the solid state either evaporate into a gaseous form or condense back to a solid state. The higher the vapour pressure (at normal temperatures), the more volatile the compound is N (11.866 Pa) and is more volatile and would readily evaporate more rapidly than DBA (2.80×10^{-9} Pa) at normal temperatures (Mackay and Callcott, 1998). PAHs vapour pressures are important for determining risk associated with sediments, transfer between two resources (soil and air) as well as field sampling and lab safety. Vapour pressures of select PAH are presented in Table 2.1.

2.3.6. Priority PAHs

The U.S. EPA has placed 16 PAH compounds on the Priority Pollutant List created under the Clean Water Act (U.S. EPA 2002). Pollutants are chosen for this list because of potential for toxicity and frequency of occurrence in hazardous waste. Seven of these 16 PAHs are considered to be possible or probable carcinogens (U.S. EPA 2002). Molecular structures of these PAHs can be found in Figure 2.2.

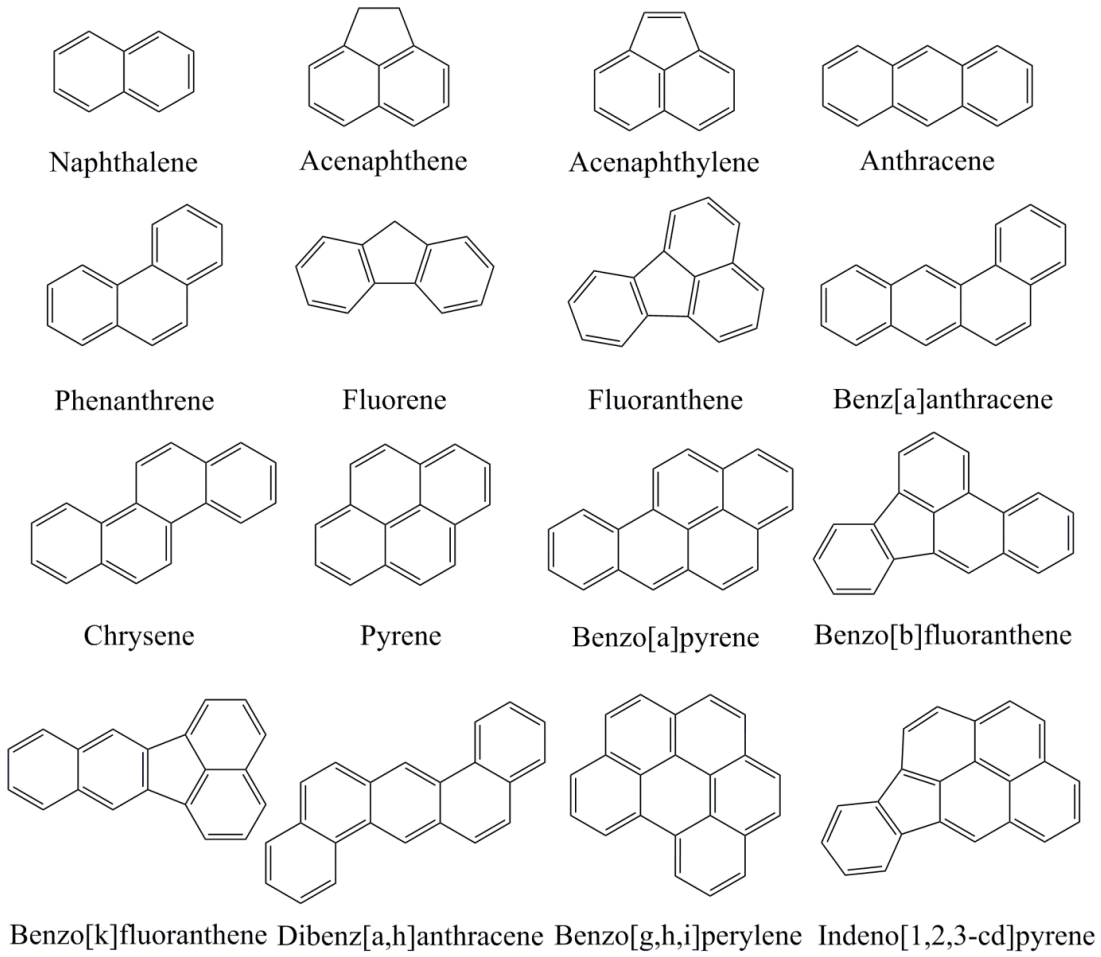

Figure 2.2. Structures of US EPA's 16 priority pollutant PAHs (ChemBioDraw Ultra 14.0).

2.3.7. PAHs in the environment

PAHs are widespread and found in air, water, terrestrial and biological systems (Cerniglia, 1992; Haeseler et al., 1999). Additionally, PAH compounds transfer between these resources, i.e. leaching of PAH from a soil resource into ground water, or transport of particulate soil PAH in the atmosphere. The following section describes contamination in atmosphere, water, sediment, soil and biological (plants and humans) systems and ranges of contamination found in each system.

PAHs in the atmosphere

Many PAHs enter in water, sediment, soil and biological resources through the atmosphere. Wide ranges in atmospheric PAH concentrations have been measured, with the highest concentrations occurring in urban areas. Atmospheric PAH levels are usually higher in the winter because of combustion products from heating and reduced thermal- and photo-decomposition (Greenberg et al., 1985; Harvey, 1997; Juhasz and Naidu, 2000).

In North America, PAH concentrations in the air range from 3.7 - 450 $ng \cdot m^{-3}$. Phenanthrene, fluoranthene and pyrene usually dominate the atmospheric PAH profile (Arey and Atkinson, 2003). Nitrated PAH compounds are formed by the gas-phase reaction of PAHs with nitrous oxides in the atmosphere (Arey, 1998). Nitrated PAHs are found at lower concentrations than non-nitrated PAHs in the atmosphere (typically in the low $ng \cdot g^{-1}$ range), but are of concern because of their persistence in the environment and their mutagenic and carcinogenic potential, which is generally higher than that of non-nitrated PAHs (Bamford et al., 2003).

PAHs in the atmosphere are either present in the gaseous phase or associated with particulates and tend to condense onto particles at temperature below 150°C temperatures (Schure and Natusch, 1982). At ambient temperatures, most atmospheric PAHs are in the particulate phase. The partitioning of PAHs into gas and particulate phases also depends on vapor pressure of the specific PAH (Wania and Mackay, 1996). At ambient air temperatures, 2- to 4-ring PAHs and 2-ring nitrated PAHs are predominant in the gas phase, while PAHs with 5 or more rings and 4-ring nitrated PAHs are associated with particulates (Arey and Atkinson, 2003). The fate of atmospheric PAHs is influenced by whether the PAHs are in the gaseous or particulate form. The residence time of small particles in the atmosphere can be one to two weeks, which would permit long range transport assuming there is no precipitation to cause wet deposition (Atkinson and Arey, 1994).

PAHs in water

The main sources of PAHs in water bodies are atmospheric particulate matter deposition, runoff of polluted ground sources and pollution of river and lakes by

industrial effluents, municipal wastewater discharge, and oil spills (Latimer and Zheng, 2003; Dabestani and Ivanov, 1999). Since PAHs have low solubility and tend to adsorb to particulate matter, they are usually found in low concentrations in water bodies. Some PAH concentrations that have been measured in water include: marine waters with levels of non-detected to 11 μg/L, wastewater in North American and European municipalities with levels of < 1 to 625 μg/L and urban runoff in the US with levels of < 0.05 to 560 μg/L (Latimer and Zheng, 2003).

PAHs in sediments

PAHs tend to accumulate in sediments rather than water (Meador et al., 1995; Juhasz and Naidu, 2000). Concentrations of PAHs in a particular sediment can range from $\mu g \cdot kg^{-1}$ to $g \cdot kg^{-1}$ levels depending on the proximity of the area to PAH sources such as industries, municipalities, and on water currents. In North America, total PAH concentrations in marine sediments usually range from 2.17 to 170,000 ng per g of sediment (Latimer and Zheng, 2003). Sediment core studies have shown an increase in PAH concentrations in the past 100-150 years with concentrations peaking in 1950 (Meador et al., 1995). PAHs profiles in sediments are usually dominated by the more hydrophobic 4-, 5-, and 6-ring compounds.

PAHs in soils

Accumulation of PAH in soils without direct industrial contamination is believed to result mainly from atmospheric deposition after long-range transport (Greenberg et al., 1985). Forest fires and airborne pollution deposition are the main source of soil PAHs in remote areas. Soil levels of PAHs resulting from natural processes are estimated to be in the range of 1-10 $\mu g \cdot kg^{-1}$ (Edwards, 1983). Jones et al. (1989a) reported a total PAH concentration of 0.1-55 $mg \cdot kg^{-1}$ in Welsh soils that resulted strictly from atmospheric deposition with no direct industrial pollution. Levels of PAHs in soils have increased in the past 100-150 years because of growing industrial activities.

PAHs concentrations in urban industrial soils can be 10-100 times higher than in remote soils (Wild and Jones, 1995). In soils at industrial sites, PAH concentrations

and type of PAH found vary depending on the type of industry. For instance, Juhasz and Naidu (2000) in a review of the literature, reported total PAH concentrations of 5,863 $mg \cdot kg^{-1}$ at a creosote production site, 18,704 $mg \cdot kg^{-1}$ at a wood preserving site, 821 $mg \cdot kg^{-1}$ at a petrochemical site, and 451 $mg \cdot kg^{-1}$ at a FMGP site. The major pathway of PAH loss in soil is degradation by microbial metabolism. In a recent study of Scottish surface soil, benzo[a]pyrene was the predominant PAH encountered (Rhind et al., 2013). The preponderance of heavier HMW PAHs in Scottish soil samples probably reflects the deposition of particle-bound PAHs together with microbial degradation and volatilisation of the lower molecular weight PAHs (Rhind et al., 2013).

The physical and chemical properties of the particular PAH compound being degraded will affect this process, as well as environmental factors such as soil temperature, moisture, pH, and oxygen concentration (Manilal and Alexander, 1991; Weissenfels et al., 1992). Sims and Overcash (1983) concluded that photolysis, hydrolysis and chemical oxidation processes did not contribute measurably to loss of PAHs from soil, but Wild and Jones (1993) reported some abiotic loss of LMW PAHs from soil by volatilization. Degradation by various mechanisms will be discussed in the next section.

PAHs in plants

PAHs accumulate in vegetation mainly through atmospheric deposition on and uptake by above-ground parts of the plant. Concentrations of PAHs in plant tissue in non-industrialized regions range from 50-80 $\mu g \cdot kg^{-1}$ (Edwards, 1986), although specific plant tissue concentrations will depend on plant species, type of PAH, and environmental conditions (Salanitro et al., 1997). Vegetation in urban areas can have up to 10 times higher PAH levels than rural vegetation (Juhasz and Naidu, 2000). Sims and Overcash (1983) and Edwards (1986) found that PAHs can be adsorbed onto plant roots, but translocation to the above-ground parts was negligible because plants are unable to transport hydrophobic compounds such as PAHs in xylem.

PAH in humans

The carcinogenicity of PAHs was first discovered in the mid 1930's and gave way to new research on the influences of PAHs on human health and the environment. Data concerning human exposure and carcinogenicity of PAHs have been from occupational workers exposed to PAHs during coke production, roofing, oil refining and coal gasification (Wynder and Hoffman, 1967; Maclure and MacMahon, 1980). However, human exposure is not limited to inhalation, absorption or ingestion through working environments. Nearly every person is exposed to PAHs on a daily basis due to widespread atmospheric distribution. As stated earlier, PAH concentrations are generally higher in the winter months as a result of fossil fuel combustion (Harvey, 1997), making human exposure during this time higher than in summer months.

Particulate matter containing PAHs are a major contributor to accumulation of PAHs in urban areas. A study in Taiwan found human exposure through inhalation to be between 0.4 and 1.55 ng per day (Kuo et al., 2003). Menzie et al. (1992) found PAH levels to be 15-50 $ng \cdot m^{-3}$ in urban areas. In addition to particulate matter, inhalation of cigarette smoke greatly increases human exposure to PAHs. Tobacco smoke contains more than 150 compounds in the gas phase and > 2000 in the particulate phase (Harvey, 1997), making tobacco use the single greatest factor contributing to respiratory cancers. Humans are also exposed to PAHs through ingestion and absorption. Drinking water has been found to contain 1-10 ng of $PAH \cdot L^{-1}$ and high levels (10's of ng of $PAH \cdot kg^{-1}$) have been found in leafy vegetables, grains, fats, oils, grilled and smoked meats (Menzie et al., 1992). It is suspected that humans inhale, ingest and absorb 1-5 ng PAH per day from food, 0.02-3.0 ng PAH per day from air, and 0.0002-0.12 ng PAH per day from water (Menzie et al., 1992).

2.4. Biodegradation of PAHs

PAHs transport, degradation and sequestration in the environment are dependent upon their bioavailability. There is no universally accepted definition of bioavailability (Harmsen, 2007; Semple et al., 2007); however, it is generally acknowledged to be related to the possibility of a substance to negatively or positively affect an organism (Pierzynski et al., 2005). Reliable estimates of PAH bioavailability are extremely important for understanding the adverse effects of PAH, but adequate tests to evaluate bioavailability in dredged sediments and contaminated soils are lacking. PAH bioavailability is affected by (Harmsen, 2007):

- physical properties of the specific PAH compound (LMW, HMW);
- soil characteristics (clay and organic matter content, structure);
- receptor organisms (bacteria, earthworms, arthropods etc).

First of all, HMW PAHs are more recalcitrant and less bioavailable in the environment than LMW (Cerniglia, 1992). Low molecular weight PAHs are removed faster by physicochemical and biological processes due to their higher solubility and volatility and the ability of different microorganisms to use them as a sole carbon source (Alexander, 1999). Bioavailability will change with time and weathering stage (Uyttebroek et al., 2007).

Soil properties like organic matter content, texture and aggregation also influence PAH bioavailability. In addition, heterogeneity of soil greatly influences PAH bioavailability (Eweis et al., 1998). Nam and Alexander (1998a) found declining PAH bioavailability to PHE degraders with time in soils with > 2% OM. Hundal et al., (2001) reported on the retention of large amounts of PHE by smectite clays. Soil structure, such as aggregation has also been found to decrease PAH availability through physical protection of PAHs on the interior of aggregates (Wu and Gschwend, 1986). However, PAH availability is not always correlated to single or multiple soil properties such as organic matter content and/or clay content, making it hard to predict (Chung and Alexander, 2002). Bioavailability also varies based on

receptor organisms (Alexander, 1999). For example, in an environment dominated by a certain microbial population, a compound may be bioavailable and readily degraded; however, in that same environment with a different microbial population, that same compound may not be bioavailable to that specific group of microorganisms and the compound will persist in the soil (Alexander, 1999). Interestingly, some microorganisms have evolved mechanisms to overcome decreased bioavailability in soils and sediments such as production of biosurfactants to increase contaminant solubility or adhesion to the surface of the contaminant making it more accessible to degradation. Consideration of PAH bioavailability based on physical characteristics of the PAH compound, sediment/soil characteristics and receptor organisms is important in the remediation of dredge sediments. The composition of PAH compounds might change during dewatering, where some LMW PAHs volatilize and HMW persist, sorption to organic matter and clays may occur as the sediments age and the microbial communities will undoubtedly change as the sediment shifts from anaerobic to aerobic.

2.4.1. PAH degradation processes

PAH compounds in the environment are subject to a number of processes that may influence their behaviour, chemical composition and isotopic signature. In particular, PAHs can be transferred, degraded and sequestered in soils and sediments (Table 2.2). Transfer is the process by which PAHs are relocated without altering their structure, degradation is the process where PAH structures are altered from their original form, and sequestration occurs when PAHs are removed from bioavailable pools and stored for long periods of time.

Table 2.2. Movement and fate of organic chemicals, such as PAHs, in the environment

Process	Consequence	Factors
Transfer (processes that relocate PAHs without altering their structure)		
Volatilization	Loss of PAHs due to evaporation from soil, plant, or aquatic ecosystems	Vapour pressure, wind speed, temperature
Absorption	Uptake of PAHs by plant roots or animal ingestion.	Cell membrane transport, contact time, susceptibility, plant species
Leaching	Translocation of PAH either laterally or downward through soils	Water content, macropores, soil texture, clay and organic matter content, rainfall intensity, irrigation
Erosion	Movement of PAH by water or wind action	Rainfall, wind speed, size of clay and OM particles with adsorbed PAH on them
Degradation (processes that alter the PAH structure)		
Biological	Degradation of PAHs by microorganisms, biodegradation and cometabolism	Environmental factors (pH, moisture, temperature, oxygen), nutrient status, OM content, PAH bioavailability, microbial community present, molecular weight of PAH (LMW or HMW)
Chemical	Alteration of PAHs by chemical processes such as photochemical (i.e. UV light) and oxidation-reduction reactions	pH, structure of PAH, intensity and duration of sunlight, exposure to sunlight, and same factors as for microbial degradation
Sequestration (processes that relocate PAHs into long-term storage without altering structure)		
Adsorption	Removal of PAHs from bioavailable pools through interaction with soils and sediments	Clay and organic matter content, clay type, moisture
Diffusion	Diffusion of PAH into soil micropores where it is unavailable for microbial degradation	Hydrophobic nature of micropores and PAH

Degradation rates of PAHs vary depending on molecular weight and solubility of the PAH compound (Alexander, 1999). The ultimate goal of degradation is the complete mineralization of PAHs to CO_2 and water (Lundstedt, 2003). Unfortunately, degradation of PAHs may result in the accumulation of metabolites (mainly ketones, quinones, dicarboxylic acid anhydrides and coumarins) that can be more toxic and/or more soluble than the parent compound (Lundstedt, 2003). For example, FLT degradation has been found to produce more soluble and potentially leachable metabolites (Vessigaud et al., 2007). Haeseler et al., (1999) showed enhanced, but incomplete, degradation of PAH compounds in a field study and noted a brief spike in leachate toxicity due to the accumulation of more soluble metabolites. However, after remediation was complete, final toxicity was negligible because the metabolites tended to be less stable and more soluble than the parent compounds, making them more available to degraders (Haeseler et al., 1999). Intermediates of PAH degradation are not always bioavailable and can also be incorporated into the humic fraction of soil, making them less available and less toxic (Kastner et al., 1999).

2.4.2. Biological degradation (biodegradation)

Biodegradation is the transformation of organic compounds as a result of microbial activity and can be enhanced with specific management. Since PAHs are widespread in the environment, their degraders can be found in both natural or contaminated sediments and soils. However, the number of degraders and their capabilities are much higher in contaminated soils than uncontaminated soils (Carmichael and Pfaender, 1997).

Before discussing remediation techniques, it is important to identify the different microorganisms capable of degrading PAHs. Bacterial, fungal and algal species have been found to degrade PAH compounds, with bacteria constituting the most important group of degraders (Cerniglia et al., 1992; Kastner et al., 1994). Numerous bacteria genera and species can degrade 2- or 3-ring PAHs have been identified, but few genera have shown ability to degrade HMW PAHs (Table 2.3). However, some

Pseudomonas species have been found to degrade 4-ring and 5-ring PAHs (Juhasz and Naidu, 2000).

Table 2.3. Some genera of PAH-degrading microorganisms (adapted from Frick et al., (1999)).

Bacteria	**PAH compounds degraded**
Acidovorax	PHE, ANT
Alcaligenes	PHE, FLU, FLT
Arthrobacter	Benzene, N, PHE
Mycobacterium	PHE, PYR, BaP
Pseudomonas	PHE, FLT, FLU, BaP
Rhodococcus	PYR, BaP
Sphingomonas	PHE, FLT, ANT

As stated earlier, biodegradability of PAHs slows as the number of rings increase (Alexander, 1999). HMW PAHs are more recalcitrant because of their very low solubility, decreased bioavailability and high stability (Cerniglia, 1992; Wilson and Jones, 1993; Juhasz and Naidu, 2000). HMW PAHs have a higher Log K_{ow} and tend to partition to solids. Low bioavailability of residual PAHs after initial rapid losses of LMW PAH compounds often limits further degradation (Erikson et al., 2003). In soil, PAHs generally undergo a two-phase loss process (Alexander, 2000; Reid et al., 2000a; Semple et al., 2007; Niqui-Arroyo and Ortega-Calvo, 2007):

- rapid initial loss of LMW PAHs to degradation and transfer to the atmosphere through volatilization. This removes the more labile fraction and results in a HMW PAH-dominated profile;
- slower loss as contact time increases (PAH aging).

For example, Uyttebroek et al., (2007) reported a biphasic loss upon spiking weathered contaminated soil with PHE and PYR. There was rapid degradation and volatilization of PAHs during the first 30 days, followed by slow but continuous degradation rates so that almost all the spiked amount was mineralized during the

140-day experimental period. For rapid biodegradation, PAH compounds must be in the aqueous phase where microorganisms can utilize them as a substrate (Ogram et al., 1985; Rijnaarts et al., 1990; Volkering et al., 1992; Bosma et al., 1997). Mass transfer through diffusion to the aqueous phase is the rate limiting step in biodegradation in soils because it often controls PAH availability to microorganisms (Volkering et al., 1992). Diffusion of PAHs into the aqueous phase can be modelled by Fick's First Law of Diffusion (Eq. 2.2).

Fick's First Law of Diffusion:

(Eq. 2.2) $$Q / t = -DA(C_o-C_x) / x$$

Where:
Q = quantity of substrate (mol)
A = area (m^2)
t = time (seconds)
(C_o-C_x) = concentration gradient, Co is the concentration at the source ($mol \cdot m^{-3}$) and C_x is the concentration at the sink ($mol \cdot m^{-3}$)
x = diffusion path length (m), distance between source and sink
D = diffusion coefficient (m^2/s) for resistance of environment to diffusion

Ideal biodegradation of PAHs, where they are the sole bioavailable carbon source, can be broken into three distinct phases based on diffusion of PAH into the aqueous phase (Johnsen et al., 2005):

I. Exponential growth phase
II. Pseudo-linear growth as the dissolved PAH concentrations stabilize but microbial biomass still increases linearly
III. Pseudo-stationary phase where biomass, PAH dissolution and concentrations all stabilize

This ideal situation is complicated in soil because of adsorption of PAH to organic matter and diffusion into small pores (both mechanisms of PAH sequestration). It is suspected that microbial communities in soils remain in Phase III (pseudo-stationary) as a result of the rate limiting diffusion of PAHs into the aqueous phase (Johnsen et al., 2005) (Fig. 2.3). Transfer to the aqueous phase is especially important for HMW hydrophobic compounds with low aqueous solubility which tend to partition to the solid phase (especially organic matter) in soils and sediments.

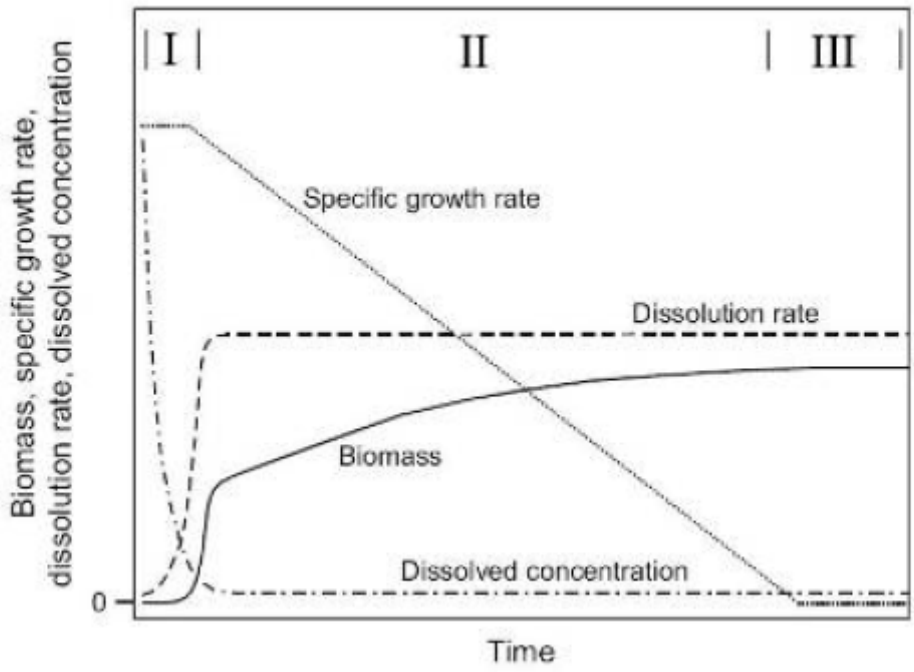

Figure 2.3. Schematic diagram for microbial biomass, specific microbial growth rate, PAH dissolution rate and dissolved PAH concentration of bacterial batch grown on solid PAHs (Johnsen et al., 2005).

Microbial communities (including bacteria and fungi) can biologically degrade PAHs during direct microbial metabolism of carbon and energy sources or by cometabolism while consuming another substrate (Lundstedt et al., 2007). Biological degradation is highly dependent upon several abiotic soil factors, including (Eweis et al., 1998) nutrients, pH, metals, temperature, moisture, salts.

The amount of nutrients present and the state of the nutrients (organic, inorganic) is important for biodegradation. Addition of nutrients through fertilizers can enhance biodegradation. Soil pH also impacts microbial activity and can alter the community composition (i.e. fungal vs bacterial dominated). The ideal range for bacteria is generally between 6 and 8 while fungi dominate degradation at pH < 5.5 (Eweis et al., 1998). However, some species of bacteria such as sulphur-oxidizing bacteria are well adapted to acidic environments.

Soil pH also influences the mobility of nutrients and metals. For example, phosphorous solubility is maximized at pH 6.5 and metal mobility minimized at pH>6 (Sims et al., 1990). Availability of metals, which are toxic to some microorganisms, can greatly reduce biodegradation of PAHs. Soil pH can be increased using lime and decreased with elemental sulphur or sulphur containing compounds (sulphuric acid, liquid ammonium polysulphide and aluminium and iron sulphates (Dupont et al., 1988).

The ideal soil temperature range for biodegradation is between 15 and 45°C with maximum rates of biodegradation occurring from 25 to 35°C (Sims et al., 1990).

Moisture is also an important component for biodegradation. A major component of bacterial cells is water which also serves as the transport medium for PAHs in the soil. Most microbes function optimally when soil water is 50 to 75% of field capacity (Eweis et al., 1998).

Salts, often present in dredge sediments, have a negative impact on microorganisms. With increasing levels of salinity, rates of hydrocarbon metabolism decrease (Ward and Brock, 1978). Excessive salts can be removed by leaching the soil. cometabolism.

2.4.3. Aerobic biodegradation

Aerobic PAH biodegradation is well studied and widespread in soil due to the presence of large numbers of aerobic degraders (Pothuluri and Cerniglia, 1994; Shuttleworth and Cerniglia, 1995; Juhasz and Naidu, 2000; Kanaly and Harayama, 2000). Bacteria and fungi degrade PAHs through different pathways shown in Figure 2.4. During aerobic degradation by bacteria, PAHs are oxidized to cis-dihydrodiols through incorporation of an oxygen molecule into the PAH. The cis-dihydrodiols are further oxidized to aromatic dihydroxy compounds (catechols) and then PAH rings are cleaved with intracellular dioxygenases (Cerniglia, 1984; Alexander, 1999; Johnsen et al., 2005).

Oxidation of unsubstituted PAHs with very high thermodynamic stability, often results in PAH compounds that are less stable than the parent compounds and more susceptible to cleavage (Volkering and Breure, 2003). Oxygenase production by bacteria can be increased using biostimulants: for example, salicylic acid is a known inducer of N dioxygenase. Yi and Crowley (2007) found that linoleic acid is a powerful stimulant of PYR and BaP degradation by gram positive bacteria. The addition of humic substances also greatly enhances microbial degradation of PAHs (Holman et al., 2002; Bogan and Sullivan, 2003).

White rot fungi have also been shown to oxidize some PAHs (i.e. ANT, FLT, and BaP) through destabilizing the rings using nonspecific enzymes, lignin peroxidase and Mn-dependent peroxide enzymes. These enzymes produce highly reactive intermediates which are then oxidized to produce quinones (Cerniglia et al., 1992; Juhasz and Naidu, 2000). Cytochrome P450, an enzyme found in almost all life forms, uses PAHs as a substrate for a monooxygenase reaction where oxygen is inserted into the benzene ring during biodegradation by white-rot fungi. The accumulation of fungal degradation by-products of some PAHs, like BaP, is of concern since monooxygenase oxidizes these PAHs to diol epoxides and trans dihydrodiols (the active molecules implicated with the carcinogenicity of PAHs). For instance, Cerniglia and Gibson (1980) found that fungal transformation of BaP

produced BaP-7,8-diol-9,10-epoxides, which are the carcinogenic form of BaP in higher organisms. This occurred within 12 hours during an incubation experiment. Though this transformation has been documented, researchers are still unsure of what happens to the metabolites in a soil environment.

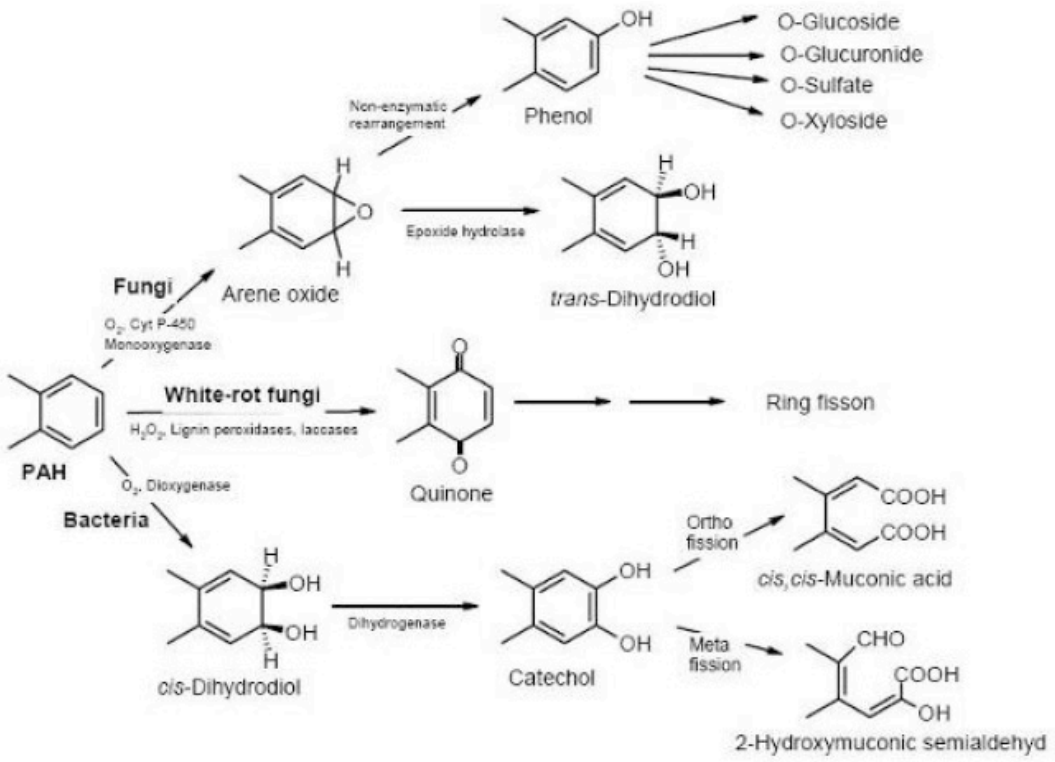

Figure 2.4. Aerobic degradation of PAH by general fungi, white-rot fungi and bacteria (Cerniglia, 1992).

Complete mineralization is common for LMW PAHs; however, partial degradation and transformation of PAHs to metabolites also occurs and leads to an accumulation of by-products (Cerniglia, 1992). Ketones and quinones are common products of aerobic degradation (Lee and Hosomi, 2001; Kochany and Maguire, 1994; Mallakin et al., 1999), and hydroxylated polycyclic aromatic acids result from the partial degradation and/or transformation of some HMW PAHs (Kelley et al., 1993; Roper and Pfaender, 2001) (Table 2.4).

Table 2.4. Products of biodegradation (Alexander, 1999).

Substrate	Products	Reference
Acenaphthylene	1,8-Napthylenedicarboxylic acid	Komatsu et al. (1993)
Anthracene	3-Hydroxy-2-naphthoic acid	Rogoff and Wender (1957)
Fluorene	Phthalic acid	Grifoll et al. (1994)
Naphthalene	2-Hydroxybenzoic acid	Liu et al. (1992)
Phenanthrene	1-Hydroxy-2-naphthoic acid	MacGillivray and Shiaris (1994)

2.4.4. Anaerobic biodegradation

Anaerobic biodegradation of PAHs is not as well documented as aerobic biodegradation. PAHs often persist or undergo slow degradation in anaerobic conditions (Alexander, 1999). Complete mineralization of PAHs usually involves three or more species of microorganisms in anaerobic conditions, where in aerobic sediments, only one species of microorganism is often necessary (Alexander, 1999). Abundance of electron acceptors in anaerobic conditions is usually the limiting factor for degradation (Alexander, 1999); however, transformation of 2- and 3-ring PAHs under methanogenic, iron-reducing and sulphate reducing conditions have been reported (Volkering and Breure, 2003).

2.4.5. Anaerobic biodegradation in unconventional gas systems

Microbial or biogenic methane accounts for approximately 20% of the world's natural gas resources (Rice and Claypool, 1981). Biogenic methane is produced during the immature stage of gas formation as a result of the sequential degradation of complex organic matter by the action of a community of microorganisms. The

anaerobic decomposition of organic matter requires the syntrophic cooperation of anaerobic fermenting bacteria and methanogenic Archaeabacteria, which represent the terminal step of this food chain.

Evidence from the phylogenetic analyses of 16S rRNA, has shown that methanogens are an ancient lineage of the phylum *Euryarchaeota* (Hedderich and Whitman, 2006). They are strictly anaerobic, methane-producing Archaea, though within the phylum *Euryarchaeota*, there are also non-methanogenic microorganisms. The current taxonomy, based on 16S rRNA gene sequences, describes five orders of methanogens: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanopyrales*. Organisms from different orders have less than 82% 16S rRNA sequence similarity. Methanogens of different orders possess different cell envelope structure, lipid composition, substrate range, and other biological properties. Orders of methanogens are further divided into 10 families and 31 genera. Organisms with less than 88-93%, and less than 93-95% 16S rRNA sequence similarity are respectively separated into different families and genera (Whitman et al. 1998). However, 16S rRNA libraries of environmental DNA, suggest that there is a large number of taxa are yet to be described. Thus, the current taxonomy is considered a work in progress with plenty of opportunities for improvement (Hedderich and Whitman, 2006).

Despite the limited range of substrate that methanogens can utilise, they are genetically very diverse. They share similar substrates and electron donors, leading to the same final product, but their cellular structure, metabolic regulations and pathways could be remarkably different. Table 2.5 summarise some general characteristics that discriminate among the five orders of methanogens.

Table 2.5. General characteristics of the five orders of methanogens, highlighting the variety of substrates utilisation and cellular lipids. Modified from Liu et al. (2011).

Order	Shape	Substrates	Cell wall	Cellular lipids	
				Core lipids	Polar lipids
Methanobacteriales	Rods, cocci	H_2 + CO_2 (formate, CO, methanol, secondary alcohols)	Pseudomurein protein	Caldarchaeol, Archaeal, hydroxyarchaeol	Glucose, N-acetylglucosamine, myo-inositol, ethanolamine, serine
Methanococcales	Cocci	H_2 + CO_2, formate	Protein	Archaeol, Caldarchaeol, Hydroxyarchaeol, macrocyclic archaeol	Glucose, N-acetylglucosamine, Serine, ethanolamine
Methanomicrobiales	Cocci, rods, spirals, sheathed rods	H_2 + CO_2, formate. (secondary alcohols)	Protein, glycoprotein	Archaeol, caldarchaeol	Glucose, Galactose, Aminopentanetetrol, glycerol
Methanosarcinales	Pseudosarcina, cocci, sheathed rods	Methanol, methylamine, Acetate. (H_2 + CO_2)	Protein, glycoprotein	Archaeol, hydroxyarchaeol, caldarchaeol	Glucose, galactose, mannose, myo-inositol, ethanolamine, serine, glycerol
Methanopyrales	Rods	H_2 + CO_2,	Pseudomurein	Archaeol	nd

In short, biopolymers are hydrolysed and fermented, and the products formed are funnelled to compounds that are used by methanogens. The general pattern of anaerobic mineralisation of organic matter is that fermentative bacteria consume easily degradable compounds such as sugars, amino acids, purines, pyrimidines and glycerol to a variety of fatty acids, CO_2, formate and hydrogen. Then acetogenic bacteria degrade (higher) fatty acids to acetate, formate, CO_2 and hydrogen. These compounds are the substrates for methanogens. These processes take place simultaneously, but because of the different growth rates and activities of the microorganisms involved, the different growth processes are partially uncoupled, resulting in the accumulation of organic acids. Methanogenesis is a dynamic process,

methanogens strongly influence the metabolism of fermentative and acetogenic bacteria by means of interspecies hydrogen transfer (Schink and Stams, 2006; Stams and Plugge, 2009).

Methane is mainly produced through two different metabolic pathways. The first path is via CO_2-reducing prokaryotes, which use hydrogen as the electron donor or energy source and CO_2 as the electron acceptor. The second path is by acetate fermentation. In this case, acetate and hydrogen are used to produce methane and carbon dioxide. For the conversion of more complicated organic substrates to methane, other microorganisms, such as acetogenic and fermentative bacteria, are needed. Figure 2.5 shows the overall process of anoxic decomposition, demonstrating how different anaerobes work syntrophically to convert complex organic compounds into simple molecules such as CH_4 and CO_2.

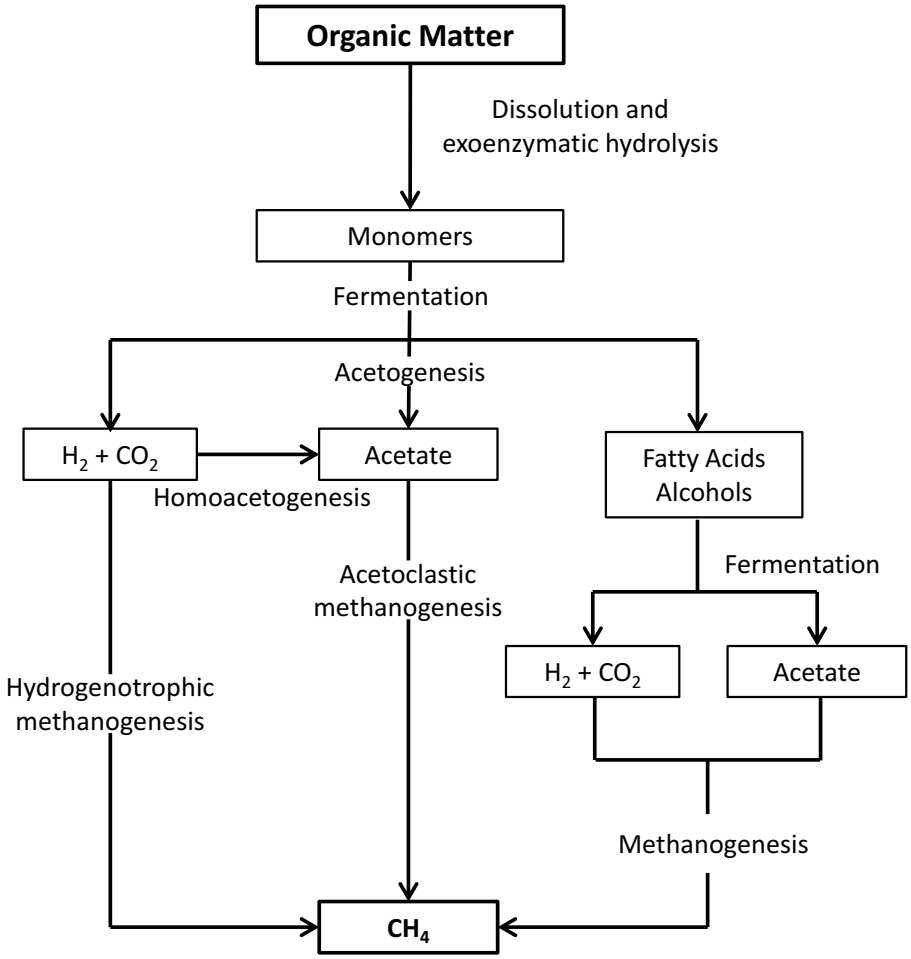

Figure 2.5. Methanogenic pathways. Modified from Liu and Sulfita (1993)

The origins of these microorganisms in the subsurface remain unclear; the most accredited theories regarding the presence of these microorganisms are two: (*i*) the continued presence of ancient microorganisms co-deposited with the sediment and/or (*ii*) the advective transport of surface microbial communities into deep reservoirs due to geological events.

Little is known about the biodegradation steps and complex microbial relationships required to transform organic matter into natural gas as acknowledged by Jones et al., (2008; 2010), Strapoc et al., (2008), McInerney et al., (2009) and Orem et al., (2010). The terminal steps in biodegradation of organic matter is microbial methanogenesis via acetate fermentation (acetoclastic methanogenesis) and CO_2 reduction using H_2 as an electron donor (hydrogenotrophic methanogenesis).

Nowadays there is an increasing interest to this complicated and nearly non accessible environment (Parkes et al., 2000), especially for natural gas exploration and for a better understanding of the global cycling of carbon. Our current understanding of the deep biosphere is poor; this environment is characterized by a highly complex interplay of biological, chemical, and hydrostatic processes operating over a wide range of spatial and a characteristic range of temporal scales (Arndt et al., 2005). The driving force behind the biogeochemical reaction network is the degradation of organic matter. The availability of ready degradable organic matter is often limited to the shallow subsurface, but the organic matter buried in deep shale provides a suitable substrate for microbial activity (Krumholz et al., 1997, 2002).

Further information regarding the microbial degradation of complex organic matter in shale gas and coal bed methane are presented in Colosimo et al., (2016) as published in the thorough review that comprises Chapter 4.

Despite the growing interest on microbial methane generation in organic rich sedimentary rocks, few studies focus on the biogeochemistry of shale. Even though microbial consortia involved in the methanogenic degradation of organic matter in the deep subsurface have been characterized for substrates such as coal formation

waters (McIntosh et al. 2008; Warwick et al. 2008; Jones et al. 2010), or petroleum reservoirs production waters (Magot et al., 2000; Head et al., 2003; Roling et al., 2003; Aitken et al., 2004; Grabowski et al., 2005), there is almost no data available for organic-rich shales.

The research in this field have been done with two main methodology: (*i*) the first is using geochemical indicators such as carbon and deuterium isotopes found in the carbon source, produced waters, methane and carbon dioxide to establish whether the gas is biogenic or thermogenic in origin (Martini et al., 1998, Shurr and Ridgley, 2002). (*ii*) The second are methane generation experiments of biodegradation using samples of coal in laboratory to determine methane production rates (Gieg et al., 2008; Jones et al., 2008).

All the above-mentioned studies have examined the microbial population present in liquid samples, including fracking fluids, formation water and flowback, but none of these was specifically aimed to characterize the microbial population trapped in the solid matrix of the shale. From a microbiological point of view, it is very complicated and laborious to analyse a solid sample such as a shale rock or a shale core, as the microbial population attached to the particles of shale and then trapped in the rock results difficult to be extracted and quantified. This is especially relevant due to the small size of the pores and the nature of the rocks, which allow very low number of bacterial and archaeal cells, making the direct detection of methanogens nearly impossible. Also, the high concentration of organic compounds requires additional steps aiming to clean the samples from organic acids and inorganic particles.

Developing an efficient method for the detection and monitoring of the microbial consortia involved in the production of methane from organic-rich shales is a crucial step to evaluate methane generation potential. Bacteria and Archaea in this case can rarely be quantified directly, as a result of low initial numbers, then probably a culture-based approaches such as biostimulation, could be a method to increase the bacterial and archaeal counts above the detection levels, as well as increase the

methanogenic activity of indigenous micro-organisms, in order to perform kinetic quantifications.

Biostimulation has been used successfully by Jones et al., (2010) for the detection of methanogenic consortia from coal samples, although this approach showed reproducibility issues. Indeed, only a fraction, between 1 in 4 and 1 in 2, of the microcosms evolved towards the production of methane (Jones et al., 2008). The low initial methanogen counts, the heterogeneity of their spatial distribution, as well as the fragility of the methanogenic consortia, which have been shown to be easily disrupted by manipulation (Hatamoto et al., 2007), may explain this variability.

Basically, the number of Archaea and Bacteria in the rocks is too low to allow direct enumerations by the most widely accepted methods such as self-fluorescence observations or qPCR assays, then a culture-based approach is the only possible way to overcome this limit, despite the numerous biases that could bring. In addition, the organic matter in shale and coal is a complex substrate: several aliphatics, aromatics and heteroatoms have been detected in shale and coal, and its composition remains still unknown. The organic fraction of shale and coal is subject to multiple chemical, physical and biological processes, which influence and transform its composition. Determine the composition of the organic matter is essential to establish the biodegradation pathways, especially the pathways that lead to methane production. Figure 2.6 shows different methodologies for the analyses of the microbial community in unconventional gas system

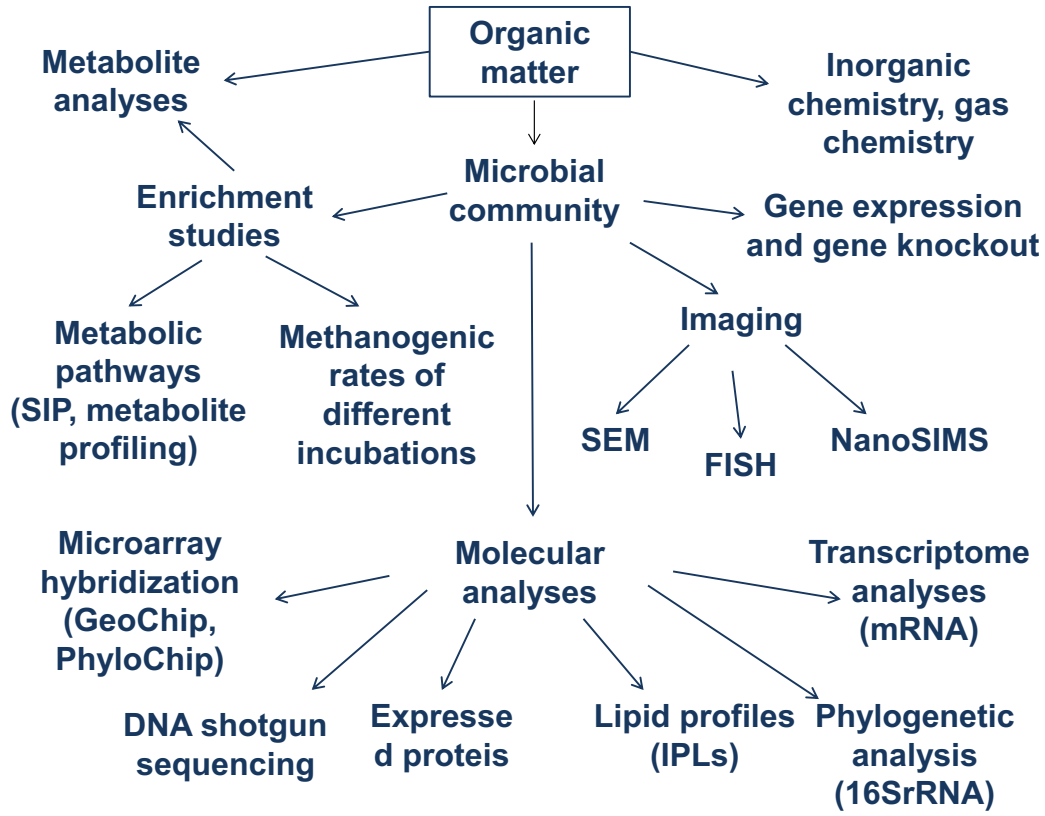

Figure 2.6 Methodologies for the analysis of microbial communities in unconventional gas systems.

2.4.6. Biodegradation in hydrocarbon contaminated environments

Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Ulrici, 2000). Bioremediation technologies are cheaper than other remediation technologies (Leahy and Colwell, 1990). which are usually constrained by mass transfer limitations (Harms and Bosma, 1997) and for PAHs with low aqueous solubility this result in low bioavailability, hence low biodegradation rates (Ramaswami et al., 1997). Coal tar, when released into the

subsurface, migrates downward as a result of gravity until encounters a low permeability layer: contact of groundwater with the coal tar DNAPL results in dissolution of some tar constituents and generation of contaminated groundwater plumes (Birak and Miller, 2009). Inside contamination plumes, the degradation potential for aromatic hydrocarbons is limited or absent due to depletion of dissolved electron acceptors and toxicity of contaminants (Thornton et al., 2001; Takahata et al., 2006). Recently, the plume fringe was assumed to be a hotspot for biodegradation thanks to overlapping countergradients of electron donors and acceptors (Winderl et al., 2008). In this transition zone, hydrocarbons dissolve from the DNAPL to the ambient water and mix with electron acceptors (Fig. 2.7) (Watson et al., 2005; Tuxen et al., 2006). Dissolution and diffusion of some hydrocarbon in water, reduce toxic concentration of contaminants in the plume fringe (Bauer et al., 2008). Moreover, the plume fringe is characterised by high microbial activities and biomass, creating a hotspot for biodegradation (Berkowitz et al., 2004; Anneser et al., 2008). NA of hydrocarbon contaminated sites relies on the use of microorganisms to remediate or attenuate pollution in soil and groundwater; the essential prerequisite is the presence of indigenous microbial population with biodegradative capabilities. The microbial degradation of hydrocarbon, and in particular of PAHs, is mediated by a range of microorganisms, the most often studied are bacteria. Some of the most common bacteria genera encountered during biodegradation of PAHs are *Alicaligenes*, *Pseudomonas*, *Sphingomonas*, *Bacillus*, *Burkholderia*, *Cyanobacter*, *Vibrio* and *Mycobacterium* (Wilson and Jones, 1993; Kastner et al., 1994; Mueller et al., 1997; Ho et al., 2000; Bastiaens et al., 2000; Johnsen et al., 2005). Low molecular weight PAHs are usually biodegraded first, although polyaromatic such as Phenanthrene,

Fluorene and Fluoranthene have been shown to be biodegraded too (Weissenfels et al., 1990). To date, hydrocarbon-degrading bacteria have been isolated and described from a number of polluted environments such as soils, sediments, aquifers, fresh and marine waters. Not surprisingly, the presence of PAH degrading microorganisms has been confirmed at several FMGP sites (Lingle and Brehm, 2003; Bakermans et al., 2002; Zamfirescu and Grathwohl, 2001; D'Affonseca et al., 2008).

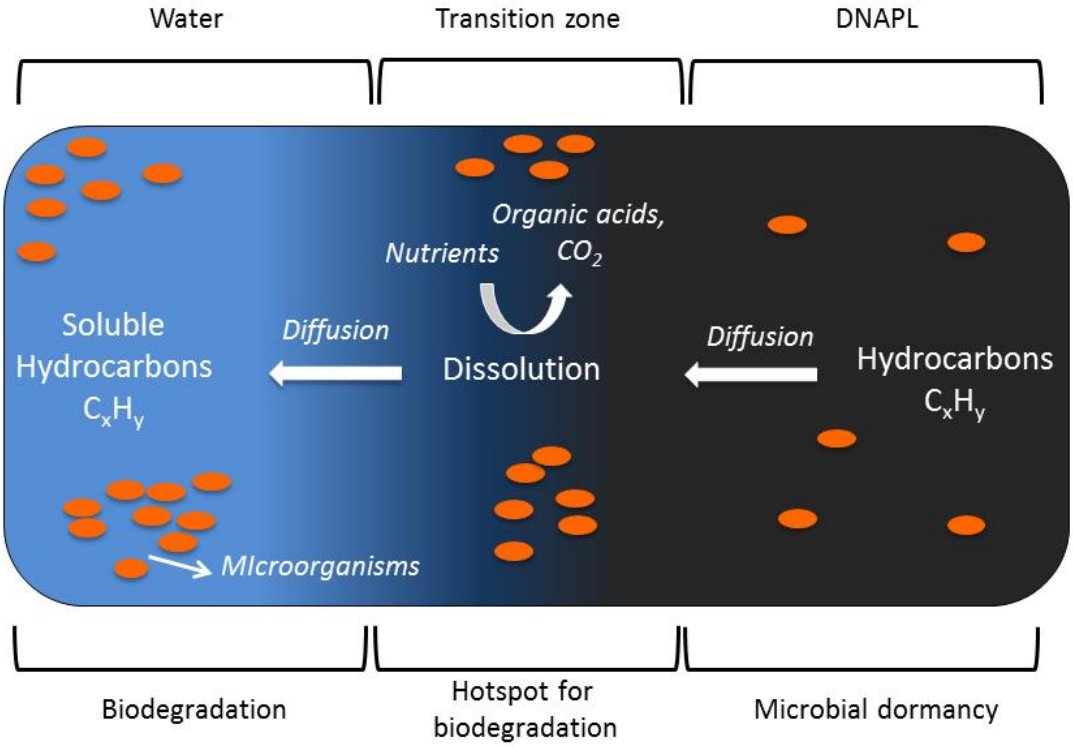

Figure 2.7. Schematic representation of biodegradation at the fringe zone (water/DNAPL interface). Hydrocarbons are utilised by microorganisms as energy source (electron donors) for their metabolic processes. Different electron acceptors such as oxygen, nitrates, sulphates or ferric iron are required for microbial activity. Microorganisms transform hydrocarbons into metabolites such as organic acids and CO_2.

2.4.7. Cometabolism

Cometabolism is the process by which microorganisms transform organic compounds simultaneously despite their ability to use only one of the substrates for energy or nutrition (Alexander, 1999). Organic by-products of cometabolism are not incorporated into the degraders' cells as carbon and remain in the soil matrix for possible utilization by another microbial group or merely persist and accumulate. PAHs are cometabolized when the non-specificity of microbial dioxygenase enzymes leads to the metabolism of a different PAH besides the ones used as a carbon source. For example, a microorganism may be utilizing naphthalene (which is easily degraded) or just simple organic matter as an energy or carbon source and, at the same time, degrading benzo[a]pyrene into other organic substrates or metabolites which then accumulate in the soil.

Cometabolism is explained by three basic reasons (Alexander, 1999):

I. initial microbial enzymes are used to convert a substrate into an organic product which is not further transformed by other enzymes specific to that microorganism. No metabolic intermediates used for biosynthesis or energy production are produced in this scenario and accumulation occurs;
II. initial substrate is transformed into enzyme inhibiting or growth suppressing organic products;
III. an additional substrate that is not available at the time of the reaction is needed by the microorganism to complete the degradation of both substrates.

Cometabolism is considered just another type of microbial transformation (Alexander, 1999). There are two constants in the cometabolism process: (*i*) accumulation rates of metabolic products are consistent as the microbes are not utilizing the substrates as a carbon or energy source, and (*ii*) accumulation of secondary products is evident (Alexander, 1999). In an ideal situation, microorganisms capable of breaking down secondary products are present in soils and completely mineralize the material into CO_2 and biomass (Lundstedt, 2003).

2.5. Biological techniques for enhancing PAHs biodegradation

Biodegradation is the biological transformation of molecules into smaller molecules with less toxicity (preferably water and carbon dioxide which is then termed "mineralization"). Bioremediation is the application of biodegradation to decrease pollutant concentrations (Olson et al., 2003). In this section, techniques to enhance biodegradation of PAHs during bioremediation will be discussed. Several factors may limit the biodegradation of PAH in contaminated soils including (Alexander, 1999; Olson et al., 2003; Straube et al., 2003; Harmsen et al., 2007):

- limited supply of bacterial nutrient or carbon sources;
- non-optimal abiotic conditions of temperature, pH, salts, oxygen concentration and toxins;
- lack of bacterial species that can degrade PAH compounds or low microbial biomass in general;
- low PAH bioavailability to degrading organisms;
- physiochemical characteristics of PAH compound.

Manipulations of the above limitations are the basis for bioremediation with the subsequent goals of: (*i*) improving soil microbial habitat through fertilizer additions, tillage, liming, and/or (*ii*) plant establishment to promote microbial functional groups capable of degrading PAHs, while at the same time (*iii*) increasing the bioavailability of the PAHs. Biostimulation techniques are used to improve the soil microbial habitat and bioaugmentation strategies manipulate the microbial community structure to make it more capable of degrading PAHs.

2.5.1. Biostimulation

Lack of sufficient carbon and nutrient sources to sustain the growth of biodegrading microorganisms may affect bioremediation success (Odokuma and Dickson, 2004; Ward and Singh, 2004). Nutrient and carbon additions can enhance microbial activities which may promote cometabolism. Abiotic conditions can also affect bioremediation, since oxygen is often the most limiting factor in microbial growth. Oxygen can be manipulated through physical (landfarming, composting) and chemical (injection of manganese peroxide) techniques to stimulate microbial communities (Ward and Singh, 2004).

2.5.2. Bioaugmentation

Bioaugmentation is the introduction (or inoculation) of a specific competent microorganism or group of microorganisms to improve the metabolic capacity of the indigenous population of microbes (Gentry et al., 2004). For example, Mycobacterium sp. are known to have extremely lipophilic surfaces so they are better equipped to directly take up organic hydrophobic contaminants such as PAHs (Bogan et al., 2003).

Bioaugmentation can be especially useful in sites with high PAH concentrations, in recently polluted soils with limited adapted microbial populations, or in aged soils/sediments where the PAH profile is dominated by HMW PAHs (Mueller et al., 1989). Successful field-scale applications of bioaugmentation are limited (Alexander, 1999). Bioaugmentation is still experimental with most successful cases reported in confined systems where conditions are controlled to favour the growth of added microbes. There are several reports of the usefulness of bioaugmentation in enhancing bioremediation of contaminated soils (Lendvay et al., 2003; Silva et al., 2004), and others reporting that the procedure has failed to improve biodegradation (Bouchez et al., 2000).

2.5.3. Composting

Composting is a form of biostimulation because it consists of nutrient additions, moisture and oxygen control in a contained system. In a properly-maintained compost pile, temperatures in excess of 55°C are reached which kills most pathogens (Eweis et al., 1998) and is a technique most commonly used for treatment of municipal solid wastes.

In applications for hazardous waste, contaminant organic compound concentrations and extremely high temperatures (> 55°C) cannot support microbial activity in a composting environment and additional organics must be added and temperatures controlled (Eweis et al., 1998). Like all bioremediation techniques, composting is effective only if implemented properly. Optimization of four parameters is necessary for successful PAH degradation during composting; aeration, temperature, moisture and pH.

Composting is a generally aerobic process with microsites of anaerobic activity; therefore, the pile must be aerated through mechanical mixing or additions of bulking agents to improve structure and porosity. Temperature of the pile must remain between 30 and 60°C for optimal microbial community structure, solubility and mass transfer rates (Eweis et al., 1998). Addition of organic material, while maintaining a C:N ratio for 25:1, facilitates higher temperatures in compost piles and biodegradation.

Moisture must be manipulated through water and amendment additions. Most compost piles are able to reach high temperatures with low moisture contents. To obtain optimum moisture content in the pile (50- 80%), a bulking agent (wood chips and sewage sludge) can be added (Epstein and Alpert, 1980).

There are three types of composting systems; windrow, static piles and closed reactors (Eweis et al., 1998). Open systems (windrow and static piles) are more commonly used than the closed reactors. In a windrow system, the compost mixture

is placed on an impermeable layer to prevent leaching of contaminants. The cross section of the pile must be such that high temperatures are maintained on the interior of the pile with minimal heat losses on the outside of the pile. Aeration is achieved through mechanical mixing (front end loader or turner). If the pile is too large to mix, pipes can be installed to increase aeration in the pile. Windrow piles are often covered with a high density, polyethelyne material or wood chips in outside environments to prevent leaching from the pile during rainfall events (Eweis et al., 1998). In static piles, compost material is placed on an impermeable platform with perforated pipes connected to a blower system. Piles are aeriated at either a fixed rate or variable rate depending on the microbial activity. Static piles are also covered with plastic sheeting, but the cover must allow air to enter the pile. Closed reactors are the least used systems because of size limitations, but are the most controlled. Closed systems are constructed as the name implies and contain mixing devices through drum rotation or mixing instruments within the tank (Eweis et al., 1998).

Extensive degradation of 2-, 3- and 4-ring PAHs has been observed during composting at a bench scale; however, five- and six-ring degradation was not observed (Potter et al., 1999). In one study, there was an initial increase in HMW PAHs after 12 days of composting, followed by a decrease after 50 days (Johnson and Gosh, 1998). The initial increase was attributed to contaminant mobilization with soil shearing in the reactor. In general, soil to compost ratios of 2:1 on a dry weight basis produce the highest degradation of PAHs compared to those with higher ratios (Stegmann et al., 1991; Dooley et al., 1995).

2.5.4. Landfarming

Landfarming is a commonly used, passive remediation technology for PAH removal from contaminated soils (Gray et al., 2000; Harmsen et al., 2007). This method became popular in the 1950's for treatment of hazardous material through leaching and volatilization; however, in the 1970's landfarming methods and ideas changed

with increased regulations and the focus switched to biological removal of contaminants (Eweis et al., 1998).

In landfarming, contaminated material is spread evenly over an impermeable layer and treated with standard agricultural techniques (i.e. tillage). It is important to maintain oxygen diffusion throughout contaminated material throughout the treatment, so depth of material is an important consideration as well as tillage strategies. Land treatment units must be established during landfarming of highly contaminated material. These units must have: (1) an impermeable layer, (2) a drainage system, (3) a soil treatment zone, (4) berms and swales, (5) a water storage pond, and (6) a monitoring system. The impermeable layer prevents water movement from the contaminated material to groundwater and can be a synthetic or clay liner or compacted soil. The drainage system collects the leachate and is a series of pipes in a sand layer with drainage to a tank. Soil treatment zones are not applicable to dredge sediments as they are being pumped into a basin and not on top of other soil. Berms and swales protect against cross contamination and are used to contain the material over the nonpermeable layer. Storage ponds collect the leachate and are treated according to contaminants. A monitoring system is necessary to ensure contaminants are retained within the unit. Monitoring systems include wells, and air monitoring systems (Eweis et al., 1998).

The purpose of landfarming is to stimulate indigenous microorganisms to degrade PAHs via (Straube et al., 2003):

- adding nutrients and a carbon source (amendments);
- mixing soil to better distribute amendments;
- introducing oxygen into soil at depth;
- increasing the chance of microbial contacts with contaminants.

The addition of inorganic nutrients compensates for limiting nutrients values in the waste material. The optimum C:N:P ratio for degrading hazardous wastes is reportedly between 100:10:1 (Straube et al., 2003) and 300:10:1 (Eweis et al., 1998).

Adding too much of any nutrient can greatly increase costs. Mixing, associated with tillage, breaks apart soil aggregates, exposes PAHs once entrapped within the aggregates to biotic and abiotic degradation and homogenizes PAH levels in the soil.

During landfarming, it is possible to enhance the bioavailability and degradation rate of the rapidly desorbable fraction in large pores, but not the slowly desorbable fractions associated with organic matter and smaller pores (Harmsen, 2007). Microorganisms cannot enter into pores smaller than their own size, so PAHs strongly sorbed to organic matter are slowly degraded. The biodegradation of the slowly degradable fractions is thus controlled by diffusion (Harmsen et al., 2007). Reducing PAHs to acceptable levels through landfarming can be a lengthy process and often does not result in levels low enough to meet EPA standards. In one study in Holland, rapid degradation of PAH compounds (550 $mg \cdot kg^{-1}$) in dredge sediment occurred within the first year, followed by slow degradation for the next seven years. After 15 years, PAH levels (22 $mg \cdot kg^{-1}$) were similar to the background soils in the same area (Harmsen et al., 2007).

2.5.5. Phytoremediation

Phytoremediation is an on-site remediation strategy that uses plants to reclaim contaminated areas mainly through increasing microbial activity in the rhizosphere while breaking down organic compounds in contaminated soils by metabolic processes (Liste and Alexander, 2000; Binet et al., 2000; Dzantor and Beauchamp, 2002). The rhizosphere is the small volume of soil immediately surrounding plant roots which has much higher concentrations of root exudates, CO_2 pressures, and microbial activity (20x) than surrounding bulk soil (Rovira and Davey, 1974; Hutchinson et al., 2003).

Root morphology factors of various plant species impact phytoremediation success, including: length, surface area, mass, depth of penetration, quantity and composition of dead roots and exudates, root hairs, and bacterial and fungal associations

(Hutchinson et al., 2003). Selecting species with varying root properties can be beneficial during phytoremediation efforts. Plants are thought to enhance PAH degradation mainly through mechanisms such as mobilization into the rhizosphere (thus increasing bioavailability) and enhancing bacterial populations in the rhizosphere (Liste and Alexander, 2000; Binet et al., 2000). Plant roots provide an easily degradable source of carbon to the soil (both through root turnover and exudates) which encourages microbial activity in the rhizosphere and promotes biodegradation of organic contaminants (Olson et al., 2003; Xu et al., 2006), but can also decrease PAH bioavailability through sorption (Hutchinson et al., 2003).

Root contributions of carbon to the transfer 30-60% of new fixed photosynthetic carbon to the roots of which 40-90% is transferred directly into the rhizosphere (Olson et al., 2003). A similar range of photosynthetic carbon, 30-70%, is transferred to the soil in perennial plants, where 25-80% is transferred from the roots to soil (Olson et al., 2003). These contributions of carbon from plants to the soil stimulate microbial communities and thus the degradation of PAHs (Liste and Alexander, 2000; Binet et al., 2000; Xu et al., 2006). Although stimulation of microbial communities in the rhizosphere is the main process for PAH degradation, some studies have revealed that potential uptake and metabolism of PAHs is also possible (Harms, 1996). Uptake is controlled by molecular configuration and size as well as the capability for uptake by a specific plant species (Harms, 1996).

Organic compounds with low water solubility (high log K_{ow}) tend not to be transported within a plant, but organics with log K_{ow} between 0.5 and 3.0 are likely to be transported within a plant (Burken and Schnoor, 1998; Olson et al., 2003). Additionally, plants typically transport LMW PAH compounds or HMW compounds that have been transformed outside the plant in the rhizosphere (Olson et al., 2003).

Phytoremediation also helps reduce the offsite transfer of contaminants by controlling runoff, wind erosion and leaching, and is a non-disruptive self-sustaining process which requires relatively little management. However, phytoremediation is a very lengthy process (1-3 years) that is often limited by phytotoxic contaminant

levels, and is usually used as a secondary treatment in soils contaminated with residual levels of PAH (Joner et al., 2002). The success of the process will depend on environmental factors such as adequate supply of oxygen, water, and nutrients, as well as edaphic factors like the soil texture, pH, EC, and the levels of pollutants (Cunningham et al., 1995).

When selecting phytoremediation species, there are several factors to consider; climate, water availability, salinity, and the presence of other phyto-toxins (Hutchinson et al., 2003). Climate includes the length of growing season, rainfall and temperature patterns. Water availability is influenced by soil texture, bulk density, hydraulic conductivity and is related to climate. Dense clay soils have less water movement or lower infiltration, while sandy soils have greater infiltration and hydraulic conductivities. Sandy soils also drain quickly and have lower water storage. Salt resistant species selection for soils or sediments with high salinity is extremely important for establishment and health of plants. Other toxins are also a concern, as some contaminants can be too toxic in soils to support plants. Obviously, if the contaminant levels are too high, phytoremediation will not be successful (Hutchinson et al., 2003).

2.5.6. Surfactants

Mass transfer of PAH compounds to the aqueous phase in the soil solution can be a major limiting factor in the bioremediation of PAHs (Volkering et al., 1992). Compounds like surfactants, cyclodextrins, and vegetable oil may be used to enhance PAH solubility. Properly applied surfactants have been shown to improve desorption, apparent aqueous mobility and bioavailability of hydrophobic organic compounds such as PAHs (Bragg et al., 1994; Mata-Sandoval et al., 2002).

Surfactants are amphiphilic (possess both hydrophilic and hydrophobic properties) molecules with a hydrophilic polar head (Gao et al., 2007). They can be classified by the charge on their polar head as anionic, cationic, non-ionic or zwitterionic

(Mulligan et al., 2001). At or above a certain concentration level called critical micelle concentration, the hydrophobic parts of the surfactants will tend to associate together to form a micelle (an aggregate of surfactant molecules dispersed in a liquid colloid) with a hydrophobic core (Santharam et al., 1997). Surfactants solubilize hydrophobic contaminants by partitioning them into the hydrophobic core of the micelle. If the concentration of surfactant exceeds the critical micelle concentration, solubility of hydrophobic compounds can increase by an order of magnitude over normal aqueous solubility (Edwards et al., 1991; Mulligan et al., 2001; Gao et al., 2007). The critical micelle concentration of a specific surfactant depends on temperature, ionic strength and surfactant chemistry.

In mixed pollutant systems, the extent of solubilisation will differ from those in single solutes. For example, Guha et al. (1999) found that N solubilized by surfactants increased the solubilisation of other PAHs such as PHE. Surfactants are thought to be able to solubilize sorbed organic compounds in a soil-water system only after critical micelle concentration is attained (Laha and Luthy, 1991; Gao et al., 2007). However, several interactions affect the solubilisation of hydrophobic compounds by surfactants. These include the micellular phase-organic interactions, surfactants monomer-organic interactions in the aqueous phase, and the interactions of surfactants and organic compounds with the solid phase (Mata-Sandoval et al., 2002).

Some of the negative effects of surfactants on biodegradation may be due to toxicity to microorganisms, prevention of bacterial access to contaminants through sequestration of micellar solubilized organics, or preferential biodegradation of the surfactant rather than the contaminant. For instance, PHE solubilized by the surfactant Tween-80 was found to be unavailable for biodegradation by *Sphingomonas paucinobilis* because this microorganism preferred to use the hydrophobic portion of the surfactant as a carbon source rather than destabilizing the micelles.

The determination of a critical micelle concentration for a surfactant in a complex medium such as soil can be difficult. Desorption occurs at surfactant concentrations greater than the critical micelle concentration, but at lower concentrations, admicelles (surface aggregates of surfactants, also called hemimicelles) may form, sorb onto soil, and act as additional sorption sites that can enhance PAH sorption instead of reducing it (Doong et al., 1996, Santharam et al., 1997). Anionic and non-ionic surfactants are more commonly used in remediation because they are less likely to sorb onto soil surfaces (Mulligan et al., 2001). Non-ionic surfactants are also advantageous because they have a low critical micelle concentration, high cold water solubility, and low microbial toxicity (Kim and Weber, 2003; Zhao et al., 2005). Conte et al. (2005) have shown that PAHs can be effectively desorbed using non-ionic surfactants like dodecylbenzene sulfonate.

Biosurfactants

Some synthetic surfactants can be toxic to microorganisms, which may decrease the number of degraders in the soil (Sandbacka et al., 2000). The addition of biosurfactants (either surfactant producing microorganisms or natural compounds that act as surfactants) is one way to get around the problem of toxicity. Biosurfactant-producing microbes have been proposed as an alternative to chemical surfactants to enhance availability of hydrophobic compounds (Hunt et al., 1994; Oberbremer et al., 1990). Surfactants that are produced by microorganisms tend to have lower toxicities and are effective at wider temperature, pH, and electrical conductivity ranges (Bordas et al., 2005). Atlas, (1993) found that biosurfactant-producing indigenous bacteria achieved higher hydrocarbon degradation rates than those achieved by nutrient addition alone.

Cyclodextrins

Cyclodextrins are cyclic oligosaccharides that are able to form complexes with hydrophobic molecules. They are the product of the action of cyclodextrin glycosytransferases on starch and are non-toxic and biodegradable in the environment. Cyclodextrins can be added to washing waters to solubilize PAHs and increase desorption from soil (Stokes et al., 2006).

Vegetable oil

Vegetable oil has been proposed as an economic and environmentally friendly solvent to dissolve PAHs and has been shown to be as effective as organic solvents like acetone and dichloromethane (Gong et al., 2005).

2.5.7. Bioreactors

Treatment of contaminated sediments in a slurry phase is completed in a bioreactor. Advantages of the slurry phase include: complete mixing of nutrients into sediment, increased contact between microorganism and contaminant, control (Eweis et al., 1998). Bioreactors are commercially available for this type of treatment but are expensive. Sediment is often put into the bioreactors in small batches; however, continuous flow operations are possible. With treatment, the slurry is mixed with nutrients and microbial cultures and aerated. The sediment then settles and the water is transferred to a treatment plant while the sediment is returned to a contained area (Eweis et al., 1998). For large quantities of sediment, this is a difficult option.

2.6. Isotopes in PAHs

Molecular compositions of PAH mixtures are frequently determined using gas chromatography mass spectrometry (GC-MS) and gas chromatography with a flame ionization detector (GC-FID) (McRae et al. 2000). These analytical methods are capable of quantifying concentrations and ratios of particular PAH compounds. Molecular signatures can be inconclusive in the absence of other information, however, these molecular analyses are often paired with a newer technique called compound specific stable isotope analysis (CSIA).

CSIA has become an increasingly common and reliable analytical method for PAH source apportionment over the past twenty years. It exploits the isotopic rather than the molecular signature of PAH compounds, a signature which tends to be less subject to interference by weathering processes. CSIA generates isotope ratio data; the main ratio of interest is the $^{13}C/^{12}C$. This ratio is reported using delta notation, which gives the permil (‰) deviation of the isotope ratio of a sample from that of a standard.

The Vienna Peedee belemnite standard (VPDB) is the most commonly used standard for this type of analysis, defining 0‰ on the -scale (O'Malley et al., 1994; Kim, et al., 2005). The primary geochemical concept underlying CSIA involves kinetic isotope effects. These effects determine which isotopes are preferentially incorporated into PAHs during formation or into their organic precursors during photosynthesis. Kinetic effects alter the isotope ratios of the resulting PAHs at each major stage and pathway to formation, as described below. These situations collectively demonstrate that the isotopic and molecular signatures of PAHs are determined both by the isotopic composition of the precursor compounds and by formation conditions (McRae, et al. 1996; Schmidt, et al. 2004; Kim et al., 2005).

2.6.1. Assimilation of CO_2

A kinetic isotope effect alters the isotopic composition of precursor organic materials as a consequence of CO_2 assimilation by autotrophs through either a C3 or C4 photosynthetic pathway. Both photosynthetic pathways discriminate against ^{13}C, but to different extents: C3 plants assimilate heavier isotopes slower than C4 plants. Thus, C3 plants have isotope values ranging from -22 to -30‰, while C4 plant values range from -10 to -18‰ (O'Malley et al., 1996). The resulting isotope ratios are reflected to varying degrees in petrogenic PAHs made from plant matter and pyrogenic PAHs formed from the incomplete combustion of plant-derived fuels.

2.6.2. Petrogenic PAHs

Crude oil and coal are produced by the thermal maturation of organic material of marine or terrestrial origin (McRae, et al. 1996; Schmidt, et al. 2004; Kim et al., 2005). PAHs also form during these processes and can be found in crude oil and refined petroleum products. Crude oil typically contains between 0.2 and 7% total PAHs; refined petroleum products such as diesel fuels and gasoline contain a combination of these parent crude oil PAHs and trace amounts of PAHs formed during refining processes (Saber et al., 2005). The isotopic compositions of different petroleum products will vary from one another because they originate from different sources of crude oil (McRae, et al., 2000).

2.6.3. Pyrogenic PAHs

Pyrogenic PAHs are formed during the incomplete combustion of fuels. Organic compounds are first cracked into smaller, unstable hydrocarbon fragments during pyrolysis and then undergo a series of radical reaction pathways involving carbon-carbon bond formation, cyclisation, and ring fusion to form more stable aromatic

compounds (McRae, et al. 1996; Schmidt, et al. 2004; Kim et al., 2005; O'Malley et al., 1994). As these radical reactions occur, ^{12}C is preferentially incorporated into bonds over ^{13}C in accordance with a normal kinetic isotope effect. Thus, PAHs get progressively depleted in ^{13}C as the number of rings in the molecular structure increases (Okuda et al., 2002). Kinetic isotope effects, rather than equilibrium isotope effects, predominate in PAH formation because these processes occur rapidly and do not achieve equilibrium.

2.6.4. Degradation

In contrast to formation pathways that generate pyrogenic and petrogenic PAHs, degradation pathways through weathering and other natural processes do not significantly affect the isotopic signature of PAHs. O'Malley et al., (1996) found hat the isotope ratios of PAHs are preserved during processes such as volatilization, photolytic, and microbial degradation reactions (i.e., the isotopic signature of PAHs is conservative) (Kim, et al., 2008). These conditions make it possible to use isotopic fingerprints to implicate a source in the creation of PAHs because the fingerprint can be assumed to remain constant.

2.6.5. Molecular characteristics

Petrogenic compounds can be distinguished from pyrogenic compounds based on different molecular "fingerprints". The aromatic rings of petrogenic PAHs frequently contain alkyl substituents. Alkylated PAHs are more abundant than the parent PAH compounds in petrogenic mixtures, whereas alkylated PAHs are far less abundant than the unalkylated parent compounds in pyrogenic mixtures (Saber et al., 2005). Additionally, low molecular weight compounds are more common in petrogenic PAHs while high molecular weight compounds are more common in pyrogenic PAHs (Walker et al., 2005).

2.6.6. Isotopic signature

CSIA pairs a gas chromatography separation method with an isotope-ratio mass spectrometer (GC-IRMS, Fig. 4.1) to yield the isotopic ratios of individual compounds in a heterogeneous sample. The gas chromatograph separates organic components from one another in complex mixtures and is attached to a combustion reactor which combusts the organic components into CO_2 (O'Malley et al., 1994). The CO_2 will have a mass of 45 or 44 depending on whether it contains ^{13}C or ^{12}C. The CO_2 then passes continuously through an isotope ratio mass spectrometer where the isotope ratios of the compounds are determined by comparison with the 45:44 mass to charge ratio of reference CO_2 (O'Malley et al., 1994, 1996, 1997).

Purification procedures vary from laboratory to laboratory but usually include an extraction step and a column chromatography separation procedure. These procedures must maintain the isotopic integrity of the PAH samples in order to be useful for source apportionment. Dichloromethane is often used for the extraction, and a silica gel column is typically used for the column chromatography step (Okuda et al., 2002; McRae, et al., 1999). The column purification step is necessary to separate the aliphatic fraction from the PAH fraction because the two would otherwise coelute during GC-IRMS analysis (Muccio et al., 2007). Kim et al. recommended that additional high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) purification steps be employed as well. The authors reported no isotopic fractionation as a consequence of the purification procedures, even in cases of low yields (Muccio et al., 2007). O'Malley et al., (1994) also reported a sample processing strategy involving extraction and column purification that did not alter the isotopic signature of standard compounds, even when less than 50% of the starting material was recovered (Kim, et al., 2009).

In addition to the ^{13}C analysis, hydrogen stable isotopes can be used to further elucidate the sources of PAHs. Sun et al., (2003) reported D values in conjunction with ^{13}C values, and found that the combination allowed a much greater ability to differentiate among PAHs derived from petrol, jet fuel, and different coal conversion

processes than using ^{13}C alone. Sun et al., (2003) further noted that deuterium enrichment takes place simultaneously with ^{13}C depletion. This deuterium enrichment is consistent with expectations based on PAH formation mechanisms which typically involve dehydration steps. Dehydration allows lighter hydrogen isotopes to preferentially leave the molecular structure because C-H bonds are weaker than C-D bonds (Okuda et al., 2002).

2.6.7. Investigated sources and applications of CSIA

CSIA, often used in conjunction with molecular methods, has been successfully used to allocate a broad range of sources and production conditions for PAHs from air, water, sediment, and soil samples. O'Malley et al. (1994) first demonstrated that CSIA could be used for source apportionment by showing that PAHs emitted by wood burning exhibit a different isotopic signature than those found in car soot (Kim, et al., 2009). O'Malley et al., (1994) noted that low molecular weight PAHs enriched in ^{13}C are characteristic of pyrogenic mixtures, and high molecular weight PAHs depleted in ^{13}C are characteristic of petrogenic mixtures, providing a precedent for using isotopic data to source environmental PAHs (Kim, et al., 2009). McRae et al. further demonstrated the utility of CSIA by reporting that PAHs generated by coal and biomass pyrolysis, and in diesel particulates, contained substantially different ^{13}C values (O'Malley et al., 1994). A separate study found differences in ^{13}C of atmospheric PAHs which allowed the authors to conclude that automotive exhaust contributed the most to atmospheric PAHs in Beijing, while coal combustion was the major contributor to air in Chongqing and Hangzhou (He et al., 2002).

CSIA has also been applied to tar identification (Okuda et al., 2002). Environmental PAHs with extremely low, variable ^{13}C compositions were linked to biodegradation, suggesting that PAHs from microbially generated PAHs tend to be more depleted in ^{13}C than those derived from other processes (McRae, et al., 1999). Analysis of ^{13}C has also been proposed for studying paleo-fire activity to learn about climate-biosphere interactions because it has been demonstrated that C3 and C4-derived

PAHs formed by combustion are isotopically distinct. O'Malley et al., (1994) discovered that PAHs formed from biomass burning during forest fires largely retain the isotopic composition of the original plant material. In addition to research investigating which types of sources can be distinguished using isotope ratios, many successful investigations relating to particular environmental sites are published. For example, CSIA was used to allocate PAHs in sediments from St. John's Harbor in Newfoundland to a primarily wood-burning source, rather than from crankcase oil or other petroleum products (Kim, et al., 2009).

In another study, molecular methods attributed PAHs in an urban estuary in Virginia to wood-treatment facilities, while CSIA revealed an additional contribution by coal transport, a source that had not been revealed by previous techniques or been anticipated by the authors (Walker et al., 2005). ^{13}C measurements have also been used to analyse product versus source PAHs in order to learn about the mechanisms of PAH formation. These mechanisms can provide insight into sources that utilize different reaction conditions. For example, it was demonstrated that PAHs formed from different coal conversion processes could be differentiated due to a progressive enrichment of ^{12}C accompanying higher temperatures of formation (O'Malley et al., 1994). This research indicated that the isotopic values of PAHs from coal are likely a function of the extent of ring growth required to form PAHs during processing, meaning that mild processes such as low temperature carbonization yield two or three ring PAHs with alkyl substituents and isotopic signatures similar to the signatures of the parent coals, while high temperature carbonization, gasification, and combustion exhibit distinct ranges of -25 to -27‰, -27 to -29‰, and -29 to 31‰, respectively, as ring condensation increases (O'Malley et al., 1994).

2.6.8. Limitations

As CSIA becomes an increasingly common source apportionment tool, several considerations need to be addressed. There is currently a lack of standardized methods for CSIA with respect to PAHs. Purification procedures are continuously being modified and are inconsistent across studies. For example, differences in purification procedures were recently listed as a possible explanation for disagreements between two studies seeking to source PAHs derived from creosote wood preservatives (Walker et al., 2005). Furthermore, although GC-IRMS is a fairly sensitive instrument, it requires at least 10 mg/L of an individual PAH for each injected sample, which is a relatively high concentration for natural samples.

New techniques need to be developed to improve CSIA for the analysis of environmental samples with low concentrations of organic pollutants, such as particulate matter for air pollution studies. One promising advance within the past year to circumvent this problem was the development of a large volume temperature programmable injector technique for GC-IRMS analysis of PAHs, to be used in place of the more common splitless injector method. This technique was demonstrated to measure samples with concentrations as low as 0.07 mg/L (Sun et al., 2003). Although a vast number of ^{13}C values for pyrogenic compounds have been published over the past sixteen years, two other source apportionment ratios of potential utility have been neglected. ^{13}C values for petrogenic PAHs are relatively limited in the literature (McRae et al. 1996; Schmidt et al., 2004; Kim et al., 2005). Furthermore, although Sun et al. (2003) revealed that analysing PAH delta values in combination with ^{13}C values appears to be a promising strategy for differentiating similar sources, δD data are not yet commonly measured or published (Okuda et al., 2002). This is an area where future research should be directed to expand the capability of CSIA as an environmental forensics device. CSIA of PAHs can be inconclusive on its own when isotope ranges from different sources are similar. To overcome this limitation, many studies combine carbon CSIA with other molecular analyses of chemical fingerprints, including alkylated ratios, isomer ratios, low molecular weight to high

molecular weight ratios, or a statistical analysis called principal component analysis (Walker et al, 2005; C. McRae et al. 2000; Kim et al., 2009).

Source apportionment using chemical fingerprints faces even greater limitations than CSIA, however, and is not always reliable on its own. Molecular signatures are far more subject to interference from weathering than isotope ratios. For example, one study found that after only eighty days of weathering, parent PAHs predominate over alkylated species meaning that the two types of compounds weather at different rates (McRae et al., 1999). This change could lead to an incorrect conclusion in some cases that a mixture of PAHs was pyrogenic rather than petrogenic. Moreover, the molecular characteristics for many potential sources are not unique, further limiting the value of chemical fingerprinting independent of CSIA (Ref). The reliability of GC-FID and GC-MS methods for measuring molecular signatures also decreases when PAHs originate from multiple sources (McRae et al., 2000).

2.6.9. Conclusions

PAHs can be found virtually everywhere in the environment and often demonstrate human pollution. Source apportionment of PAHs using molecular methods has been common practice for many years, used for the identification of responsible parties in mystery oil spills or environmental remediation efforts, among other applications.

The field of environmental forensics was revolutionized sixteen years ago by the application of compound specific stable isotope analysis to these investigations, using relatively new GC-IRMS technology. CSIA has proven valuable in yielding PAH source apportionment information because isotope ratios tend to remain more constant over time and provide more information than molecular techniques alone. 13C data have already been reported for a wide variety of PAH sources and reaction conditions, but certain isotope ratios of potential use have been neglected. These include 13C values of petrogenic sources and δD values for PAHs from all sources.

Furthermore, standardized methods still need to be established for the field, and sensitivity and concentration limits could benefit from future technology research. Moving forward, it appears that a combination of molecular and isotopic techniques rather than sole reliance on one over the other provides the greatest assurance of apportioning the correct source.

Chapter 3

Material and methods

The following chapter will describe in detail the experimental methodologies applied and will be partially repeated in the three papers imbedded in the thesis. The experimental part consists of molecular, chemical and isotopic methods, which will be described separately in this section and then combined for the specific aim of each paper. The methodologies are further divided in microbial, molecular and chemical analysis of shale samples and molecular, chemical and isotopic analysis of coal tar samples.

3.1. Shale samples

Six cores were obtained from the collection of the British Geological Survey (BGS, Keyworth, UK). Samples consisted of shale cores from different depth and origin, as listed in the Table 3.1. The samples were stored in a dry room at 20°C in the collection of the BGS.

Table 3.1. Samples ID

Geographic Origin	**Age**	**Sampling Depth (m)**
Rosecote_1	Carboniferous	600
Rosecote_2	Carboniferous	690
Duffield_1	Carboniferous	400
Duffield_2	Carboniferous	450
Dorset_1	Jurassic	100
Dorset_2	Jurassic	80

3.1.1. Samples preparation

The aim of this experimental session was to develop and validate a protocol for handling and analysing shale cores. In general, when trying to characterise a core or a shale rock for microbial and molecular analyses, there are two major issues: (*i*) the first is ensuring anaerobic conditions in order to maintain the microbial population active and alive, and (*ii*) the second is related to cross-contamination of the sample, which could occur in different times after the sampling, handling and storage. Unfortunately, there is no way with current technology to ensure both anaerobic conditions and sterility: the first is guaranteed using an anaerobic cabinet, while the second is obtained using a laminar flow cabinet.

In the attempt to preserve the indigenous microbial community alive, it was decided to ensure anaerobic conditions. To maintain sterility, the anaerobic cabinet was cleaned three times with Virkon (1%) and EtOH (70%). Episodes of contamination can take place both during the preparation and the analysis in the laboratory, but also in earlier stages, such as during sampling and storage. In order to prevent cross-contamination in the laboratory, each material and tool used was autoclaved if possible and/or sterilized with Ethanol (70%) and Virkon (1%). To avoid any prior contamination occurred during sampling process and handling of the cores, a protocol *ad hoc* for the future analyses of 16S RNA gene was developed.

To prepare the various core sections of shale rocks for DNA analysis and incubation experiments, each core was exposed to UV lights for 12 hours before handling. The external surface of the core samples has been removed under anaerobic conditions using a sterile hammer and chisel. The interior of the core sample was crushed in the anaerobic cabinet using a sterile mortar and pestle until it was in a coarse-powdered form. For the analysis of 16S RNA gene and to make enrichments cultures, ~ 15 g of powdered core from each samples were prepared in anaerobiosis and placed into an anaerobic jar. 0.2 g of this powdered samples from each samples were used for DNA analyses, the remaining part was stored at 4°C for future incubation (Most probable number, MPN) and enrichment cultures.

3.1.2. DNA isolation and spectrophotometry for nucleic acid measurements

DNA was extracted using two different kits (MoBio Power Soil DNA Isolation Kit and MP Fast DNA Spin Kit for Soil) following the manufacturing instructions and with more classic procedures such as Chloroform-Phenol extraction. The efficacy of the different extraction methods was checked with a set of PCR assays, electrophoretic runs and quantification of environmental DNA using the Nanodrop 1000 (Thermo Scientific). The MP Fast DNA Spin Kit for Soil yielded high quality DNA, but the amount of DNA extracted was ~11 ng/µL, which is the minimum required to perform a pyrosequencing run. The more classical chloroform/phenol extraction was suitable to eliminate organic contaminants from the samples, and more than 15 ng/µL of environmental DNA were extracted. However, the signal (checked through several electrophoretic runs) resulted to be of low quality. The low quality of the extracted DNA could be attributed to a possible damage of the strong solvents employed. The adapted protocol used to perform chloroform/phenol extraction is described in the Appendix of Chapter 3 (Section A13). The Power Soil DNA isolation kit was found to be the more effective to extract DNA from complex samples, since the signal was always of high quality and the amount of DNA extracted was always above 11 ng/µL. The Power Soil DNA isolation kit is used for the standard extraction of DNA from enrichment media and environmental samples. This extraction kit is slightly more stringent than the older UltraClean DNA Isolation Kit, and is suitable for difficult samples, such as sediments containing higher organic content and humic acids.

The efficacy of nucleic acids extraction was also checked using a spectrophotometric assay. When using spectrophotometric analyses to determine the concentration of DNA or RNA, the Beer-Lambert law is used to determine unknown concentrations without the need of standard curves. Basically, the law relates the amount of light absorbed to the concentration of the absorbing molecule. The following absorbance unit to nucleic acid concentration conversion factor was used to convert OD to concentration of unknown nucleic acid samples: A260 dsDNA = 50 µg/mL. In this

case, the A260 unit is the amount of nucleic acid contained in 1 mL and giving an OD of 1.

3.1.3. PCR amplification of 16S rRNA gene

The 16S rRNA was amplified from the environmental DNA using universal primers 8F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3′) for a full 16S DNA profile. The archaeal population was screened using the 109F (5′-ACK GCT CAG TAA CAC GT-3′) and 915R (5'-GTG CTC CCC CGC CAA TTC CT-3') primers (Grosskopf et al., 1998). A different set of primers was also used in order to improve the chance of amplifying bacterial DNA: the two primers used for the PCR were meant to target shorter sequences: 515F (515F 5'-GTG CCA GCM GCC GCG GTA A-3') and 1061R (5'- CRR CAC GAG CTG ACG AC -3'). PCR conditions were set as follow for the full 16S rRNA amplification of bacteria and *Archaea*: an initial denaturation step at 94°C for 4 minutes, followed by 30-35 cycles of melting (94°C for 30 Seconds), annealing (50°C for 30 Seconds) and extension (72°C for 1.5 minutes). The final extension was achieved with the last step (72°C for 5 minutes).

The PCR conditions used with the 515F and 1061R primers were slightly different: a shorter extension of 30 seconds (72°C) has been adopted, according to the manufacturing guidance of the 500 bp primers, and in order to prevent the formation of primer dimers. In addition, the final extension has been increased at 10 minutes. A longer final extension has been applied to the 500 bp primers because the objective was to clone the amplicone (PCR product) into TA cloning vector, since this will be helpful in incorporating A residues at the 3’ end of the amplicone.

3.1.4. Agarose gel electrophoresis

The electrophoresis was prepared using either a 1.5% or 2% agarose gel, the run was performed at 400 mA, 80 mV for 60 minutes. The buffer used was either a 50x TAE buffer or a 40x TAE buffer. All the extracts and templates were analysed directly after the extraction of nucleic acids, or kept at -80°C for no more than 24 hours. An example of gel electrophoresis is given in the Appendices of Chapter 3.

3.1.5. MPN experiments

The MPN method is an important technique to estimate viable microbial populations in soil, water, food, agricultural products, medical and other samples. Samples of the different dilution solutions are filled in vials, tubes or in microwell plates, mixed with nutrient solution, and incubated several days to several months, depending on the growth rate of the microorganism group examined. The MPN method is based on the assumption that viable cells theoretically can grow from one cell in an aliquot in the nutrient solutions, and thus show a positive growth reaction. If at higher dilutions no further cells are in the aliquots, no growth can occur and subsequently no positive reaction. The evaluation of the results is based on the application of the theory of probability to the numbers of observed positive growth responses to the standard dilution series of the sample inoculum (aliquots) placed into the set number of culture media tubes. The dilution should be done up to a step where also negative reactions is observed. The results are used to derive a population estimate based on the mathematics of Halvorson and Ziegler (1933) (Eq. 3.1).

(Eq. 3.1) $[a_1p_1/(1-e^{(-a)}\,1^x)] + ... + [a_kp_k/(1-e^{(-a)}\,k^x)] = a_1n_1 + ... + a_kn_k$

Where:

a = the dilution level of each dilution;

n = the number of inoculated units at each dilutions level;

p = the number of positive units within each dilution level;

k = the highest dilution level of the series;
e = the base of the natural logarithm.

There are two assumptions underlying the mathematical solution. First, it is assumed that organisms in the initial and subsequent dilutions are randomly distributed. Secondly, it is assumed that one or more organisms contained within an inoculant volume are capable of producing a positive result. If the second assumption is not satisfied, unusual patterns of positive and negative occurs. The MPN techniques also assume that all test organisms occupy a similar volume.

For the aim of the research, and considering the preliminary results of the 16S rRNA gene amplification, the target microbial populations chosen were SRB and nitrate-reducing bacteria (NRB). The media used in this culturing work are the Lovley (1991) medium B for NRB and the "Postgate medium B" for SRB. Tenfold dilutions were carried out in triplicate using the grounded shale and the sterilized media described below (Table 3.2 and 3.3).

Table 3.2. Growth media for SRB

Ingredient	**Formula**	**Amount per Litre**
Potassium dihydrogen orthophosphate	KH_2PO_4	0.5 g
Calcium sulphate	$CaSO_4$	1.0 g
Ammonium chloride	NH_4Cl	1.0 g
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	2.0 g
Sodium lactate	$CH_3CH(OH)COONa$	3.5 g (4.49 mL of 60% Syrup)
Yeast extract		1.0 g
Iron sulphate, heptahydrate	$FeSO_4 \cdot 7H_2O$	0.5 g
Ascorbic acid	$C_6H_8O_6$	0.1 g
Sodium thioglycollate	$HSCH_2COONa$	0.1 g
Tap water		Top up to 1000 mL
pH		7.0 – 7.5

After adding all the reagents and making up to 1 litre by the addition of autoclaved deionized water, the pH of the media was adjusted to between 7 and 7.5 by the drop by drop addition of 1 or 10 M sodium hydroxide. The gas bottles were sealed with a rubber stop and an aluminium crimp cap and incubated at 25°C. Pictures of MPN experiments are given in the Appendices of Chapter 3.

Table 3.3. Growth media for NRB.

Ingredient	**Quantity**
$NaHCO_3$	2.5 g/L
NH_4Cl	0.25 g/L
$NaH_2PO_4.H_2O$	0.6 g/L
KCl	0.1 g/L
Mineral Mix (DL)	10 mL
Vitimin Mix (DL)	10 mL
CH_3COONa	0.82 g/L *
KNO_3	1.01 g/L +
Purified Water	800 mL
Final pH	6.8 – 7.0

* This quantity provides a 10 mM concentration of Sodium Acetate as electron donor.

+ This quantity provides a 10 mM concentration of Potassium Nitrate as electron acceptor.

The media was made anaerobic by bubbling out with an 80/20 N_2/CO_2 mix. The liquid phase of 100 mL bottles was bubbled out for 10 minutes and headspace for a further 5 minutes.

3.1.6. Sample preparation techniques for chemical analyses

After collections, the six shale cores were treated in order to avoid contaminations from the previous storage: since the six cores were not collected to perform microbial or chemical analyses, the cores where washed 3 times with DCM and sonicated for 4h to get rid of contaminants attached to the surface of the cores.

In general, it is important to minimize the number of external surfaces that touch rock/core samples. Each surface represents an opportunity for contaminant hydrocarbons to be introduced into the samples. Surfaces that are in contact with the samples are either cleaned with organic solvents or furnaced at 450°C for 8 hours. Additionally, materials used to process high maturity samples are reserved only for these samples and are kept separate from general laboratory supplies. Laboratory solvents including hexane, dichloromethane (DCM) and methanol (MeOH) are high purity and organic free (OmniSolv, EMD Chemicals). Prior to use, glassware, glass wool, aluminium foil and silica gel are furnaced at 450°C (8h) and quartz sand (Accusand, Unimin Corp.) is furnaced at 450°C (12h). Metal tools used to process samples are rinsed with DI water and cleaned (5 each) with MeOH, DCM and hexane. Crushing tools are scrubbed with furnaced quartz sand, rinsed with DI water and sonicated for 30 min each in MeOH and DCM.

Similarly to the sample preparation for microbial analyses, the external part of the cores was removed using hammer and chisel, but in this case, the shale was grounded using a ball mill (Mixer Mill MM 400), until a very fine powder was obtained. The Ball Mill was set dry grinding mode to a frequency of 3 - 30 Hz with cycles of 1 min. The Ball Mill is too disruptive for microbial cells, while it is more suitable for downstream chemical analyses, where a very fine powder determines an increased surface/contact area, allowing a more efficient extraction of organic compounds.

3.1.7. Total organic carbon (TOC)

Total organic carbon (TOC) was analysed using the Apollo 9000 TOC Analyzer integrated with the Teledyne Tekmar 183 Boat Sampler for soil and sediments. Apollo 9000 is divided into three compartments: critical components can be viewed during operation in the front compartment for convenient system monitoring and maintenance. The right compartment houses the furnace and the valves needed to route the gas and liquid flow. The left compartment includes the electronics for controlling the system.

For TOC analysis, the sample is acidified with H_3PO_4 (20%) and incubated at 70°C for 15 min to dissolve carbonates. The sample was sparged with carrier gas to remove the inorganic carbon (IC) by venting, then the sparged sample was injected into the furnace.

Solid samples are weighed (~ 40 mg) into the removable boat, which is readily accessible through the hatch-covered port. The boat is manually advanced into the furnace, where the sample is combusted at 800°C.
Figure n illustrates the linear (R^2 value of 0.9974) wide range of different orders of magnitude for Potassium Hydrogen Phthalate (KHP, $C_8H_5KO_4$) standards using the method TOC 100-1000 ppm of carbon.

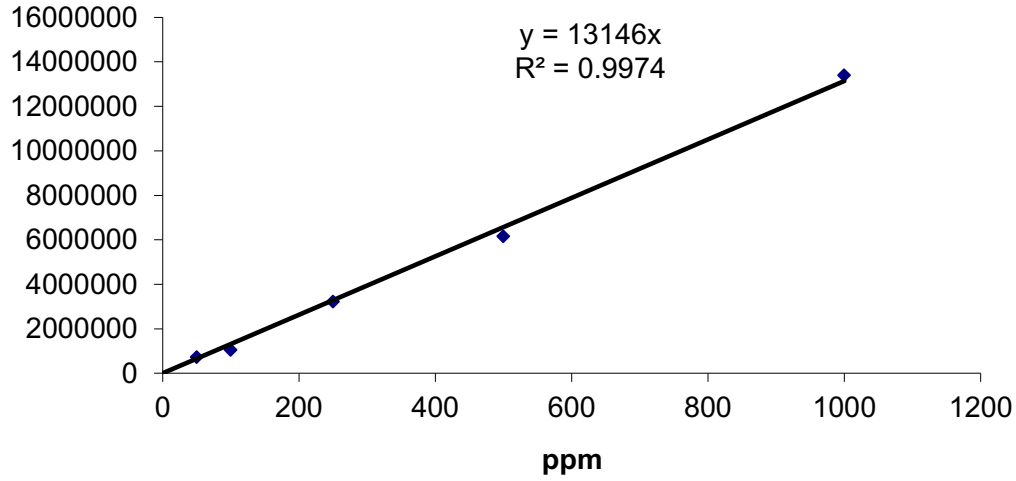

Figure 3.1. Calibration curve for 50-1000 ppm of carbon

Due to the high carbon content of shale and coal, the samples were diluted with an inert material prior the analyses. A dilution of 1:100 has been applied for shale samples, while 1:1000 was used for coal. The inert material used to dilute the powdered shale and coal was furnaced sand (SiO_2 50-70 mesh particle size) at 450°C for 8h to eliminate possible organic contaminants. The sand was furnaced because preliminary analyses showed that laboratory grade sand exhibits a TOC content of ~ 0.01 wt.%, while after combustion the TOC drop to 0.005 wt.%. 40 mg of the mixture has been acidified, incubated at 70°C for 15 min and analysed.

The Rosecote samples exhibit a TOC value of ~ 5 wt.%, while the Duffield samples have TOC value of ~ 2 wt.%. Coal shows much higher organic carbon content: ~ 80% (Table 3.4)

Table 3.4. Average TOC value of the two Carboniferous shale.

Samples	**TOC (wt.%)**	**mg carbon · g^{-1}**	**SD**
Rosecote_1	5.37	53.79	0.542951
Rosecote_2	5.13	51.30	0.525231
Duffield_1	2.18	21.88	0.039101
Duffield_2	2.21	22.18	0.010852
Sand	0.01	0.16	0.006456
Pre-furnaced sand	0.005	0.05	0.138434

3.1.8. Accelerated solvent extraction

Accelerated solvent extraction (ASE) is an automated method for the batch extraction of solid and semi-solid sample matrices (Appendices of Chapter 3). The technique uses elevated temperatures and pressures to aid the breakage of analytes to sample matrix bonds (Richter et al., 1996). Elevated temperatures cause a decreased solvent viscosity, which enhance the penetration of the solvent into the sample matrix. Elevated pressures are employed to ensure the solvent remains in the liquid phase throughout the extraction (Richter et al., 1996).

Samples are packed within individual, stainless steel extraction cells. Each extraction cell is lined with glass-fiber paper to ensure that undesired particulate matter does not collect in the sample. The sample is then added to the cell and any remaining cell volume packed with an inert solid, such as diatomaceous earth (DE). A portion of activated silica gel can also be included below the sample to provide simultaneous extraction and clean-up. The packed extraction cell is placed onto an autosampler carousel which transports the cell into a pre-heated oven. After the designated heating time, the cells are pressurised with nitrogen gas (to approximately 10 MPa) and the solvent is injected at the top of the cell.

The resulting sample extract is forced from the base of the cell into a collection vial. Extractions may be performed in either dynamic or static extraction mode, or a combination of the two modes. In static extractions, the solvent remains within the cell until the extraction reaches equilibrium, then is rapidly washed into the collection vial by further solvent and high pressure. In dynamic extraction mode, the fresh solvent continuously flows through the cell at a controlled rate (Richter et al., 1996).

Different methodologies of extractions were used to isolate the organic fraction of shale and coal. The most effective was an extraction combining hexane, methanol (MeOH) and dichloromethane (DCM). 10 mL stainless steel extraction cells, packed with 3 g of silica gel, 5 g of powdered shale and filled with diatomaceous earth for the remaining volume, were used. The extraction conditions adopted are listed in the Table 3.5.

Table 3.5. ASE extraction conditions.

Solvents	**Hexane**	**MeOH**	**DCM**
Temperature	150°C for 7 min	100°C for 5 min	100°C for 5 min
Rinse volume	150%	150%	150%
Purge time	60s	60s	60s
Pressure	10 mPa	10 mPa	10 mPa

Other extractions were performed using hexane, toluene, hexane/toluene (8:2) at different conditions, but none of them yielded enough organics to be identified. DCM alone is effective, but a combined extraction using hexane and MeOH results in a wider fraction of extracted organics. For all the different extracting conditions, a different amount of samples was used until the extraction was effective: from 0.5 g of powdered shale/coal to a maximum of 10 g. The amount of samples to be extracted is crucial for downstream analyses: an excessive amount could be problematic in term of clean-up of the samples. Preliminary analyses showed that about 5 g of shale and 2.5 g of coal are the optimum for an effective extraction. The

utilisation of different filling agents, like sand or diatomaceous earth was non-influential to the amount of EOM.

3.1.9. Sample evaporation

Prior to instrumental analyses, environmental extracts may require concentration by evaporation of excess solvent. This was achieved using a parallel work-up station (BUCHI syncore®, rotatory poly-evaporator), consisting of four main sections: a heated sample rack (with shaking mechanism), a vacuum pump, a condenser and a freezer unit (Appendices of Chapter 3). Multiple samples can be concentrated simultaneously by placement in suitable polyvap tubes within the sample rack. The heated rack and vacuum pump encourage the evaporation of solvent, which is then collected by a condenser unit. A freezer unit is required to circulate coolant around a small aliquot of the sample held within a tapered section at the base of each sample ask; this prevents the sample from being evaporated to dryness. In addition, the sample rack incorporates a shaking mechanism to ensure a strong vortex in the sample is produced, thereby preventing evaporation retardation. The temperature and pressure are controlled by the operator and are chosen based on the volatility of sample solvent, for hexane the conditions employed are detailed in Table 3.6, for MeOH and DCM, the evaporation conditions are shown in Table 3.7.

Table3.6. Evaporation conditions utilised for Hexane

Steps	Start pressure (mbar)	End pressure (mbar)	Time
Step1	500	200	1
Step2	400	200	2
Step3	200	200	40

Table3.7. Evaporation conditions utilised for MeOH and DCM.

Steps	Start pressure (mbar)	End pressure (mbar)	Time
Step1	500	400	1
Step2	300	200	2
Step3	200	170	20

The heated rack temperature was set to 50°C, the sample lid temperature at 60°C, while the freezer unit was set between -5°C and -10°C.

3.1.10. Nitrogen blow down

When volatile compounds were not the target of the analyses, a further concentration was achieved using a Nitrogen Sample Concentrator, which efficiently concentrate dozens of samples simultaneously. Two processes working together by heating from the bottom (30°C) and nitrogen blowing on the surface accelerate the evaporation and sample concentration. After the samples were completely dry, they were re-suspended in 500 μL of hexane and analysed at the GC-MS. Some of these extracts were further treated using silica-gel separation in order to improve the detection at the GC-MS.

3.1.11. GC-MS pre-treatment: silica-gel chromatography

Silica gels are used as an adsorbent in numerous applications, including chromatographic separations and removal of impurities through adsorption. In chromatography or column chromatography, the stationary phase is most often composed of silica gel, and due to its polarity, non-polar components elute before the polar components (normal phase chromatography). On the other hand, silica gels with C18 type hydrophobic groups attached to its surface allow elution of polar

components first and are used in reversed-phase chromatography. Silica is also used in thin-layer chromatography. Silica gel exhibits strong desiccant properties and, therefore, was also useful for control of relative humidity.

Stationary phase

Column chromatography proceeds by a series of steps: the stationary phase or adsorbent in column chromatography is a solid. The most common stationary phase for column chromatography is silica gel, followed by alumina. Cellulose powder has often been used in the past. Also possible are ion exchange chromatography, reversed-phase chromatography (RP), affinity chromatography or expanded bed adsorption (EBA). The stationary phases are usually fine ground powders or gels and are microporous for an increased surface. There is an important ratio between the stationary phase weight and the dry weight of the analyte mixture that can be applied onto the column. For silica column chromatography, this ratio lies within 20:1 to 100:1, depending on how close to each other the analyte components are being eluted.

Mobile phase (eluent)

The mobile phase or eluent is either a pure solvent or a mixture of different solvents. It is chosen so that the retention factor value of the compound of interest is roughly around 0.2 - 0.3 in order to minimize the time and the amount of eluent to run the chromatography. The eluent has also been chosen so that the different compounds can be separated effectively. There is an optimum flow rate for each particular separation; a faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation. However, the maximum flow rate is limited because a finite time is required for analyte to equilibrate between stationary phase and mobile phase, (Van Deemter's equation). A simple laboratory column runs by gravity flow. The flow rate of such a column can be increased by extending the fresh eluent filled column above the top of the stationary phase or decreased by the tap controls. Faster flow rates can be achieved by using a pump or by using compressed gas (e.g. air, nitrogen, or argon) to push the solvent through the column (flash column chromatography).

The particle size of the stationary phase is generally finer in flash column chromatography than in gravity column chromatography. For example, one of the most widely used silica gel grades in the former technique is mesh 230 - 400 (40 - 63 µm), while the latter technique typically requires mesh 70 - 230 (63 - 200 µm) silica gel.

Prior to GC-MS analyses, the samples were fractionated using silica-gel chromatography. A known amount of silica was furnaced at 450°C for 8 hours, along with beakers, tweezers, Pasteur pipettes, burettes (10 mL) and spatulas. When the silica was completely dry, it was activated by adding 1% of DI water and mixed for half hour. When water is uniformly distributed, the silica-gel was saturated with hexane.

Burettes were placed vertically using clamps, a small amount of glass-wool is placed at the bottom of the burette and silica gel was added. The solvents used for the fractionation were hexane, DCM, a solution of n-hexane/DCM (2:1) and a solution of DCM/MeOH (95:5), as shown in the Table 3.8.

Table 3.8. Fractions of the silica gel chromatography.

Number	**Volume (mL)**	**Solvent**	**Ratio**	**Organics**
N1	4	Hexane	/	Aliphatic
N2	2	n-hexane/DCM	2:1	Aromatic
N3	4	DCM	/	Aldehydes and chetons
N4	5	DCM/MeOH	95:5	Alcohols

The fractionation has been carried out by adding 1 mL of solvent at the time for every fraction. Once the fractionation was completed, the samples were dried using the nitrogen blow down. In order to recover the dry samples, a small amount of DCM was added to the tube 3 time for each sample, and transferred in GC-MS vials. The excess of DCM was dried again with nitrogen blow down, and the samples were re-

suspended in 500 μL of hexane. The samples were stored at -80°C prior the analyses at the GC-MS.

3.1.12. Derivatization

Sample derivatization is a general term used for a chemical transformation designed to improve analytical capabilities. The chemical structure of the sample can be modified to a form consistent with a better outcome for separation from other mixture components, or into a form that is more easily introduced to the measuring instrument, or into a chemical form that provides an enhanced response in terms of either improved selectivity or sensitivity. Derivatization reactions are chemical reactions and, as such, there are many variables that control reaction completeness, speed and specificity. These include temperature, solvents, catalysts, and supports. Each of these variables can be optimized within a specific application.

Derivatization reactions used in MS often overlap with those used in other venues of analytical chemistry. However, there are some derivatization reactions specifically designed for MS, such as those that enhance ionization or introduce a specific mass shift to the sample ions that becomes evident in the mass spectrum. The general goals of derivatization in MS are outlined in Table 3.9. Most of these goals should be self-explanatory, and they are not mutually exclusive. For instance, derivatization to create a new compound as a surrogate for the original almost always changes the physical and chemical properties of the compound (thereby changing the volatility and the thermal stability), almost always changes the mass, and usually affects the limits of detection that can be achieved. Constant demands for better selectivity and sensitivity in MS analysis, the use of a wide variety of different ionization methods, and the expanding areas of application of MS combine to ensure a steady stream of literature reports of new derivatization reagents or modifications of past procedures. Reviews of new and more established reactions are especially valuable.

Table 3.9. General goals of derivatization in mass spectrometry.

Aims	Process
Change of physical/chemical characteristics	Increased volatility. Greater thermal stability. Change of higher-order structure
Structural determination	Location of structural features such as double bonds. Functional group identification. Charge localization and directed fragmentation
Mass shift	Shift of masses of sample ions away from interfering signals. Shift of sample signal to mass range with lower chemical noise. Incorporation of isotopic labels. Incorporation of mass defect isotopes
Sensitivity enhancement	Increased ionization efficiency. Selective ionization. Charge tagging

After the silica gel chromatography, three of the four fractions obtained were subject to derivitazion. N1 fraction was not derivatized because of the absence of functional group to be derivatized and because nearly all the organics found in significant abundance were easily detected after the silica gel chromatography. 20 μL of BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane) were used to derivatize the N2, N3 and N4 fractions of the Rosecote, Duffield and Dorset samples.

3.1.13. GC-MS analyses

A Thermo Scientific (Hertfordshire, U.K.) Trace Ultra GC fitted with a DSQII quadrupole mass spectrometer and Triplus autosampler was used for all GC-MS analyses. Helium (BOC Ltd., 99.999% purity) was used as the carrier gas at a flow rate of 1 mL/min (Appendices of Chapter 3). A J&W Scientic DB-5 column with dimensions 30 m x 0.25 mm id x 0.25 μm lm thickness was used for all GC-MS analyses. The oven temperature was programmed as follow: 60°C for 3 min, 60°C to 80°C at a rate of 1°C/min, then 80°C to 230°C at a rate of 5°/min, 230°C to 310 at 10°C/min, followed by an isothermal period of 8.50 at 310°C.

Data processing was performed using the Xcalibur® software (Thermo Scientific Corporation, Massachussetts, USA). The mass spectra of unknown organic compounds were compared against those within the National Institute of Standards and Technology (NIST) Mass Spectral Library (Gaithersburg, MD, USA).

3.2. Coal tar samples

Coal tar DNAPL samples (Table 3.10) were provided by Parsons Brinckerhoff from a range of FMGPs in United Kingdom and United States of America (sample no. 6). All samples were obtained as free phase coal tar DNAPLs, except sample no. 5 (tar mixed with soil and fine gravel used for comparison), which were sealed in amber glass bottles and stored at 4°C prior to analysis. The gas manufacturing processes used at each site, sampling location and additional information are summarized in Table 3.11. At sites where multiple samples were obtained (site S1), different sampling locations were used; further details of the manufacturing processes and history of the sites can be found in the Supporting Information of Paper III (Chapter 6).

Table 3.10. Summary and description of DNAPL samples investigated in this study[a].

Sample no.	Site name	Colour	Viscosity	Additional comments
1	S1	Dark brown	Very low	Fluid at top, but sludge-like at the base of the vial
2	S1	Dark brown/black	Very low	Fluid at top, but sludge-like at the base of the vial
3	S2	Dark brown/black	Low	Easily poured liquid
4	S3	Dark red/brown	Very low	Easily poured liquid
5	S4	Black	Very high	Tar mixed with soil and fine gravel
6	S5	Black	Very high	Very sticky and dense
7	S6	Dark/brown	Low	Easily poured liquid

[a]FMGP sites have been anonymized for confidentiality reasons.

Table 3.11. Summary of manufacturing processes employed at FMGP sites investigated in this study.

Sample no.	Site class[a]	MGP process(es)	Sampling location	Years of Operation
1	VR	VR, potential traces of CWG, oil reforming, and early horizontal retort tar	Former tar tank. Obtained from downstream from the tar tank	1836-1971
2	VR	VR, potential traces of CWG, oil reforming, and early horizontal retort tar	Former tar tank. Obtained from downstream from the tar tank	1836-1971
3	HR	Horizontal retort	Within tar tank	1886-1971
4	CR	Wood preservation site, distillation from creosote oil	Sump, which contained tar/oil	Unkn.
5	LTHR	HR (early, low-temperature)	Unkn.	1854-1946
6	CO	Coke ovens (steelworks)	Tar tank	1970 to present day
7	Unkn.	Unkn.	Unkn.	Unkn.

[a] The probable site classes (VR = vertical retort, HR = horizontal retort, LTHR = low-temperature horizontal retort, CR = creosote, CWG = carburetted water gas, and CO = coke oven) were assigned on the basis of historical site data which indicated the periods of operation for each process.

3.2.1. Isotopic analysis: sample preparation and fractionation

To isolate the aromatic fraction of the coal tar samples, an automated fractionation method was used for the ASE 350 accelerated solvent extraction system (Dionex, Camberley, UK) equipped with 10 mL stainless steel extraction cells. The extraction

cells were lined with two cellulose filters (to ensure unwanted particulate matter did not collect in the extraction bottles) and packed with 3 g silica gel 60 (10% deactivated w/w using deionized water) to provide simultaneous sample extraction and clean up. For all extractions, ground mixtures of coal tar, diatomaceous earth and sodium sulphate (to remove residual water) were prepared with a 1:1:1 ratio. Approximately 1.5 g of the ground DNAPL mixture was added to the extraction cell and the remaining cell volume was packed with diatomaceous earth. To allow sample fractionation, two separate ASE methods were employed to sequentially extract the same cell using solvents of increasing polarity. In order to obtain the first fraction, hexane (50% cell volume) was used to extract the cell. The oven and static times were switched off to allow the solvent to flow straight through the cell and encourage only the aliphatic portion to elute. The second fraction was eluted with hexane:toluene in a 9:1 ratio (70% cell volume). The oven temperature was maintained at 50°C with the cells heated for 5 minutes prior to extraction. The extracts were concentrated to approximately 1 mL using the rotatory evaporator Büchi Syncore® Analyst (Oldham, UK). The extracts were then transferred into autosampler vials and hexane was added to reach a final volume of 1.5 mL for each sample.

3.2.2. GC-MS and GC-IRMS analyses

Isotope ratio mass spectrometry (IRMS) is a technique used to measure the ratio of speci c stable isotopes within a sample. Elements of interest are those which have two naturally occurring stable isotopes such as carbon, hydrogen, oxygen, sulphur and chlorine. Carbon is the most widely used, while hydrogen is the less studied due to analytical difficulties in obtaining stable signals: in this thesis, both carbon and hydrogen isotopes were investigated. A number of review papers have been published detailing the principles and applications of IRMS (Meier Augenstein, 1999, Nic Daeid et al., 2010).

Isotopic ratios are calculated by measuring the ratio of heavy to light isotopes; for example, $^{13}C/^{12}C$ represents the ratio (R) of stable carbon isotopes while $^{2}H/^{1}H$ represents that of hydrogen. The values are always expressed in delta notation (δ) in units of per mille (‰) by measuring the abundance of each isotope within a sample relative to a reference standard of known isotopic value (Eq. 3.2)

$$\delta^{A}X = 1000 \times \frac{{}^{A}R_{sample} - {}^{A}R_{std}}{{}^{A}R_{std}} \quad \text{(Eq. 3.2)}$$

Unlike most mass spectrometers, an IRMS is not able to scan a range of m/z values (mass to charge ratio), it is restricted to only those m/z values applicable for stable isotope calculations. This means it has unique collectors for only these specific values and can therefore achieve greater precision and sensitivity (Meier Augenstein, 1999). The molecules entering the IRMS are not directly analysed, instead they are converted into small gases; H_2 and CO_2 for hydrogen and carbon isotope analyses respectively (Figure 3.16). This results in a reduced number of collectors required by the IRMS. In carbon analysis mode, three separate collectors are required for the ions of m/z 44, 45 and 46 corresponding to $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{18}O^{16}O$ respectively (Hoefs, 2008). Commercial IRMS systems are capable of analysing several different isotopes by incorporating multiple sets of collectors (e.g. carbon, hydrogen, nitrogen and oxygen analyses by a single instrument). A schematic representation of the IRMS system utilised in this study is shown in Fig. 3.2. The apparatus used for this experiments is shown in the Appendices of Chapter 3.

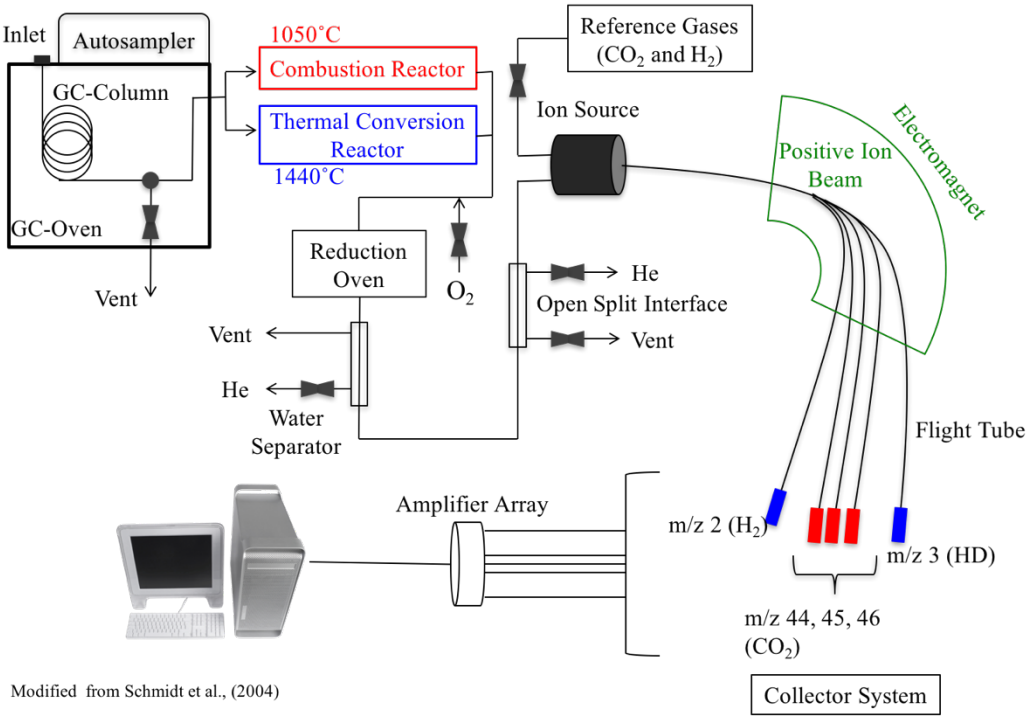

Figure 3.2. Schematic representation of the GC-IRMS system used for the experiments carried out in this study. Modified from Schmidt et al. (2004).

The gas chromatograph was fitted with a 30 m ZB-SemiVolatiles capillary column (0.25 mm ID, 0.25 µm Film thickness). The helium flow was kept constant at 2 mL/min. The initial oven temperature was set to 65°C and held for 2 minutes before the temperature was ramped at 5°C/min to 320°C, with a final temperature hold time of 2 minutes. 0.5 µL of sample was injected using a Triplus (Thermo Scientific) autosampler. The split ratio was varied between 5 and 250 in order to obtain a signal size higher than 0.2 V for the m/z 44 ion (carbon) and m/z 2 ion (hydrogen) of each of the PAHs investigated. All sample isotope values were calculated relative to standard gases with known isotopic composition: carbon dioxide ($\delta^{13}C$ = -37.0(‰) vs. VPDB) for GC-C-IRMS (gas chromatography – combustion – isotope ratio mass spectrometry) analyses and hydrogen ($\delta^{2}H$ = -261.8 (‰) vs. VSMOW) for GC-TC-IRMS (gas chromatography – thermal conversion – isotope ratio mass spectrometry) measurement (Agroisolab GmbH, Germany). The standard gases were injected at the beginning of each sequence to assess stability and linearity. Additionally, for GC-TC-IRMS analyses, the H_3 factor was calculated at the beginning of every sequence

and after cycles of 10 analytical runs, the average value was of 6.5. Each analytical run was programmed to include five reference gas pulses prior to the sample peaks and an additional two reference gas pulses immediately after the last sample peak. The analytes of interest were identified prior to isotope analysis using a Thermo Scientific (Hertfordshire, U.K.) Trace Ultra GC fitted with a DSQII mass spectrometer and Triplus autosampler. The same column was used for both GC-MS and GC-IRMS analyses. All injections were of 0.5 μL and were carried out using the same split ratio used for isotopic analyses. Helium was used as the carrier gas, with a flow rate of 2 mL/min and extracts were analysed using the same oven temperature program as for GC-IRMS analyses. The ion source and transfer line were maintained at 200°C and 320°C respectively. The electron ionization voltage was set at 70 eV for all analyses. A stock solution consisting of the 16 US EPA priority PAHs, was injected for both GC-MS and GC-IRMS analyses. Triplicate injections of the solutions were performed and the isotopic values monitored to ensure results were consistent. Coal tar extracts were separated by the analysis of blank (hexane) samples to prevent cross contamination.

3.2.3. GC-MS and GC-IRMS data analysis

GC-MS data processing was performed using the Xcalibur® software (Thermo Scientific Corporation, Massachussetts, USA). The mass spectra of unknown organic compounds were compared against the National Institute of Standards and Technology (NIST) Mass Spectral Library (Gaithersburg, MD, USA). The system used for compound specific carbon isotope analysis (CSIA) comprised of a Trace GC, GC Isolink and Conflo IV interfaces and a Delta V advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, MA, USA) (Appendices of Chapter 3). The combustion reactor for carbon analyses was maintained at 1040°C and reoxidised every 50 analytical runs using a stream of oxygen gas. The pyrolysis reactor for hydrogen measurement was maintained at 1420°C and conditioned every 50 analytical runs with Isooctane (Sigma Aldrich). Data processing was performed using the Isodat® software (Thermo Scientific). The complex nature of isotope

analysis required the application of additional quality control procedures to ensure accurate and precise isotopic values are obtained. The calibration was performed by comparing the isotopic ratio of a sample with a reference gas calibrated. Stability of the isotopic values was monitored with a run that consisted in the isotopic analysis of ten identical reference gas pulses (Fig. 3.3).

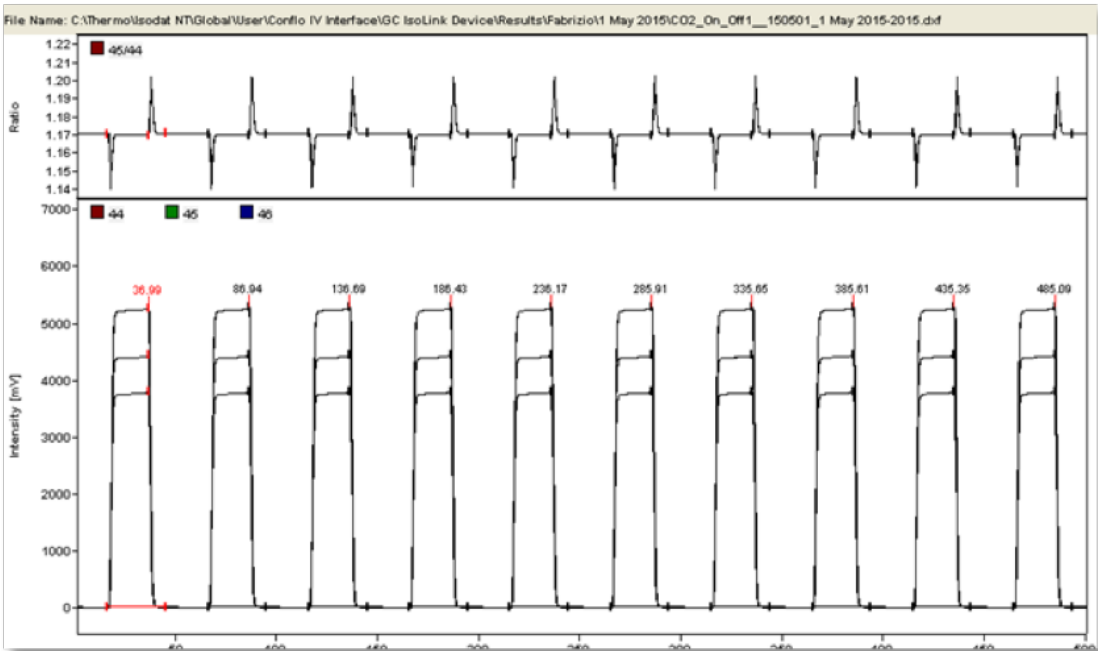

Figure 3.3. Example of stability test of the isotopic values assessed through ten identical reference gas pulses.

Measuring the standard deviation of the delta (δ) values assessed the repeatability of the instrument and stability was considered achieved when the standard deviation of the δ values was less than 0.06 for carbon measurement and less than 1.0 for hydrogen analyses. The linearity of the δ values was monitored to ensure δ values are independent of peak intensity. Using a series of reference gas pulses with varying intensity assessed the linearity of the instrument: the values were considered linear if the δ values had a standard deviation of less than 0.06 for carbon analyses (Fig. 3.4).

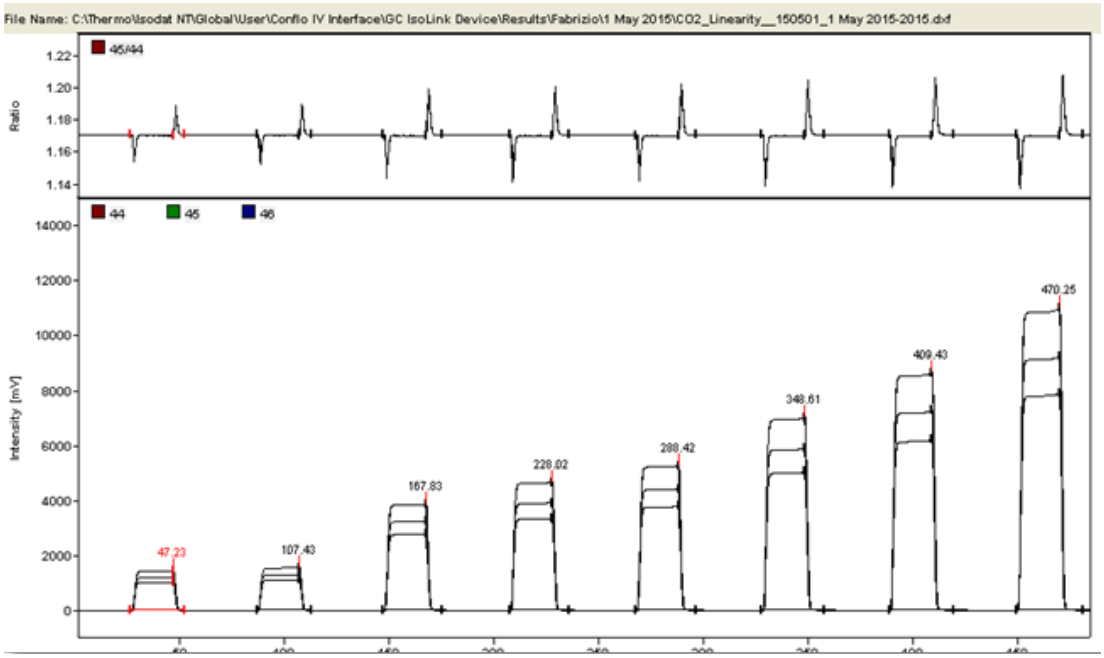

Figure 3.4. Example of linearity test.

The linearity test is not applicable to $^{2}H/^{1}H$ measurements, which requires the H^{3+} factor determination. The H^{3+} factor is applied to the $\delta^{2}H$ data as a correction for the contribution of H^{3+} species formed by ion/molecule reactions in the ion source at increasing gas pressures (Eq. 3.3).

(Eq. 3.3) $H_2^+ + H_2 \quad H_3^+ + H$

The reaction constant is proportional to both (H_2^+) and (H_2) and, for a given IRMS, the number of ions formed is proportional to the number of molecules present. The ratio (H_3^+)/(H_2^+) is a linear function of the m/z 2 intensity and the correction simply subtracts a portion of the m/z 2 intensity from the m/z 3 intensity. The H_3^+ factor was determined by measuring the intensity of m/z 3 as a linear function of m/z 2, performed with working gas. A series of gas pulses with increasing intensity were introduced in the IRMS by adjusting the gas pressure. The instrument software can then calculate the H_3^+ factor.

The isotopic composition of an element was expressed as a ratio between the abundances of two stable isotopes. The ratios, expressed in δ notation, are in units per mille (‰). Carbon and hydrogen isotopic ratios are reported relative to the international reference standards VPDB (Vienna Pee Dee Belemnite) and VSMOW (Vienna Standard Mean Ocean Water) respectively.

3.2.4. Molecular analyses: 16S rRNA gene extraction procedure

The high viscosity and the low density of most of the DNAPLs, did not allow the samples to be processed with conventional DNA extraction kits or with more classical procedures such as chloroform-phenol DNA extraction (also due to the low-quality yield of DNA, not suitable for sequencing analyses). Therefore, because of the physico-chemical properties of coal tar DNAPLs, an ad-hoc protocol was developed in order to make DNA extraction feasible.

Prior the isolation of environmental DNA, the DNAPLs were mixed with sand (30-70 mesh particle size, Sigma-Aldrich) in various proportion (Table 3.12) to decrease the viscosity of the samples. By mixing with sand, the physico-chemical properties of a polluted soil were simulated, allowing DNAPLs to be extracted with common DNA extraction kits for soils. Every tool and glassware were autoclaved 3 times and/or furnaced at 450°C for 8h. To avoid bacterial contamination, individual glass vials (20 mL) were filled with 5 g of sand and autoclaved three times. The vials were then furnaced at 450°C for 8 hours to eliminate any presence of DNA and/or organic contaminants. After furnacing, sand was transferred directly under Microbiological Safety Cabinet Class II (Contained Air Solutions Ltd, CAS, Manchester, UK) and exposed to UV light until reached room temperature of 20°C and prior to mixing with DNAPLs.

Liquid DNAPLs were transferred in the glass vial using Pasteur pipettes, while more dense tars were mixed with sand using sterilised spatula. DNA was isolated from

DNAPL/sand mixture (0.25 g) using the PowerSoil DNA extraction kit (MOBIO Laboratories, Carlsbad, CA, USA) with few modification of the manufacturing protocol. Sterilised sand was also included in the DNA extraction to verify the presence of contaminant DNA. Quality of the DNA extracts was verified by gel electrophoresis.

Table 3.12. Relative weight of DNAPLs mixed with sand.

Sample no.	**Sand mixed with DNAPL (g)**	**Weight of DNAPL (g)**	**% of DNAPL**
1	5	2,31	46,2
2	5	5,13	102,6
3	5	2,51	50,2
4	5	2,33	46,6
5	0	5,22	NR
6	5	2,85	57
7	5	1,46	29,2

3.2.5. 16S rRNA gene amplification and pyrosequencing

PCR of the V1-V6 hypervariable region of the bacterial 16S rRNA gene was performed using amplicon fusion universal bacterial primers 27F (Lane, 1991) and 907R, (Lane, 1995) synthesised by IDTdna (Integrated DNA Technologies, BVBA, Leuven, Belgium). The forward primer (5′ CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNAGAGTTTGAT CMTGGCTCAG 3 ′) consisted of a fusion containing the 454 Life Sciences ‘Lib-L Primer A’, a four-base ‘key’ sequence (TCAG), a unique ten-base barcode ‘MID’ (Multiplex Identifier Adaptors, Roche) sequence for each sample (Table 3.13), and bacterial primer 27F.

Table 3.13. Roche's 10-base MID set sequences.

Sample no.	MID ID	Barcode	DNA concentration prior sequencing (ng/µL)
1	MID-1	ACGAGTGCGT	54.7
2	MID-2	ACGCTCGACA	52.2
3	MID-3	AGACGCACTC	29.4
4	MID-4	AGCACTGTAG	12.5
5	MID-6	ATATCGCGAG	11.1
6	MID-7	CGTGTCTCTA	43.3
7	MID-8	CTCGCGTGTC	25

The reverse fusion primer (5′ CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCGTCAATTCMTTTRAGTTT 3′) contained the 454 Life Sciences 'Lib-L Primer B', a 4 base 'key' sequence (TCAG), and bacterial primer 907R. The PCR amplification was performed in 75 µL volume reactions using 47.1 µL Roche PCR Grade Water, 7.5 µL Reaction Buffer (without $MgCl_2$), 9 µL 25 mM $MgCl_2$, 1.5 µL Nucleotide Mix, 3.75 µL DMSO, 1.2 µL 25M 907R Primer, 0.75 µL High Fidelity Enzyme Blend, 3 µL of sample DNA and 1.2 µL 25M Different MID labelled Forward Primer 27F for each sample.

Furnaced sand was prepared for PCR with the DNAPL samples. A negative and a positive control were also prepared for the PCR reaction, containing respectively 2 µL sterile H_2O and DNA extracted from *Geobacter sulfurreducens* (from the collection of The University of Manchester, School of Earth, Atmospheric and Environmental Sciences). The PCR conditions included an initial denaturing step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a final elongation step at 72°C for 5 min. At the end of the run, the whole PCR product was mixed with 12.5 µL of 5x gel-loading dye, and 35 µL of the mixture was loaded on a 2% Tris-Acetate-EDTA/agarose gel. A 2000-100 bp ladder was also loaded on the gel that was run at 80 mV for ~ 2 h.

At the end of the run, the DNA bands were observed on a Gel Doc 2000 Gel Imaging System (Bio-Rad Laboratories). Following gel electrophoresis, bands of the correct fragment size (~ 410 bp) were excised, purified using a QIAquick Gel Extraction Kit (Quiagen, Limburg, Netherlands) according to the manufacturer's protocol, and eluted in 30 μL of DNAse free H_2O. The purified PCR products were quantified using the NanoDrop ND-1000 (Thermo Scientific) (Table 6). The DNA products were then stored at 4°C until it was sequenced. The emulsion PCR was performed at The University of Manchester, School of Earth, Atmospheric and Environmental Sciences, the pyrosequencing run was performed at the sequencing facilities of Faculty of Life Science of The University of Manchester using a Roche 454 Life Sciences GS Junior.

3.2.6. Pyrosequencing data analysis: bioinformatics

The 454 pyrosequencing reads were analysed using the Quantitative Insights Into Microbial Ecology pipeline (Qiime 1.8.0 release) (Caporaso et al., 2010b). The pyrosequencing reads of this study have been deposited in the NCBI Sequence Read Archive (SRA).

Raw sequences were first assigned to the different samples by using the barcode sequences provided: sequences outside the 300–500 bp range were removed along with the reverse primer sequence, using the *split_libraries.py* script. The usearch 6.1 programme (Edgar, 2010) was used to perform filtering of noisy sequences and chimera checking.

Operational taxonomic units (OTUs) were picked from and compared at 97% similarity with the May 2013 release (13_5) of greengenes OTU reference using the usearch 6.1 programme through the *pick_otus.py* script. The most abundant OTU sequence was chosen as a representative using the *pick_rep_set.py* script. Taxonomy assignment was based on the greengenes reference database (McDonald et al., 2012)

using the Ribosomal Database Project Naive Bayes (RDP) classifier v 2.2 (Wang et al., 2007), with the confidence level set at 80% through the *assign_taxonomy.py* script.

An OTU table was built using the *make_otu.py* script and a biological observation matrix file (BIOM) was built using *convert_biom.py* script. A OTU heatmap file was generated with the *make_otu_heatmap.py* script. The sequences were then aligned to the greengenes core reference alignment (De Santis et al., 2006) using PyNAST (Caporaso et al., 2010a) through the *align_seqs.py* script. Aligned sequences were then filtered using the *filter_alignement.py* script, and a phylogenetic tree was built through the *make_phylogeny.py* script (Price et al., 2009).

Alpha diversity, alpha rarefraction and beta diversity were calculated using respectively the *alpha_diversity.py*, *alpha_rarefraction.py* and *beta_diversity_through_plots.py* scripts. Jackknifing was performed with the *jackkinfed_betadiversity.py* script to directly measure the robustness of individual Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clusters and build jackknifed 2D and 3D PCoA plots. Sequences (average length of 500 bp) were analysed against the NCBI (USA) database using BLASTn program packages and matched to known 16S rRNA gene sequences to retrieve the closest relatives.

Chapter 4a

Chemical characterisation of shale and coal

4a.1. Organic compounds in shale and coal

Organic-rich shales represent one of the most abundant carbon-rich rocks in Europe and worldwide (Klemme and Ulmishek, 1991). A significant part of those source-rocks has not undergone a sufficient burial to generate the pressure and temperature conditions necessary for the complete transformation of the organic matter into oil or coal. As such, their organic content is called immature, and is characterised by a higher proportion of biologically reactive molecules. These immature source-rocks represent a huge and yet untapped fossil carbon resource, which exceeds both the oil and coal resources (Rice and Claypool, 1981). Thus, there is today a considerable economical and geopolitical interest in these novels, unconventional energy resources to try and generate fuel of interest for human consumption. Among those, microbiologically assisted methanization of organic matter is one of the most promising technologies investigated (Suflita et al., 2004). Field and laboratory data showed that biogenic methane production occurs in shale and coal sedimentary basins (Krumholz et al., 1997; Martini et al., 2008; McIntosh et al., 2008). Prior to any field exploitation, several major questions need to be addressed regarding the structure of the microbial populations in the rock and the fraction of the organic matter effectively transformed into methane.

The microbial characterisation of shale rocks (Chapter 3, Section 3.1 to 3.1.5) showed no microbial activity in the samples analysed. The results of these experiments confirmed, through 454-pyrosequencing and culturing techniques, the validity of the protocol developed. Conversely, the chemical characterisation of shale

rocks (Chapter 3, Section 3.1.6 to 3.1.13), performed with GC-MS techniques, showed that a large fraction of the compounds detected could be biodegraded by an appropriate microbial consortium. With these experiments, more than 100 compounds have been identified (at 97% match identity) using the characteristic m/z fragments. Many of these compounds could be biodegraded to methane. The chemical characterisation of highly mature Carboniferous/Jurassic organic matter along with a commercial coal presents unique challenges and necessitates extraordinary attention to concerns of contamination.

Silica-gel chromatography techniques were used to obtain a better separation of the compounds of interest and as a tool for cleaning the samples before injection in the GC-MS. Four fractions (corresponding to aliphatic, aromatic, aldehydes and chetons and alcohols) were obtained from each sample. After separation, the samples were derivatized and then injected in the GC-MS. Triplicate analyses were carried out for each sample. An example of the chromatograms obtained is shown in Fig. 4a.1.

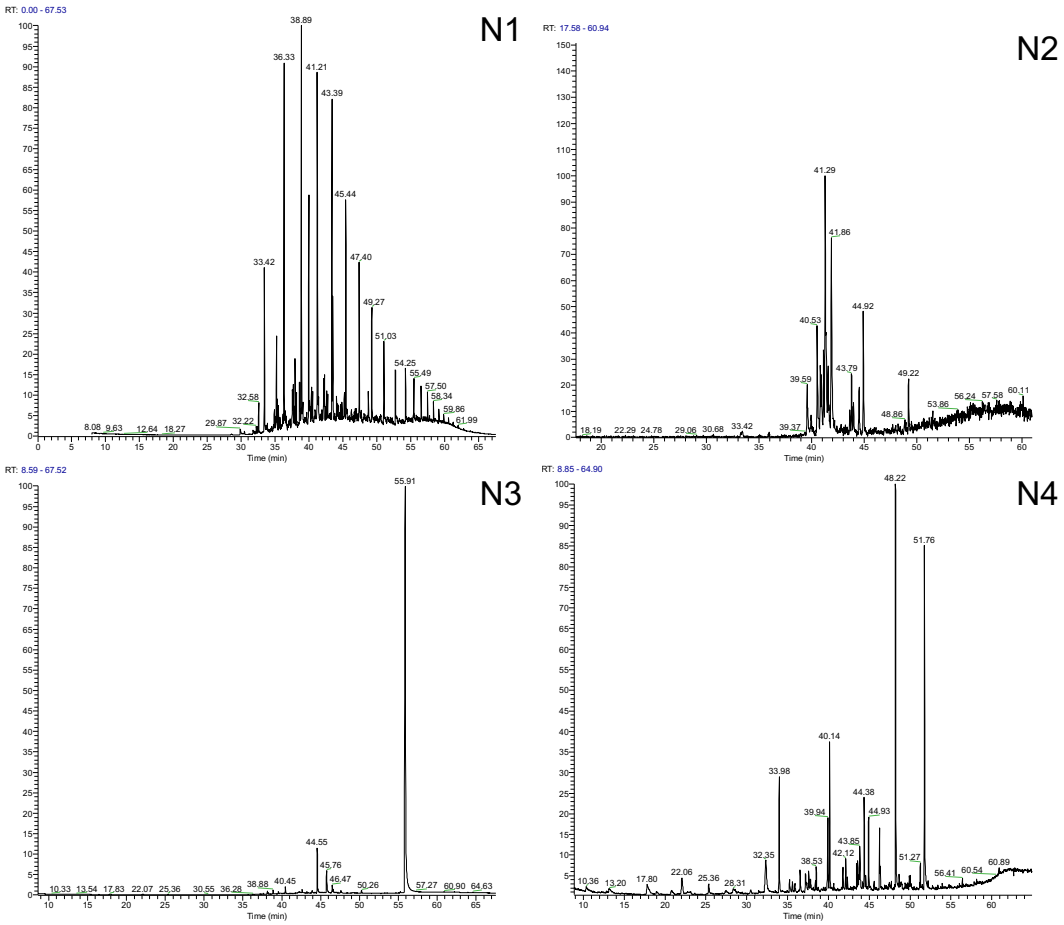

Figure 4a.1. Chromatograms obtained from each fraction of silica gel chromatography. Sample: Rosecote 1 (Carboniferous shale).

The mass spectra of unknown organic compounds were compared against those within the National Institute of Standards and Technology (NIST) Mass Spectral Library (Gaithersburg, MD, USA).

The characteristic m/z fragments described in Table 4a.1 were used to identify the compounds of interest.

Table 4a.1. Characteristic m/z fragments for the different class of organic compounds detected.

Classification	m/z	Compounds
Non-aromatics	57 + 73	n-Alkanes, isoprenoids and branched alkanes
	109 + 123 + 193 + 233	Bicyclic alkanes
	113 + 169 + 197	Crocetane
	113 + 183	Phytane/isoprenoids
	109 + 123 + 193 + 233	Diterpenoids hydrocarbon
	217	Steroids
	191	Hopanes
Aromatics	142 + 156	Methyl, ethyl and dimethyl N
	170	Trimethyl N
	184	Tetramethyl N
	178 + 192	Phe, Ant, Methyl-Phe and methyl-Ant
	206	Ethyl- and dimethyl-N
	155 + 169 + 183	Isohexylalkyl N
	237 + 223	Diaromatic tricyclic hydrocarbons

Non-aromatic hydrocarbons

The two shale samples and the commercial coal extracts were dominated by a series of n-alkanes ranging from C12 to C34. The distribution of n-alkanes (m/z = 57 and 73) is similar in the two shale samples, while the commercial coal was characterized by n-alkanes ranging from C13 to C30. Branched alkanes were recognized in low abundance in the shale compared with the coal extracts. Hopanes, bicyclic alkanes and diterpenoids were clearly detected in the aromatic fraction of both shale and coal. Isoprenoids were detected only in the coal extracts.

C27-αβ-Hopanes and C31-αβ-Hopanes were detected in the aliphatic fractions of the carboniferous samples, the αβ configuration is the most common for this class of

compound and it is an indicator of biological activity. One C30-βα-Hopane was found only in the Carboniferous samples, although this a rare configuration of hopanes, due to its thermal instability.
Seven bicyclic alkanes ranging from C14 to C16 have been identified in both shale and coal extracts using the expanded m/z = 109+123+179+193 fragmentograms.
N-ketones were found in low abundance in the shale extract: only C14 to C16 ketones were detected. The coal extracts exhibit a wider fraction of n-ketones, ranging from C14 to C27.

Aromatic hydrocarbons

Aromatic compounds were easily distinguished in the N2 fraction after silica gel chromatography and derivatization. At least 50 aromatic compounds were detected by their characteristic mass fragments. The land plant- and combustion-derived PAHs were also found in significant abundance, especially in coal extracts. Alkylbiphenyls, alkylanthracenes and alkylpyrenes were recognized as minor contributors to the aromatic fractions.

The distribution of trimethyl-naphthalene (TMN) homologues was similar in the shale samples, while a higher number of different TMNs was found in the coal extracts. The 2,3,6-TMN was the most abundant in all extracts, the biomarker 1,2,5-TMN has not been detected in any of the extracts. Tetramethylnaphthalene (TeMN) isomers were detected using the m/z = 184, the fragmentograms show a major abundance of 1,4,5,8-TeMN in all samples. 1,2,3,4-TeMN was detected only in the coal sample.

The distribution of phenanthrene, anthracene, methyl-phenanthrene and methyl-anthracene isomers from all aromatic fractions was based on the m/z = 178+192 fragmentograms. Phenanthrene was the most abundant in all shale and coal extracts, with a predominance of methyl-phenanthrene in the shale. The aromatic fraction of coal shows a major diversity of dimethyl-phenanthrene, and dimethyl-anthracene. The coal extract was characterized by a greater abundance of TMN and TeMN.

Methyl- ethyl- and dimethyl naphthalene were recognized in low abundance in the shale aromatic fraction: only two were detected in the shale extracts. 4-Isopropyl-1,6-dimethylnaphthalene, also known as Cadalene was found in the Rosecote shale extracts. Cadalene is derived from generic sesquiterpenes, though it is ubiquitous in essential oils of many higher plants. Cadalene, together with Retene (1-methyl-7-isopropyl phenanthrene), Simonellite (1,1-dimethyl-1,2,3,4-tetrahydro-7-isopropyl phenanthrene) and ip-iHMN (6-isopropyl-l- isohexyl-2-methylnaphthalcne), is a biomarker of higher plants, which makes it useful for paleo-botanic analysis of rock sediments. The ratio of Retene to Cadalene in sediments can reveal the ratio of the *Pinaceae* genus in the biosphere.

Fatty acids

All extracts were found relatively abundant in Fatty Acids Methyl Esters (FAMEs) and Fatty Acids Trimethylsilylesters (FATMs). In particular, C16 to C26 FAMEs were found in coal extracts, while C12 to C20 FAME were detected in the shale extracts. FATMs concentration in all the extracts is far more abundant than FAMEs. C9 to C17 FATMs were detected in all samples. Squalene (C30 hydrocarbon and triterpene) is involved in the synthesis of all plant and animal sterols, including cholesterol, steroid hormones, and vitamin D in the human body. Squalene also occur in a wide range of Gram-positive and Gram-negative bacteria, it is a precursor of hopanoids and sterols (members of a large group of cyclic triterpenoic compounds).
Hexadecanoic acid, also known as palmitate was found in abundance in all samples, suggesting a major role in coal/shale degradation.

4a.2. Discussion and conclusions

The organic compounds detected in this study revealed a wide range of aliphatic and aromatic hydrocarbons. The results obtained are consistent with other studies of coal

biodegradation. Orem et al. (2010) observed significant concentrations of C22-C36 n-alkanes and hexadecanoic acid, which were released during their incubation studies of sub-bituminous coal. Monomethylalkanes and alkylcyclohexanes, which are typical of microbial mats and coals (Orem et al. 2007), have also been detected in our study. Extracts also contained biomarkers such as hopanes and terpenoids.

The biodegradation of aliphatic and cyclic hydrocarbons might be a source of metabolites such as fatty acids in coal and shale produced waters (Orem et al., 2007, Warwick et al., 2008). Typical products of n-alkane biodegradation via addition to fumarate are methylalkylsuccinates (Warwick et al., 2008), also detected in this study. All of these intermediates can potentially be further oxidized to methanogenic substrates. The detection of these compounds in coal samples suggests that a small fraction of shallow, low-maturity coal may serve as a potential feedstock source for microorganisms (Glombitza et al., 2009). Conversely, other studies have indicated the potential toxicity or the ability to inhibit methanogenesis of some intermediates, such as fatty acids (Jones et al., 2010). The inhibition of methanogenesis may be associated with the lowering of pH via the accumulation of organic acid intermediates.

The organic composition of the two Carboniferous samples, along with the commercial coal, can be considered a suitable substrate to study the dynamics of organic matter biodegradation in unconventional gas systems. The absence of an indigenous microbial population in the shale and coal samples might be a limit to the biodegradation study, but can be bypassed by the addition of an external consortium, evaluating in this case the potential of bioaugmentation as a tool to enhance the microbial degradation of organic matter.

Chapter 4

Paper I: Biogenic methane in shale gas and coal bed methane: a review of current knowledge and gaps

4.1. Introduction

The paper presented in Chapter 4 is an extensive review article that analyse the recent developments in biogas generation from shale gas and coal-bed methane. This review describes the main processes of microbial methane generation and accumulation in the two unconventional gas systems, giving an overview of microbial life in deep subsurface habitats.

Paper I identifies central questions regarding our understanding of microbial processes deep inside the earth, correlating the state of scientific knowledge and major advances in extraction technologies that have been made towards a sustainable development of unconventional gas resources.

In the deep subsurface microbial life is subject to numerous constraints: sediments, rocks and minerals offers environments for life that are very different from terrestrial and aquatic habitats. Water is common, but there is a very large solid surface-area-to-water-volume ratio and generally there is relatively little space for water and life per volume subsurface. The microorganisms dwell in the pores of sediments, in the fractures naturally present in hard rock and in fluid inclusions.

Almost all deep environments are anaerobic and contain methane in varying concentrations. With an increase in depth, the amount of dissolved solids in the groundwater tends to increase and so does the temperature, but the gradients of these increases vary, depending on the geological formation. The temperature trend implies that at all places on the planet, there will be a maximum depth below which life as we know is impossible, but the maximum depth for life is highly variable. Knowing more about the limits of life in the subsurface will enable a better understanding of the total amount of microorganisms present and in unconventional formations will enable a more precise calculation of the amount of biogenic methane.

Oil and gas companies with an interest in microbial methane have made significant progresses in understanding the process of biogenic gas generation; companies such as Luca Technologies Inc., Next Fuel Inc., Ciris Energy, Synthetic Genomics and ExxonMobil are moving toward a commercial implementation of enhanced gas recovery, but there are still several significant knowledge gaps remaining.

Key questions remain about the mechanisms of organic matter biodegradation and methanogenic processes. Little is understood regarding microbial processes upstream of methanogenesis, especially which microorganisms are responsible for breaking down the organic compounds, what fraction of the organic matter is most susceptible to microbial degradation, and how this process might be stimulated. Studies are needed to determine the functions of microorganisms found in syntrophic association with methanogens in unconventional reservoirs. In addition, methods need to be developed for quantifying if and how much organic matter is biodegraded by enhanced recovery processes.

Further research also should focus on issues related to implementation and sustainability of enhanced recovery processes. This review article identifies different methods that can be used to increase gas production: (*i*) delivering nutrient amendments to microorganisms in the formation, (*ii*) injecting a microbial consortium, (*iii*) increase the contact surface area of microorganisms to coal/shale organics and (*iv*) increase the bioavailability of organic matter.

Moreover, the content of the manuscript focus on anaerobic biodegradation processes and their indicators. The ultimate aim of the manuscript is also to link the well-established "*Contamination land research*" with the "*Unconventional gas resource exploration and exploitation*". This work also presents the current knowledge regarding enhanced biogenic methane generation in shale and coal strata, pointing out how current engineering technologies may be adapted to stimulate biogenic gas production and favour positive microbial processes.

4.2. Biogenic methane in shale gas and coal bed[21] methane: A review of current knowledge and gaps

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Biogenic methane in shale gas and coal bed methane: A review of current knowledge and gaps

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ABSTRACT

Biogenic CH_4 generation has been observed in many shallow, low temperature shale gas basins and coal seams. The depletion of conventional resources and the increasing demand of natural gas for human consumption have spurred the development of so-called unconventional gas resources such as shale gas (SG) and coal-bed methane (CBM). Such unconventional systems represent the opportunity for the stimulation of biogenic CH_4 generation. Biogenic CH_4 in shale and coal is produced by anaerobic biodegradation of organic matter (OM): methanogenic *Archaea* represent only the final step of biogenic CH_4 generation. Several communities of microorganisms are involved in the initial breakdown of complex geopolymers and the production of intermediate compounds used by methanogens. There are several key knowledge gaps on biogenic CH_4 production in unconventional gas systems, such as the exact fraction of bioavailable OM, the microbial communities involved and how they can be stimulated to enhance microbial methanogenesis. Progress on biodegradation studies, isotopic signatures, as well as DNA analyses and proteomics could help unravel interactions within the syntrophic community involved in the methanogenic biodegradation of OM. Questions also remain regarding the environmental impact of unconventional gas production, such as water quality and the mobility of toxic metals and radionuclides. The answers to these questions might have implications for both recovery practices and a sustainable development of unconventional resources. This review summarises the current knowledge regarding biogenic CH_4 in SG and CBM: from the nature of the rocks to the producing microbial community and the indicators of biogenic CH_4, illustrating how these two environments show remarkably similar opportunities for the stimulation of biogenic CH_4 generation.

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1. Introduction

In recent decades, unconventional gas production from fractured shales and coal seams has experienced a rapid development in many parts of the world (Pearson et al., 2012). The success of SG and CBM is linked to the increasing demand of natural gas and the progressive depletion of conventional gas resources (McIntosh et al., 2008). Historically, the first extraction of SG began in Fredonia, Pennsylvania in the USA in 1821, in shallow, low-pressure fractures (Peebles, 1980; Trembath et al., 2012). Similarly, CBM was first extracted in the USA in the late 1930s, but economic production started in 1980s, when the United States Congress enacted a tax credit for "Non-conventional energy" production (US EPA, 2004). Both SG and CBM industries have experienced rapid growth during the past 20 years, thanks to significant advances in extraction technologies (Jenkins and Boyer, 2008). The combination of horizontal drilling and hydraulic fracturing has allowed access to large volumes of SG that were previously uneconomical to extract (Yang et al., 2014). SG wells typically produce significantly more gas per well, but cost substantially more to drill than CBM wells (Ritter et al., 2015). In spite of the large use of hydraulic fracturing, very little is known about the microbiological implications of this process. Hydraulic fracturing is a well-established technology to increase the permeability of a formation and, together with other stimulation strategies, could be applied *in situ* to achieve or maintain optimised conditions for microbial activity. Stimulation strategies could be used to enhance the productive lifespans of depleted microbial SG and CBM wells, extending and/or regenerating biogenic CH_4 production. *Ex situ* treatments could involve the use of wastewater and coal/shale waste materials to produce CH_4 and reduce the environmental impact of SG and CBM production. This review is not intended to be an exhaustive review of biogenic CH_4 generation, it rather describes the current knowledge of biogenic CH_4 generation in shales and coals, illustrating the analogies and differences of the two environments, and focusing on biodegradation process of complex OM. We also demonstrate how the vast literature available from bioremediation studies can significantly improve our understanding of microbial processes in unconventional gas systems. Lastly, we present the current knowledge about enhanced biogenic CH_4 generation in SG and CBM basins, pointing out how current engineering technologies may be adapted to stimulate biogenic CH_4 production and favour positive microbial processes.

2. Biogenic CH_4 in unconventional gas basins

2.1. Generation and accumulation of CH_4

The advances of biogeochemical studies on organic-rich sedimentary rocks and gas isotopic analysis, have allowed a better understanding of the origin of CH_4 in the subsurface. These studies highlighted the microbial or mixed origin of CH_4 in both coal beds (Faiz and Hendry, 2006; Scott et al., 1994; Zhou et al., 2005; Kinnon et al., 2010; Hamilton et al., 2014, 2015; Baublys et al., 2015) and shales (Martini et al., 1996; McIntosh et al., 2008; Schlegel et al., 2011a, 2013) in many parts of the world (Fig. 1). Early estimations of microbial gas suggest that approximately 20% of total natural gas production in the world is biogenic (Rice and Claypool, 1981), and an additional 10% of the gas resources may also be of microbial origin (Grunau, 1983). In general, CH_4 in shale and coal beds, is generated from OM, sourced from the remains of organisms deposited as fine particles in sedimentary rocks, along with the mineral grains that constitute such rocks. Microbial methanogenesis in unconventional gas systems is a multi-step, syntrophic process that involves a consortium of bacteria and methanogenic Archaea. Bacteria break down complex OM into intermediate compounds (e.g. long chain fatty acids, alkanes, and low molecular weight aromatics; Orem et al., 2010), which are then biodegraded into substrates that are converted by methanogens (e.g. acetate, CO_2 and H_2, methanol, formate; Strapoć et al., 2011) into CH_4. Typically, all other potential electron acceptors (e.g. ferric iron and sulfate) must be exhausted before microbial methanogenesis will proceed (Claypool and Kaplan, 1974; Kuivila et al., 1989; Mah et al., 1977; Martens and Berner, 1974; Reeburgh and Heggie, 1977).

OM is first converted to CH_4 by bacterial processes (*primary biogenic* CH_4), and later by high temperatures and pressures as the sedimentation proceeds (*thermogenic* CH_4) (Jones et al., 2008). Further episodes of microbial methanogenesis are often observed after meteoric recharge of water or other geological events (*secondary biogenic* CH_4) (Milkov, 2011). After generation, the gas rises upward through the pore system, until it encounters a trap such as an impermeable rock, forming a conventional reservoir. However, some of the gas generated during thermogenic and biogenic process, remains trapped in the fine grained source rock (e.g. shales and coals), forming unconventional basins (Pearson et al., 2012).

2.2. Gas storage

The gas generated through thermogenic or biogenic process is stored in three modes (Curtis, 2002): (*i*) *free gas* in intergranular porosity and natural fractures, (*ii*) *adsorbed gas* onto kerogen and inorganic minerals and (*iii*) *dissolved gas* in water, kerogen and bitumen. Characterisation of porosity and pore size distribution is particularly important when considering biogenic CH_4 production, since a major constraint to microbial colonisation of organic-rich rocks would be the limited space available, as well as the contact surface area of microorganisms with OM. There are several challenges in establishing a relationship between the presence and activity of microorganisms and physical properties of the host rocks, such as the pore size, pore size distribution and permeability. Fig. 2 shows the three modes of gas storage and the main transport mechanisms from the source rock to production well. Pores in solid material can be divided in: (*i*) macropores (>50 nm), (*ii*) mesopores (2–50 nm), and (*iii*) micropores (or nanopores) (<2 nm) (Rouquerolt et al., 1994). This classification is particularly important for unconventional basins since a significant portion of gas is sorbed onto the surfaces of mesopores and micropores (Ross and

Fig. 1. World map showing locations where biogenic CH_4 from CBM (black circles) and SG (red circles) has been observed. For CBM basin names and references, see Strapoć et al. (2011) and Ritter et al. (2015). For SG: (1) Antrim shale (Martini et al., 1996, 1998, 2003). (2) New Albany shale (Martini et al., 2008; McIntosh et al., 2010; Schlegel et al., 2011a, 2011b; Strapoć et al., 2010). (3) Cretaceous Mancos shale (Krumholz et al., 1997). (4) Eastern Paris Basin (Meslé et al., 2015). (5) Alum shale (Krüger et al., 2014). (6) Po basin (Mattavelli et al., 1992). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bustin, 2009). It has been hypothesised that the primary role of macro- and mesopores is to act as a transport conduits (Moore, 2012). Micropores play a significant role in CH_4 adsorption, typically contributing the most to the total surface area, as demonstrated for both coal (Beliveau, 1993; Mastalerz et al., 2008) and shale (Chalmers and Bustin, 2007a,b; Ross and Bustin, 2009). Gas storage has long been studied for CBM, identifying a number of factors that influence gas sorption process, including maceral type (Lamberson and Bustin, 1993), ash content (Laxminarayana and Crosdale, 1999), rank (Levine, 1996; Bustin and Clarkson, 1998), moisture (Levy et al., 1997) and temperature

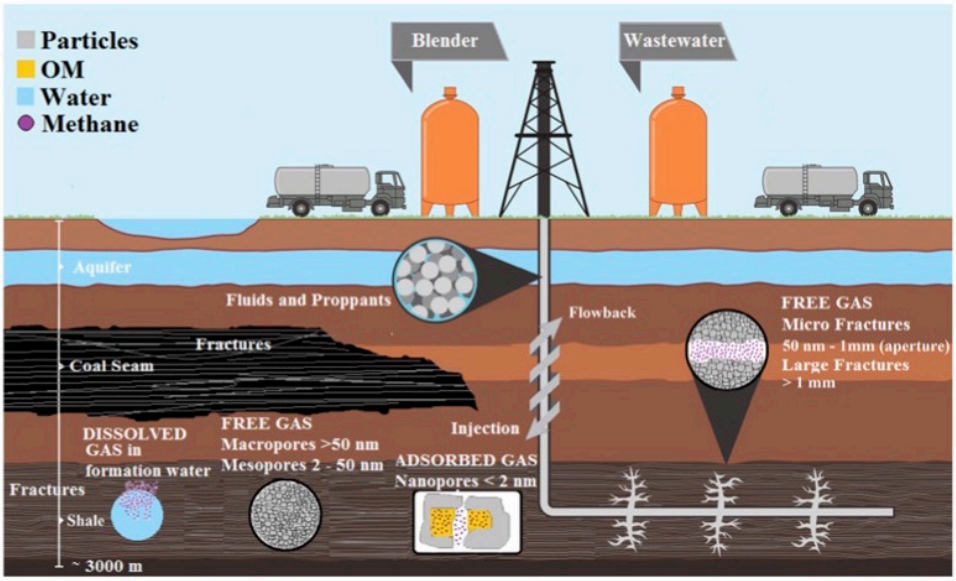

Fig. 2. Storage and transport of gas in a SG/CBM basin from CH_4 trapped in nanopores, mesopores, macropores, microfractures and large fractures to the production well.

(Bustin and Clarkson, 1998). In unconventional gas systems, most of the CH_4 is adsorbed (Milewska-duda et al., 2000), therefore pore surface area rather than pore volume is the most crucial factor for gas storage (Moore, 2012). The pore surface area is directly correlated to the pore size distribution. As pore size decreases, the ratio of free gas to sorbed gas storage capacity decreases (Bustin et al., 2008). In general, for pores less than 0.01 μm, the sorbed gas component exceeds the free gas storage (Beliveau, 1993). Micropores in shale formations contribute the most to the total surface area, as observed by porosimetry in the Haynesville, Marcellus and Doig shales (Chalmers et al., 2012). Macropores and mesopores in these formations are associated with either kerogen and clay aggregates or kerogen and carbonate aggregates (Chalmers et al., 2012). The association between mesopores and micropores with kerogen has been identified in both CBM and SG studies (Chalmers and Bustin, 2007a,b, 2008; Clarkson and Bustin, 1996; Larsen et al., 1995; Marsh et al., 1987; Unsworth et al., 1989). Interconnection between pores of different size and fractures is an important control on the matrix permeability, which influence CH_4 transport (Chalmers et al., 2012). In tight source rock, CH_4 transport occurs by different mechanisms depending on the flow and porous formation conditions (Civan et al., 2011; Roy et al., 2003). Flow in the fractures is controlled by advection and is modelled using Darcy's law. Within the micropores, transport mechanisms include diffusion (molecular flow) and advection (Darcy flow) (Schlömer and Krooss, 1997). Simple Darcy's law based analyses and interpretation are insufficient to characterise permeability and diffusivity of gas in shale (Civan et al., 2011). In fact, at the nanometer scale, the Darcy's law is no longer applicable: flow in the nanopore matrix is controlled by diffusion, molecule-molecule and molecule-pore wall interactions (Bustin et al., 2008; Javadpour, 2009; Schettler et al., 1989). In organic-rich rocks, microporosity is often correlated with total organic carbon (TOC) (Chalmers and Bustin, 2007a,b; Passey et al., 2010), the organic fraction of shale and coal is an important control on CH_4 storage, as demonstrated by positive correlations between TOC and sorbed gas (Gasparik et al., 2013; Ross and Bustin, 2007, 2009; Chalmers and Bustin, 2008). During thermal maturation, the decomposition of OM leads to the production of hydrocarbons and allows the formation of intraparticle organic pores, as observed for coal (Bustin and Clarkson, 1998; Laxminarayana and Crosdale, 1999; Levy et al., 1997) and shale (Chalmers et al., 2012; Chalmers and Bustin, 2007a,b; Jarvie et al., 2007). In general, at higher thermal maturity, the diagenesis transforms OM, creating more microporosity and decreasing the heterogeneity of the pore surface (Levy et al., 1997). This process was often observed in high rank coal, where usually there is a high sorbed gas capacity (Bustin and Clarkson, 1998). The correlation between nanopore abundance in grains of OM and Vitrinite Reflectance (VRo) is consistent with observation made by Hover (1996). They found no visible intercrystalline or intraparticle matrix porosity for low thermal-maturity rocks of the Antrim and New Albany shales. Such conclusions were also supported in a study of Cretaceous shales (Chalmers and Bustin, 2007a,b) where the highest CH_4 sorption was observed in samples with highest concentrations of inertodetrinite and vitrinite. For thermally immature Jurassic shales, no relationship between TOC and micropore volume or surface area was found (Ross and Bustin, 2009), indicating that surface area alone is not the only factor controlling CH_4 capacity. A component of solute CH_4 within the internal structure of the matrix bituminite was proposed as a dominant mechanism of gas storage in Jurassic shales (Ross and Bustin, 2009). In CBM, gas content increases with depth and coal rank (Scott, 2002). The relationship between macropores and carbon content is inversely proportional: macropores decrease and micropores increase with rank, with an unexpected increasing number of macropores at low volatile bituminous rank (Levine, 1996). In general, OM is a primary control on gas adsorption: the higher the TOC content, the greater the gas-sorption rates in organic-rich sedimentary rocks (Zhang et al., 2012). Higher gas content values are typically associated with higher rank coals in many reservoirs: for example, gas content in the Piceance Basin show an overall increase in gas content with increasing rank (Scott, 2002). These results were confirmed for low and high rank coal and for organic-rich shales of different origin (Chalmers and Bustin, 2007a,b). With increasing coalification, thermal cracking of n-alkanes, waxes, and other hydrocarbons not only generates thermogenic methane but increases methane adsorptive capacity by unplugging pores, resulting in higher sorption capacity and gas content values since methane accessibility to the micropore network is improved (Scott, 2002). Controversially, in the San Juan Basin, lower rank coals have higher gas contents than higher rank coals (Scott, 2002). In this hydrogeological settings, weathering process introduced bacteria into the coal beds that produced secondary biogenic gases by metabolizing wet gases, n-alkanes, and other hydrocarbons generated during coalification (Scott, 2002). The generation of secondary biogenic gases increases gas contents beyond that expected from coal rank and if generated in sufficient quantities can actually resaturate the coal to the sorption isotherm (Scott et al., 1994). Overall, despite the similarities between shale and coal, the direct comparison of sorption characteristics of the two rocks is complicated by factors such as type and amount of OM, the mineral composition, pore volume and pore size distribution (Ross and Bustin, 2009). The controls on resource volume and productivity in SG reservoirs are similar to those in CBM, although SG reservoirs typically have lower permeability (with values in the nano- to microdarcy range), are thicker (30 to 300 ft), have lower sorbed-gas content (<10 m^3/tonne), and contain a larger volume of free gas in the pore space (Jenkins and Boyer, 2008). Of note is that although most of the pores in SG and CBM basins seem to be too small to host microbial life, evidences of microbial activity come from enrichment and isotope experiments (Martini et al., 1996, 1998; Krumholz et al., 2002; Formolo et al., 2008; Kinnon et al., 2010; Hamilton et al., 2014, 2015; Baublys et al., 2015).

3. Microbial ecology of SG and CBM

The first evidence of active, microbial populations in deep sediments was reported about 30 years ago, when microbial activity was observed in sediment depths of about 150 m in the framework of the Deep Sea Drilling Program (DSDP) (Oremland and Polcin, 1982; Tarafa et al., 1987; Whelan et al., 1986). In the past decades, the existence of prokaryotes in deep continental sedimentary rocks was proven (Pedersen, 2000) at up to 2800m. It has been suggested that the biomass into the deep biosphere constitutes one-tenth (Parkes et al., 2000), or even one-third (Whitman et al., 1998) of the total global, living biomass. The capabilities of the subsurface microbial communities to convert shale and coal OM to CH_4 was proved in laboratory (Fallgren et al., 2013; Jones et al., 2008, 2010; Warwick et al., 2008) and field studies (see Luca Technologies Inc. and Ciris Energy websites). The pathways of biodegradation of OM are microbially and biochemically complex (Jones et al., 2010), are site-specific, and could involve several communities of hydrocarbon degraders, fermenters and methanogenic Archaea. DNA-based assessment of the microbial community structure in unconventional gas basins have shown that bacterial diversity is higher than archaeal diversity (Barnhart et al., 2013; Penner et al., 2010; Green et al., 2008). The chemical complexity of OM requires the syntrophic cooperation of these microorganisms. Syntrophic metabolism accounts for much of the carbon flux in methanogenic environments (Schink and Stams, 2006). Our understanding of the *intermediary ecosystem metabolisms* (Drake et al., 2009) is limited. Bacteria related to Proteobacteria (mostly Beta, Delta and Gammaproteobacteria), Actinobacteria, Bacteroidetes and Firmicutes seem to be widespread in CBM (Green et al., 2008; Jones et al., 2008, 2010; Li et al., 2008; Strapoć et al., 2008; Robbins et al., 2016; Warwick et al., 2008) and SG (Meslé et al., 2013a,b, 2015; Struchtemeyer and Elshahed, 2012). These taxonomic groups are known for their versatile metabolic activity and hydrocarbon degrading capabilities. The archaeal diversity in shale and coal is usually

dominated by methanogens from the orders Methanosarcinales, Methanomicrobiales and Methanobacteriales (An et al., 2013; Meslé et al., 2013a,b; Fichter et al., 2012; Kirk et al., 2012).

3.1. Actinobacteria

Actinobacteria are common in soil and sediments environments, and might play a central role in the decomposition of OM. Within the Actinobacteria, the Actinomycetales and the Rubrobacterales (Prince et al., 2010) possess known hydrocarbon-degrading capabilities. Strains of *Gordonia*, *Mycobacterium*, and *Rhodococcus* are able to remove sulfur from dibenzothiophene, yielding hydroxybiphenyl (Mohebali and Ball, 2008). Actinobacteria are typically aerobic hydrocarbon-degraders, but their role in anaerobic OM degradation remains unknown (Meslé et al., 2013a,b). Metagenomic studies have also identified high proportions of genes for enzymes involved in aerobic hydrocarbon metabolism in CBM produced water samples (An et al., 2013), suggesting that the sequential degradation of complex OM causes the partial dominance of a group of microorganism in a given interval. Other studies, for example, reported that in anoxic environment the operation of different redox conditions is not mutually exclusive (Lovley et al., 1991) or cannot be explained satisfactorily by a simple microbial competition (Conrad et al., 1987).

3.2. Bacteroidetes

Bacteroidetes are commonly found in sediments, and their metabolism is chemoheteroorganotrophs. Many of such organisms can degrade macromolecules such as protein, chitin, pectin, agar, starch, or cellulose. Many others are thought to be involved in oil biodegradation (Strapoć et al., 2008). Species within *Cytophaga* are mesophilic anaerobes able to ferment polysaccharides into acetate, propionate, succinate, H_2 and CO_2 (Haack and Breznak, 1993). The genus *Petrimonas* is mesophilic anaerobic fermenter, use carbohydrates and volatile fatty acids (VFAs) releasing acetate, H_2 and CO_2 (Grabowski et al., 2005). Within the *Prolixibacter* there are acid fermenters that produce propionate, succinate and acetate (Holmes et al., 2007). Bacteroidetes also feature in coal (Li et al., 2008; Shimizu et al., 2007; Strapoć et al., 2008) and shale (Wuchter et al., 2013) microbial assemblages, although belonging to undescribed orders and families.

3.3. Firmicutes

Within the phylum Firmicutes, Clostridia of the family Clostridiaceae include pH-neutral solvent producers, mixed acid and alcohol producers, and homoacetogenic fermenters (Wiegel et al., 2006). Species like *Clostridium* have been isolated from coal sources. For example *Clostridium* BC1 (Francis and Dodge, 1988), isolated from coal-cleaning residues, presented the ability to reduce heavy metals and fix nitrogen; *Clostridium scatologenes* is an acetogenic bacteria found in a coal mine (Küsel et al., 2000). In general, *Clostridiaceae* are widespread spore-forming, anaerobic bacteria that catalyse a wide range of metabolic reactions. *Clostridia* are known to depolymerize starch, chitin, xylan, and cellulose and are known to occur in sediments (Wiegel et al., 2006). Similarly, the *Thermoanaerobacterales* include thermophilic, anaerobic, fermentative bacteria that utilize a variety of carbon substrates and may have an important role in hydrocarbon-bearing formations (Wiegel et al., 2006). The role of *Firmicutes* in coal activation has been observed before (Jones et al., 2008; Shimizu et al., 2007; Strapoć et al., 2008; Wawrik et al., 2012). *Sporomusa*, for example, can demethylate aromatic compounds; *Acidoaminococcus sp.* can ferment simple amino acids as a sole energy source. These microorganisms may potentially participate in the recycling of microbial biomass in unconventional gas systems. Although members of the *Firmicutes* often represent a minor component of the community structure in CBM basins (Ritter et al., 2015), they play an important role in laboratory experiments (Barnhart et al., 2013; Green et al., 2008; Jones et al., 2010; Li et al., 2008; Meslé et al., 2013a,b; Penner et al., 2010). In microcosm experiments the addition of methanol stimulated Firmicutes growth compared with experiments with no carbonaceous nutrients (Wuchter et al., 2013), suggesting a role of this family in the syntrophic metabolism.

3.4. Proteobacteria

The phylum Proteobacteria constitutes at present the largest and phenotypically most diverse phylogenetic lineage (Kersters et al., 2006). Syntrophic Beta, Delta and Gammaproteobacteria are commonly found in CBM (Guo et al., 2012a; Meslé et al., 2013a,b; Penner et al., 2010; Robbins et al., 2016), but also in SG flowback water (Murali Mohan et al., 2013). Betaproteobacteria consist of several groups of aerobic or facultative bacteria that are highly versatile in their degradation capacities and often containing chemolithotrophic genera. Deltaproteobacteria include a branch of strictly anaerobic genera, which contains most of the known sulfate-reducing bacteria (SRB) (*Desulfovibrio*, *Desulfobacter*, *Desulfococcus*, *Desulfonema*, etc) and sulfur-reducing bacteria (e.g. *Desulfuromonas* spp.). Deltaproteobacteria includes SRB which are able to degrade naphthalene or other aromatic hydrocarbons (Musat and Widdel, 2008). Propane and butane degraders within the SRB were also detected in marine hydrocarbon cold seeps (Jaekel et al., 2013). *Geobacter* species are known to syntrophically degrade aromatics (Lovley and Lonergan, 1990; Rooney-varga et al., 1999) and long-chain fatty acids (Coates et al., 1995) coupled to reduction of Fe(III) as a terminal electron acceptor. *Geobacter metallireducens*, for example, is genetically similar to *Syntrophus*, which can degrade a wide range of organics with a methanogenic partner (McInerney et al., 2007). In the Bowen basin pumped coal mine waters from the subsurface were dominated by bacteria belonging to the family Rhodocyclaceae (Raudsepp et al., 2016).

Gammaproteobacteria is a very large heterogeneous class; some denitrifying toluene-degrading strains belong to the Gammaproteobacteria and are able to degrade hydrocarbons with nitrate as electron acceptor (Alain et al., 2012). Although the majority of Gammaproteobacteria are chemoorganotrophs, this group also includes several chemolithotrophs that derive their energy via hydrogen-, sulfur- or iron-oxidation (Gao et al., 2009; Kersters et al., 2006).

3.5. Archaea

The archaeal diversity in SG and CBM is mostly restricted to methanogens from three orders: Methanosarcinales, Methanomicrobiales and Methanobacteriales (Green et al., 2008; Penner et al., 2010; Strapoć et al., 2011). Within Methanosarcinales there are metabolically diverse methanogens capable of utilizing H_2-CO_2, acetate, and methyl compounds as substrates for methanogenesis (Whitman et al., 2006). In the San Juan Basin, sequence libraries analysis highlighted the presence of two families: Methanosaeta (obligate acetate utilizers) and Methanosarcina (metabolically versatile) (Wawrik et al., 2012). Methanosarcinales accounted for the majority of the methanogens in coal samples from an abandoned mine in Germany (Beckmann et al., 2011) and also predominate in a consortium enriched from a CBM well from the Powder River Basin (Green et al., 2008). Cultivated strains of these taxa can utilize methyl compounds, including methanol and methylamines, where *Methanolobus* is not known to utilize acetate or H_2-CO_2. All species within the order Methanomicrobiales are known to utilize H_2-CO_2 to generate CH_4, while none are capable of utilizing acetate (Garcia et al., 2006). The presence of *Methanosarcina* in numerous worldwide CBM may suggest acetoclastic methanogenesis but also intermittent oxygen intrusion in the formation (Ritter et al., 2015), since *Methanosarcina* can survive to short oxygen exposure when in mixed cultures. In the Antrim Shale, the main methanogenic pathway is hydrogenotrophic, as discovered by Martini et al. (1996) and later confirmed by Waldron et al. (2007). In the same study,

Martini et al. (1996) found that gases from a deeper producing well of the Antrim Shale, are thermogenic, suggesting that microbial gas could be limited to shallow formations.

4. Methanogenic pathways

The range of substrates that methanogens can utilise is limited (Table 1). Biogenic CH_4 is primarily produced via CO_2-reduction (Eq. (1)) and acetate fermentation (Eq. (2)). In the first pathway H_2 is used as the electron donor and CO_2 as the electron acceptor (Weimer and Zeikus, 1978); in the second acetate and hydrogen are used to produce CH_4 and carbon dioxide (Conrad et al., 1989).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (1)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (2)$$

The first and second pathways are called respectively hydrogenotrophic and acetoclastic methanogenesis. Methanogens can also use other substrates, such as methanol (Eq. (3)) (Deppenmeier et al., 1999) and formate (Eq. (4)) (Whiticar, 1999).

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (3)$$

$$4HCOOH \rightarrow 3CO_2 + CH_4 + 2H_2O \quad (4)$$

The possible relevance for CH_4 production of other substrates such as methylamines, dimethyl sulfide, ethanol, and isopropanol is not well documented; however, methylotrophic methanogens have been detected in coal, sandstone, produced water samples (Guo et al., 2012b) and shales (Waldron et al., 2007). These substrates might be the important compounds for the enhancement of biogenic CH_4 generation in sedimentary rocks. In particular, methylamines and dimethyl sulfide are considered non-competitive substrates: when sufficient concentration of methylamines and dimethyl sulfide are present, methanogenesis and sulfate reduction are not mutually exclusive (Mitterer, 2010). For the conversion of more complicated organic substrates to CH_4, other microorganisms such as acetogenic and fermentative bacteria are also present.

4.1. Isogeochemical indicators of biogenic CH_4 in SG and CBM

Several studies on methanogenic environments in sedimentary basins world-wide have developed a set of geochemical and isotopic indicators for biogenic CH_4 in organic-rich rocks (Whiticar, 1999; Strapoć et al., 2007; McIntosh et al., 2008; Golding et al., 2013). Dual plot of carbon ($^{13}C/^{12}C$) and hydrogen (D/H) isotope ratios of CH_4 are applied for distinguishing microbial from thermogenic CH_4 in the environment (Fig. 3) (Strapoć et al., 2011; Whiticar, 1990), as well as for apportioning pathways of biogenic CH_4 production (Burke et al., 1988). The ratios, expressed in δ notation, are in units per mille (‰), relative to the isotopic composition of the internationally agreed standards VPDB (Vienna Pee Dee Belemnite) and VSMOW (Vienna Standard Mean Ocean Water) respectively for carbon and hydrogen isotopes. Biogenic CH_4 has a wide range of $\delta^{13}C$ (from −110 to −50‰) and δD (from −400 to −150‰) (Whiticar and Faber, 1986). The $\delta^{13}C$ value of CH_4 is commonly coupled with other isotopic indicator in order to distinguish between microbial and thermogenic CH_4, since $\delta^{13}C$ values of biogenic CH_4 are sometimes similar to those of thermogenic CH_4 (Coleman et al., 1988; Jenden et al., 1988; Schoell, 1980; Whiticar and Faber, 1986). The hydrogen isotope signature of CH_4 distinguishes between gas origins and can identify secondary processes such as migration or mixing (Martini et al., 1998). The hydrogen isotope signature of H_2O and CH_4 also provides a powerful analytical tool to distinguish methanogenic pathways independently of the carbon isotope signature (Schoell, 1980; Whiticar and Faber, 1986). Despite the significance of dual carbon and hydrogen isotope signatures, different origins of CH_4 often yield overlapping characteristic isotope signals (Pohlman et al., 2009; Whiticar, 1990, 1999). The empirical-based interpretations of multidimensional isotope signatures should be used with caution and coupled with other available microbiological and geochemical data (Strapoć et al., 2011). For example, carbon isotopic differences between CH_4 and CO_2 ($\delta^{13}C_{CO2-CH4}$) can be helpful to understand gas origin (Strapoć et al., 2011): thermogenic process are characterized by low $\delta^{13}C_{CO2-CH4}$ determined by high temperatures; conversely, low-temperature microbial enzymatic process determine a ^{13}C enrichment in residual CO_2 (Conrad and Klose, 2005). In mixtures of thermogenic and biogenic gases, $\delta^{13}C_{CO2-CH4}$ can be more suitable than the absolute value of $\delta^{13}C$ for discriminating gas origin (Smith and Pallasser, 1996; Strapoć et al., 2007). Three diagnostic geochemical variables were identified by Martini et al. (2008): (*i*) alkalinity and $\delta^{13}C$ of dissolved inorganic carbon (DIC) in the coproduced water, (*ii*) δ^2H of CH_4 and coproduced water and (*iii*) $\delta^{13}C$ of CO_2. Other common indicators, such as the $\delta^{13}C$ of CH_4 and the ratio of C1/[C2 + C3], have proven to be unreliable in unconventional basins where a host of secondary effects occurs and the biogenic CH_4 generated commonly has high $\delta^{13}C$ values (approximately −48‰) which overlap with early thermogenic CH_4 values (Martini et al., 2003; Whiticar, 1999). Isotopic and geochemical indicators of biogenic CH_4 production have been proved to be more effective when accompanied by molecular/microbial methods (Raudsepp et al., 2016). For example, isotopic studies indicated that in the Wilcox Group CMB, CO_2 reduction was the dominant pathways of CH_4 production (McIntosh et al., 2010; Warwick et al., 2008), but microbiological data pointed out that methylotrophic methanogens (Doerfert et al., 2009) or acetoclastic methanogens (Jones et al., 2010) were likely to be the main pathways of CH_4 generation. In the Powder River Basin, isotopic data of CH_4 indicated that hydrogenotrophic methanogenesis was the dominant pathway (Flores et al., 2008; Rice et al., 2008), while

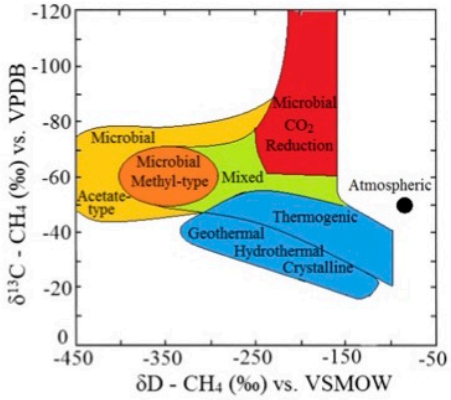

Fig. 3. Dual plot of $\delta^{13}C$ and δD of CH_4 for the classification of microbial and thermogenic CH_4. Adapted from Whiticar and Faber (1986).

Table 1
Substrates of major taxonomic groups of methanogens.

Order	Family	Substrates
Methanobacteriales	Methanobacteriaceae	H_2-CO_2, formate, methanol
	Methanothermaceae	H_2-CO_2
Methanococcales	Methanococcaceae	H_2-CO_2, formate
	Methanocaldococcaceae	H_2-CO_2
Methanomicrobiales	Methanomicrobiaceae	H_2-CO_2, formate
	Methanospirillaceae	H_2-CO_2, formate
	Methanocorpuscolaceae	H_2-CO_2, formate
Methanosarcinales	Methanosarcinaceae	H_2-CO_2, methylamine, acetate
	Methanosaetaceae	acetate
Methanopyrales	Methanopyraceae	H_2-CO_2

microbial enrichments from the same area of the basin have shown a predominance of acetoclastic methanogens (Green et al., 2008). The different conclusions of these studies indicate that the microbial communities enriched in laboratory may not be representative of the dominant microbial populations *in situ*. The relationship between carbon isotope signature of CH_4 and CO_2 ($\delta^{13}C_{CO2\_CH4}$) could be a better indicator of the extent of methanogenesis than the methanogenic pathway (Brown, 2011; Hamilton et al., 2014, 2015; Strapoć et al., 2007). The typical $\delta^{13}C_{CO2\_CH4}$ range for microbial CO_2-reduction to methane is of 60–80% (Smith and Pallasser, 1996). This carbon isotopic difference arises from preferential microbial utilization of $^{12}CO_2$. As a result, residual CO_2 becomes ^{13}C-enriched (average $\delta^{13}C_{CO2}$ of about 4.3%) and thus contrasts sharply against CO_2 in thermogenic gases with $\delta^{13}C$ values of around 20% (Smith and Pallasser, 1996).

5. Microbial processes in unconventional gas basins

The pattern of anaerobic mineralisation of OM involves the activation of complex macromolecular compounds, such as aliphatics, aromatics and heteroatoms by primary fermenting bacteria. Then secondary fermenters degrade less complex compounds to a variety of fatty acids, CO_2 and H_2. Acetogenic bacteria degrade (higher) fatty acids to acetate, formate, CO_2 and H_2, that can be used by methanogens. Acetate can also be degraded into H_2 and CO_2 via syntrophic acetate oxidation, as observed in the Yabase oil field in Japan (Mayumi et al., 2011). These processes take place simultaneously, but because of the different growth rates and activities of the microorganisms involved, the processes are partially uncoupled, resulting in the accumulation of organic acids (Stams et al., 2012). Methanogenesis is in fact a dynamic process and strongly influence the metabolism of fermentative and acetogenic bacteria by means of interspecies H_2 transfer (Schink and Stams, 2006; Stams and Plugge, 2009). Hydrogen syntrophy is hypothesised to be also responsible for anaerobic oxidation of CH_4 (Reeburgh and Heggie, 1977). In organic-rich and anaerobic sediments, SRB play a role in the anaerobic oxidation of CH_4 (Eq. (5)) (Zehnder and Brock, 1979) through a process called *reverse methanogenesis*.

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad (5)$$

Anaerobic oxidation of CH_4 coupled to sulfate reduction is assumed to be a syntrophic process, where H_2 syntrophy is the basis of the methanogen/SRB consortium (Valentine and Reeburgh, 2000). H_2 concentration is indicative of the dominant terminal electron-accepting process (Lovley and Phillips, 1987; Lovley and Goodwin, 1988; Hoehler et al., 1998). The maintenance of low H_2 allows the syntrophic oxidation of organic material through interspecies H_2 transfer (Schink, 1997; Wolin, 1982). Under sufficiently low H_2, methanogens reverse their metabolism and mediate the reversal of methanogenesis, using water as the terminal electron acceptor. The H_2 is removed and maintained at low concentrations by SRB in syntrophic association with methanogens (Hoehler and Alperin, 1996). SRB are more efficient at using H_2 as an electron donor; thus, they can create conditions that thermodynamically favour the oxidation of CH_4.

Being rich in organic carbon, shale and coal could be considered a suitable substrate for microbial activity, although kerogen is a complex and biologically recalcitrant material, composed of a mix of aliphatics (Orem et al., 2010), aromatic hydrocarbons (Orem et al., 2007, 2010; Ulrich and Bower, 2008) and heteroatoms (Orem et al., 2007). Microorganisms interact with OM in different ways, including biological depyritization, solubilisation by biologically produced alkaline materials and by biological chelators (Polman et al., 1994). The biodegrading capabilities of anaerobic bacteria were discovered in relatively recent time, as compared with aerobic-degraders (Widdel and Grundmann, 2010). The electron acceptors most frequently studied and used by anaerobic microorganism during biodegradation are nitrate and sulfate, although anaerobic degradation of hydrocarbons has been observed with Fe(III), Mn(IV) reduction (Lovley et al., 1991), and under methanogenic conditions via syntrophic interspecies electron transfer (Mbadinga et al., 2011).

However, the OM buried in sediments is a complex mixture of geopolymers and the types of organic compounds that can be oxidized to CO_2 and CH_4 are related to the different terminal electron acceptors (Fig. 4).

Few researchers have aimed to detect the degradation pathways of OM in CBM (Formolo et al., 2008; Jones et al., 2008, 2010; Warwick et al., 2008). Yet, the biodegradation pathways and the intricate microbial relationships required to convert complex OM to CH_4 are not well understood, as acknowledged by Gieg et al. (2010), Jones et al. (2008,

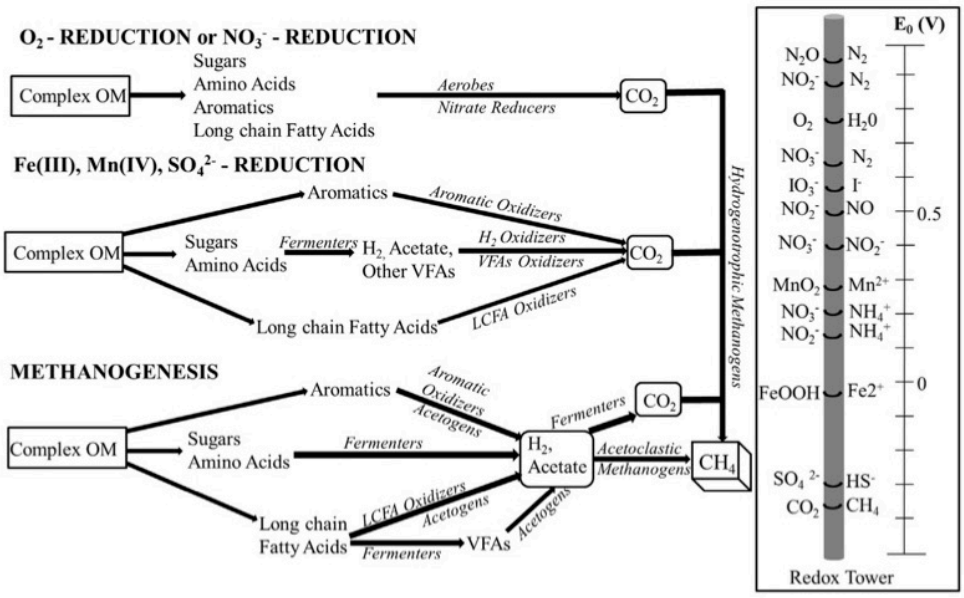

Fig. 4. Schematic of putative microbial processes in subsurface environments. The figure shows the main reactions and groups of microorganisms involved in CH_4 production, highlighting the formation of methanogenic intermediate of biodegradation. Modified from (Lovley and Chapelle, 1995). The insert on the right is the redox tower, showing the reduction potential (E_0) of the microbial processes.

2010), McInerney et al. (2009), Orem et al. (2010), Ritter et al. (2015), Strapoć et al. (2008). A number of studies for bioremediation have investigated the mechanisms by which anaerobic microorganisms activate and degrade complex hydrocarbon compounds. The anaerobic degradation of OM in shale and coal however, is expected to follow different pathways than the most studied biodegradation for bioremediation purposes. The vast body of scientific knowledge on contaminated land bioremediation can help to shed light on the complex degradation pathways of OM in unconventional gas systems. A schematic representation of the anaerobic degradation of OM from shale and coal is illustrated in Fig. 3, highlighting putative activation sites of OM, and showing the general pathways of anaerobic biodegradation of aliphatic, aromatic and heteroatom hydrocarbons.

5.1. Aliphatics

The anaerobic activation of alkanes is of particular interest since they are unreactive compounds containing only apolar σ-bonds: the most common activation is the hydrocarbon addition to fumarate, yielding alkylsuccinates (Widdel and Grundmann, 2010). The biodegradation of aliphatic and cyclic hydrocarbons can be a source of metabolites (fatty acids) that can be further oxidised to methanogenic substrates (Warwick et al., 2008); the biochemistry and subsequent degradation of alkylsuccinates is also expected to lead to fatty acid metabolism (Widdel and Rabus, 2001). Although fatty acids are a feedstock for methanogens, the accumulation of such compounds could potentially cause inhibition of methanogenesis, due to lowering of pH, (Jones et al., 2010). Alkenes activation occurs mostly from the hydration of the double bond. The biodegradation of monoterpenes and other isoprenoids in anaerobic ecosystems was observed under denitrifying conditions (Harder, 2000; Hylemon and Harder, 1998).

5.2. Aromatics

The biodegradation of aromatic compounds has been long studied, since the presence of such "contaminants" in many aquifers all around the world. These compounds are often toxic and their aqueous solubility is also an issue. The simple alkyl-substituted aromatic hydrocarbons are more readily degraded under anaerobic conditions than unsubstituted aromatics (Fig. 5). For example, the degradation of toluene under

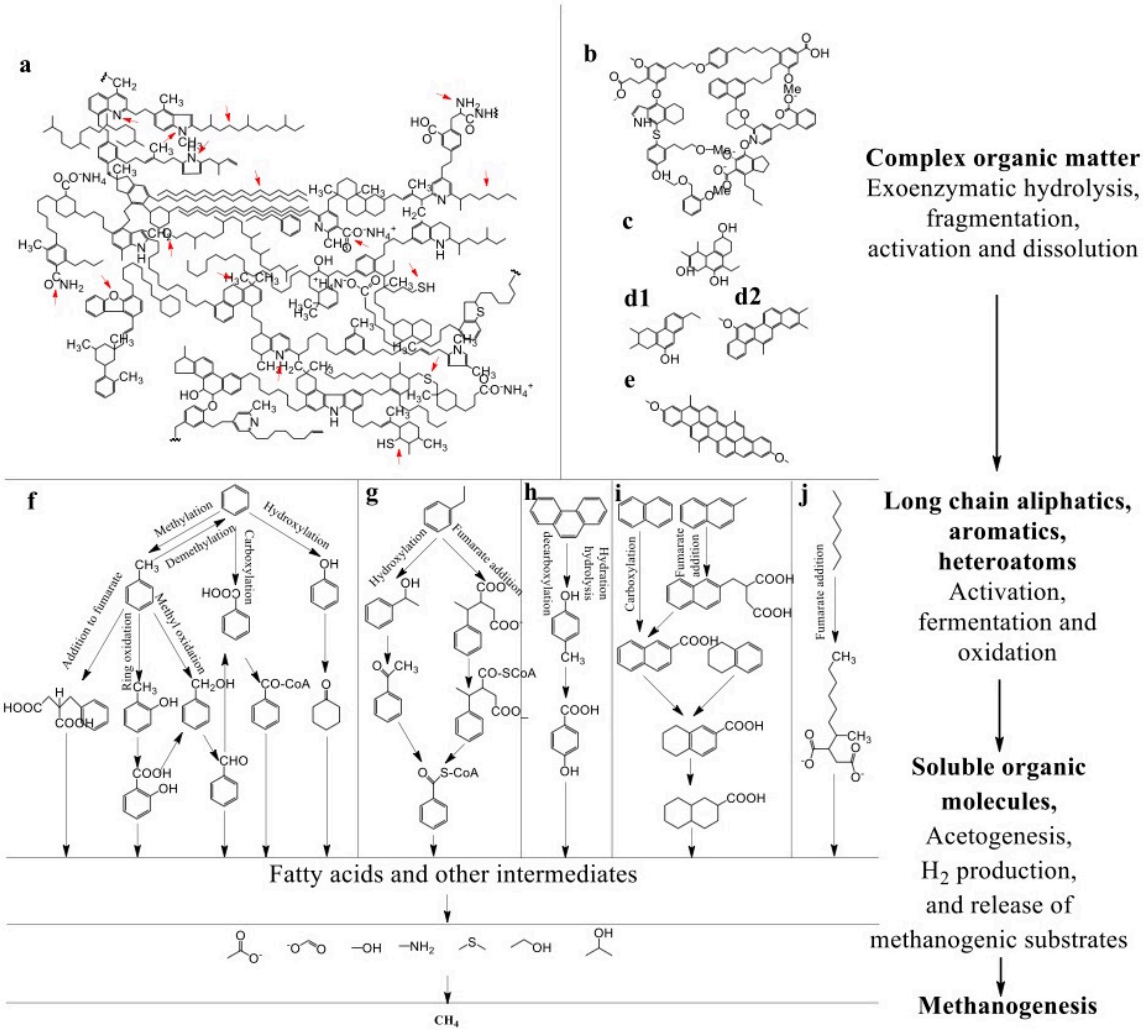

Fig. 5. General biodegradation pathways of OM from shale and coal. Schematic representation of the microbial anaerobic biodegradation of OM. Red arrows indicate the putative activation sites for the microbial transformation of OM (from Strapoć et al. (2011). (a) Structural model of the oil shale kerogen (Green River), redraw from Vandenbroucke and Largeau (2007). (b, c, d1, d2, e) Typical structures of different ranks of coal, modified from Fakoussa and Hofrichter (1999). (f) Schematic biodegradation of benzene, toluene and phenol, modified from Grbić-Galić and Vogel (1987), using McInerney et al. (2009) for benzoate degradation. (g) Representation of ethylbenzene biodegradation, redrawn from Kniemeyer and Heider (2001), (h) anaerobic biotransformation of phenanthrene, redrawn from Haritash and Kaushik (2009). (i) Naphthalene biodegradation, redrawn from Annweiler et al. (2002). (j) Short schematic degradation of heptane adapted from Strapoć et al. (2011).

methanogenic conditions require the activity of the benzylsuccinate synthase, which catalyses the addition of the methyl carbon of toluene to the double bond of fumarate (Beller and Edwards, 2000). Ethylbenzene can be completely oxidised to CO_2 by an ethylbenzene-oxidising bacterium (Strain EB1) under denitrifying conditions but not under oxic conditions (Ball et al., 1996). The final biodegradation products of ethylbenzene are potential substrates for hydrogenotrophic methanogens. The biodegradation of benzoate by a pure culture of *Syntrophusaciditrophicus* produced 1.5 mol of acetate per mol of benzoate in absence of H_2-utilizing partners or terminal electron acceptors: in co-cultures with *Methanospirillum hungatei* it produced 3 mol of acetate and 0.75 mol of CH_4 per mol of benzoate (Elshahed and McInerney, 2001). *Sporomaculatum hydroxybenzoicum* biodegraded 3-hydroxybenzoate in the absence of hydrogenotrophic microorganisms by using the crotonyl coenzyme A, which results in the final production of butyrate, acetate and HCO_3^- (Müller and Schink, 2000). The results of these studies may be significant to elucidate the degradation pathways of aromatic compounds in OM. Benzoate is a central intermediate in anaerobic degradation of many natural and xenobiotic aromatic compounds (Elshahed and McInerney, 2001). Biodegradation studies with unsubstituted aromatic hydrocarbons were carried out mostly with benzene and naphthalene under sulfate-reducing conditions: for the activation of these compounds, the mechanisms include the addition of CO_2-derived carboxyl group (Annweiler et al., 2002; Widdel and Rabus, 2001). Recent studies investigated the carboxylation of benzene and naphthalene via the putative enzymes benzene carboxylase (Abu Laban et al., 2009) and naphthalene carboxylase (Bergmann et al., 2011). Polycyclic aromatic hydrocarbons (PAHs) are commonly found in coal formation waters, coal extractable OM and methanogenic coal incubations (Strapoć et al., 2011). Many prokaryotes are capable of mineralising PAHs under anaerobic conditions; the degradation rates are usually fastest under sulfidogenic conditions, followed by methanogenic and finally nitrate-reducing conditions (Chang et al., 2002). A common bacterial strategy, which influences the PAH degradation, is the release of biosurfactants, small detergent-like molecules with a hydrophilic head and a lipophilic tail. Hydrophobic compounds become solubilized in the hydrophobic cores of the micelles, which leads to a transfer of PAHs from solid, liquid, or sorbed PAHs into the water phase (Johnsen et al., 2005). Although in the absence of nitrate or sulfate the anaerobic biodegradation of PAHs is thermodynamically unfavourable, in the presence of active methanogenic bacterial species these complex compounds may be degraded by the syntrophic food chain. The initial steps could involve the degradation of organic compound to H_2 and CO_2: subsequent utilisation of H_2 by methanogens, reducing CO_2 to CH_4, provides enough energy to make the overall reaction thermodynamically favourable (Genthner et al., 1997); thus, methanogenic bacteria serve as terminal electron sink via interspecies hydrogen transfer, and make biodegradation of PAHs thermodynamically feasible (McInerney and Bryant, 1981). The capability for anaerobic hydrocarbon degradation appears to be rather widespread in various lines of phylogenetic descent. The diversity of anaerobic hydrocarbon degraders may indicate that hydrocarbons were already used as growth substrates at an early stage of bacterial evolution and the anaerobic metabolism may be older than the aerobic (Widdel and Rabus, 2001). Altogether the pathways for the biodegradation of organic compounds can be summarized in reactions of fumarate addition, hydroxylation, C1 addition/carboxylation, and methylation (Strapoć et al., 2011). The anaerobic degradation of organic compounds is less documented as compared with the aerobic biodegradation, and most of these pathways are not completely understood. Many data, however, are available from studies of bioremediation in anoxic soil, and may help to decipher the complicated pathways of biodegradation of geologically-old OM and the microbial consortia involved in the syntrophic chain. The relevance of these studies is not only related to the planning of *in-situ* stimulation strategies, but also to remediate or mitigate accidental contamination due to drilling activities, mining and storage of wastewater.

5.3. Heteroatoms

NSO (nitrogen-, sulfur- and oxygen-containing heterocyclic compounds) were found in coal (Orem et al., 2007; Wawrik et al., 2012) and shale formations (Gross et al., 2015). Heteroatoms were long considered recalcitrant to biodegradation, and in a "susceptibility scale" classified as the last group of compounds, after normal alkanes (usually catabolized first), followed by branched alkanes, monocyclic saturated, monoaromatic hydrocarbons and PAHs (Hunt et al., 1995; Rowland et al., 1986; Volkman et al., 1983; Wenger and Isaksen, 2002). NSO compounds are not as recalcitrant as once believed and could undergo selective degradation process as complex as those for hydrocarbons (Kim et al., 2005) NSO compounds are more soluble in water than PAHs, since the replacement of a carbon atom with a nitrogen, sulfur or oxygen atom result in higher polarity and hence higher water solubility and increased bioavailability and mobility. Also, chemical bonds between carbon and heteroatoms have lower bond dissociation energies than aliphatic or aromatic C—C bonds (Savage, 2000). Thus, heteroatoms are more reactive than PAHs, characterised by C—C bonds; the mechanisms of activation of these compounds are similar to biodegradation pathways observed for PAHs, and could include demethoxylation as demonstrated by stable isotope probing (Liu and Suflita, 1993).

6. Environmental requirements for *in situ* biogenic CH_4 production

Biogenic CH_4 production is significant in nearly every shallow coal seam at temperatures less than 80°C (Jin et al., 2010; Pfeiffer and Ulrich, 2010), in SG basins the contribution of biogenic CH_4 is also depth related in the majority of basins (Golding et al., 2013; Krumholz et al., 1997; Martini et al., 2008; McIntosh et al., 2008). The relationship between methanogenesis and depth is not controlled only by temperature, but also correlates with possible events of natural groundwater recharge that enhances methanogenesis by either (*i*) transporting microorganisms into organic-rich reservoirs, providing moisture necessary for microbial activity, decreasing salinity, removing waste products, and/or (*ii*) transporting nutrients necessary for microbial growth (Jones et al., 2013; Martini et al., 1996; McIntosh et al., 2002; Strapoć et al., 2008, 2010; Zhang et al., 2013). Reduction in Cl^- concentration is crucial for promoting methanogenesis in basins with high salinities, since methanogens prefer salinity gradient between 0.5 and 4 M Cl^- (Orem et al., 2010; Osborn and McIntosh, 2010; Schlegel et al., 2011b; Waldron et al., 2007; Zinder, 1993). The observation that microbial gas generation occurs at significant rates only in shallow CBM and shale gas basins is also dependant on the bioavailability of readily degradable OM. With increasing depth, the organic compounds become more recalcitrant to biodegradation (Head et al., 2003; Wellsbury et al., 1997; Strapoć et al., 2008; Robbins et al., 2016). Recent studies showed a significant negative correlation between final biogenic methane yield and rank, suggesting that the bioavailability of the coal organic material decreases with increasing thermal maturity (Robbins et al., 2016). The chemistry of coal changes systematically with increasing rank, as oils and gases are generated and then cracked, producing abiotic methane and higher hydrocarbon gases, thus reducing the fraction of biodegradable moieties (Papendick et al., 2011). The negative correlation between biogenic methane and rank of coal does not provide an exhaustive explanation of biogenic CH_4 production, suggesting that other limiting factors such as the accessibility of microbes to OM could play a more important role. Although the transport/presence of bacteria in organic-rich rocks cannot be completely ruled out, indigenous microbial communities live mainly within fractures (cleats) in shale and coal formations, or at the interface of coal with overlying or underlying rock layers (Fredrickson et al., 1997; Martini et al., 1998; Scott, 1999). This provides limited surface area for the microorganisms to interact with OM. It has been suggested that the pore throat size must be double the diameter of cells to allow bacteria to effectively pass through (Fredrickson et al., 1997). In the Illinois Basin CBM, Strapoć et al.

(2008) reported that the dominant methanogen was on average 0.4 μm in diameter, indicating that pores and/or fractures in reservoirs supporting methanogenesis must be much greater in diameter. In sandstone formations with permeability less than 100 mD, the bacterial penetration typically occur slowly (Jenneman et al., 1985), suggesting that in shale-sandstone sequence, microorganisms are slowly, but steadily transported in the deep subsurface. Competition with other groups of microorganisms could be another limiting factor, several studies have investigated the competition between SRB and methanogens. These studies suggested that methanogenesis and sulfate reduction are mutually exclusive due to competition for carbon substrates (Claypool and Kaplan, 1974; Kuivila et al., 1989; Lovley and Phillips, 1987; Mah et al., 1977; Martens and Berner, 1974; Reeburgh and Heggie, 1977). In the absence of sulfate, SRB may play a role in the breakdown of OM into methanogenic substrates (Mah et al., 1977; Raskin et al., 1996; Wawrik et al., 2012). Depending on the redox conditions and availability of substrates, the two processes can take place simultaneously, although the sequential dominance of SRB or methanogens in a given interval is more likely to happen. The dominance of a particular class of microorganisms is dependent on many factors, such as H_2 concentration, which control also the production and oxidation of CH_4 under anaerobic conditions.

7. Stimulation of biogenic CH_4 production

Research into the stimulation of biogenic CH_4 production in unconventional gas systems is a new focus area for engineers and scientists. There are some similarities with conventional microbial enhanced oil recovery, but many research questions remain unanswered. Recently, there has been considerable work on microbial methanogenesis in CBM (Green et al., 2008; Harris et al., 2008; Jones et al., 2010; Papendick et al., 2011; Penner et al., 2010; Ritter et al., 2015; Singh et al., 2012) and SG (Martini et al., 1998; Jones et al., 2010), reflecting the potential for *in situ* sustainable regeneration of CH_4. While microbial methanogenesis in unconventional formations is complicated by a number of biogeochemical factors, a review of the relevant microbiological and geochemical literature allows the identification of key parameters for *in situ* stimulation strategies that include:

1. presence of viable methanogens and primary/secondary fermenters;
2. competition for methanogenic substrates;
3. methanogesis rates;
4. bioavailable/biodegradable OM;
5. temperature;
6. formation salinity;
7. presence of fractures and pore size distribution.

Strategies for the *in situ* stimulation of CH_4 production typically include technologies developed for the bioremediation of contaminated sites, such as (*i*) the addition of inorganic or organic nutrients in order to stimulate the native microbial populations (*biostimulation*) and (*ii*) the addition of a microbial consortium (*bioaugmentation*). Other consolidated technologies in the unconventional gas industry could potentially be used to achieve optimal conditions in the formation, including hydraulic fracturing that can (*iii*) increase the contact surface area of microorganisms to coal/shale and (*iv*) increase the bioavailability of OM. These approaches could be used separately or in combination to achieve a continuous generation of biogenic CH_4 from existing producing wells or depleted wells.

7.1. Biostimulation

Microbial stimulation involves the addition of nutrients and/or electron donors and acceptors to the formation in order to stimulate CH_4 production from indigenous microorganisms. Nutrients are typically added in formations where biogenic CH_4 generation is active or where methanogenic rates are decreasing over time in the attempt to stimulate the growth of methanogenic communities and shift redox conditions to methanogenesis (Barnhart et al., 2013; Fallgren et al., 2013; Jones et al., 2010; Ritter et al., 2015). The addition of methanogenic substrates such as CO_2-H_2 or acetate could stimulate biogenic CH_4 production, but the primary goal of microbial stimulation should be to target primary and secondary fermenters (Mahaffey et al., 2013; Schlegel et al., 2013) able to degrade the complex geopolymers and release intermediary products that can be converted to CH_4 by methanogens. This should take into account that syntrophic and fermentative bacteria, which are likely to be the main contributor to OM breakdown, survive near the thermodynamic limits of life (Elshahed and McInerney, 2001; McInerney et al., 2008) and, therefore, their growth is slow (Lovley et al., 1991) and dependant on several other factors. The introduction of electron donors/acceptors, which could stimulate microbial growth, is likely to divert electrons away from methanogenesis, since stimulation of more rapid organic release could result in toxic conditions that could limit biogenic CH_4 generation (Jones et al., 2008). Biostimulation seems to be the primary approach of current commercial stimulation projects (Luca Technologies: Mahaffey et al., 2013; Next Fuel: Fallgren et al., 2013; Ciris: Ciris Energy, 2013).

7.2. Bioaugmentation

Bioaugmentation involves the introduction of microorganisms into the target environment to increase the *in situ* metabolic activity (da Silva and Alvarez, 2010). Bioaugmentation may consist of a single microorganism or more typically a consortium of microorganisms (i.e., Bacteria and Archaea). In most cases, the microorganisms to be injected do not originate from the target environment, but are enriched and evaluated for high methanogenesis rates in laboratory experiments. When introducing an enriched consortium in the target formation, CH_4 generation rates could be much lower than in laboratory studies, where incubations are typically carried out with small chips of rock, therefore the accessibility of the microorganisms to OM is greatly increased when compared with *in-situ* conditions. This could bring biases in the results, leading to an overestimation of the CH_4 generation rates. Although the bioaugmentation method was shown to produce more CH_4 than biostimulation (Jones et al., 2010), it may be difficult, in some cases, to obtain permission from regulatory agencies to inject microorganisms into the subsurface, especially in areas where adjacent aquifers are used for drinking water. Very few research groups have pursued the microbial augmentation approach at the field scale (Ritter et al., 2015). MicGas™, for example, used a combination of biostimulation and bioaugmentation, adapting methanogens derived from termites to coal in the presence of appropriate nutrients (see http://www.arctech.com/micgas.html).

7.3. Increase the contact surface area of microorganisms to coal/shale

Since the pore matrix of coal and shale is typically too small for microorganisms, methanogenesis is often limited to fractures (Scott, 1999) and at the fringe between the source rock and more permeable formations, where the pore size is greater, as well as the availability of water (Krumholz et al., 2002; Martini et al., 1998). Increasing the surface area available for microbial colonization could be accomplished through existing techniques, such as hydraulic fracturing. Hydraulic fracturing is carried out to increase the permeability of SG formations and coal seams, and involves the pumping of large volumes of fluids into these formations under high pressure. Water and sand represent 98 to 99.5 % of the fluid used in hydraulic fracturing. Additional additives may include acids to remove drilling mud near the wellbore and biocides to prevent deleterious microbial activity (Davies, 2011). A portion of the so-called "fracking" fluids remains in the formation after the completion of the fracturing process, offering the opportunity to introduce a microbial consortium into the induced fractures, as part of a nutrient-delivery system, or more broadly, to modify the biogeochemical

conditions in the formation. Such use of hydraulic fracturing should consider alternative solutions to the addition of biocide, typically used to prevent sulfide production that potentially increase human and environmental health risks, corrosion, and costly degradation of product quality. Possible strategies to prevent sulfide production could be to eliminate sulfur-containing compounds from the drilling mud. For example, dolomite could be substituted for barite when adding weight to bentonite-based drilling mud and, lignosulfonates could be replaced with polyphosphates, leonardite, and tannins (Struchtemeyer et al., 2011). In spite of the importance of hydraulic fracturing, very little is known about the microbiological consequences of this process. Increasing permeability helps facilitate CH_4 production (i.e., enhances transport of gas to the wellbore (Solano-Acosta et al., 2007), and would likely help carry injected nutrients, water, and/or microorganisms to additional coal surfaces. Currently, there are only few studies that evaluate the change in the microbial composition of fracking fluids before and after the fracking process (Davis et al., 2012; Struchtemeyer and Elshahed, 2012; Struchtemeyer et al., 2011, 2012), but none of them aim to enhance the engineering of fracking practices to stimulate microbial processes.

7.4. Increasing the bioavailability of OM

The biotic and abiotic process of breaking down OM into methanogenic intermediates is often considered a rate-limiting step in methanogenesis (Scott, 1999; Strapoć et al., 2011; Wawrik et al., 2012). Increasing the bioavailability of complex geopolymers could be accomplished through the addition of chemicals to dissolve the coal/shale matrix (Scott, 1999). Laboratory studies have suggested that the addition of a strong oxidant, such as potassium permanganate (Huang et al., 2013) or hydrogen peroxide (Jones et al., 2013) may help to convert coal carbon to organic acids, although such chemicals could potentially be harmful to methanogens. The addition of surfactants was also tested to reduce surface and interfacial tensions between coal molecules (Papendick et al., 2011; Singh and Tripathi, 2013), however, surfactant micelles can trap substrates and actually reduce their bioavailability in some cases (Mihelcic et al., 1993).

8. Conclusions

While recent studies have helped to clarify the role of various microbial populations in conventional oil reservoir, the broader implications for SG and CBM production are still not understood. Laboratory-based and commercial projects studies regarding the stimulation of microbial methanogenesis has significantly increased our knowledge about the processes that lead to microbial CH_4 generation from complex OM. Laboratory-based research has provided insight into locations and environments where microbial CH_4 was observed, the microbial communities involved and the metabolic pathways that lead to methane production. Commercial projects showed that microbial methane production in unconventional gas basin is significant and can be stimulated *in situ*. Yet, there are very few published shale reservoir microbiology studies, highlighting the need for novel insight into guiding practical strategies for enhanced gas recovery and for mitigating undesirable microbial processes and environmental impact. Any shallow, low temperature SG and CBM basin represent the opportunity for microbial methane stimulation. Shallow gas wells are relatively inexpensive to drill compared to deep basin; as a consequence, biogenic gas systems represent an important component in the mix of natural gas accumulations that will ultimately meet high demands of gas. Shale and coal vary greatly in terms of their physical, geochemical and biological characteristics. Studies on the *in situ* stimulation of microbial methane production should consider the compilation of studies discussed in this review. Current available technologies such as hydraulic fracturing could be adapted and used to stimulate microbial methanogenesis in shallow unconventional systems. Most of the biological activity in SG and CBM occurs in fractures and at the interface between the source rock and more permeable formations, where the pore size is greater, as well as the availability of water. Hydraulic fracturing, typically used to increase the permeability and the fractures network of SG formations, could be adapted to increase the contact surface area of microorganisms with the shale/coal interface and to guarantee a greater accessibility of OM for biodegradative microorganisms. Further research should be focused on issues related to the implementation and sustainability of hydraulic fracturing process. Intensified concerns by the public have prompted some companies to develop more environmentally friendly fracturing fluids. Halliburton, for example, is testing its CleanStim® formulation, composed of ingredients sourced from the food industry. Similarly, Chesapeake Energy eliminated 18% of the chemical additives used in hydraulic fracturing fluids thanks to their GreenFrac® initiative. FracFocus, a web-based registry with support from the U.S. Department of Energy, provides details on the additives, chemicals and the amount of water typically used in the hydraulic fracturing process.

Research into the microbiology of unconventional gas systems is a new interesting topic for engineers and scientists. Despite the similarities with conventional petroleum microbiology, there are many research questions regarding the bioavailability of OM, what specific microbial communities lead to methane production and their metabolic pathways. Moreover, research on water resources and wastewater management are still an issue. The answers to these research questions have implications for both enhanced recovery of gas and sustainable development of unconventional gas resources.

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4.3. Final considerations

The extensive review presented in this Chapter showed that the current understanding of microbial processes in the deep subsurface is still in its infancy. While a number of studies and data are available for geotechnical and engineering aspects of shale gas and CBM production, our current knowledge on microbial methane in unconventional gas systems is limited.

Little is known about the key steps in anaerobic food chains that require syntrophic metabolism, as compared to the aerobic metabolism. The review presented in this Chapter suggest that one limiting step in the mineralisation of complex organic matter is to be found in the syntrophic metabolism, rather than in the last step of biodegradation, where methane is generated. Syntrophic metabolism accounts for much of the carbon flux in methanogenic environments (Schink and Stams, 2006). Most of the studies discussed in Colosimo et al., (2016) focus on the final step of biodegradation of organic matter (acetoclastic or hydrogenotrophic methanogenesis), which represents only the bottom of the reactions cascade. Methanogenesis is carried out with a restricted number of compounds (acetate, formate, CO_2/H_2, methanol, methylamines, dimethyl sulphide), while the chemical complexity of organic matter require several communities of microorganisms able to metabolize a wide range of compounds.

Our understanding of the *intermediary ecosystem metabolisms* (Drake et al., 2009) is limited. Bacteria related to *Proteobacteria* (mostly *Beta-*, *Delta-* and *Gamma-proteobacteria*), *Actinomycetes*, *Bacteroidetes*, *Cloroflexi* and *Clostridiales* seem to be widespread in CBM (Green et al., 2008; Jones et al., 2008 and 2010; Li et al., 2008; Penner et al., 2010; Warwick et al., 2008; Strapoc et al., 2008) and shale gas (Struchtmeyer et al., 2011). These taxonomic groups are known for their versatile metabolic activity. The metabolic functions of the archaeal and bacterial diversity in CBM of different origins is shown in Table 4.1 and 4.2, respectively.

Table 4.1. *Archaea* diversity and function in CBM.

Order	Genera	Metabolic activity	Functions
Methanosarcinales	*Methanosarcina*	Versatile	Methanogens
	Methanolobulus	Methylotrophic	
	Methanosaeta	Acetoclastic	
Methanomicrobiales	*Methanocalculus*	Hydrogenotrophic	
	Methanoculleus	Hydrogenotrophic	
Methanobacteriales	*Methanobacterium* *Methanocorpusculum*	Hydrogenotrophic	
Methanopyrales	*Methanopyrus*	Hydrogenotrophic	
Methanococcales	*Methanococcus*	Hydrogenotrophic Formate utilizers	

Table 4.2. Bacteria commonly found in CBM formations. The table show the main Phyla and Genera with putative role in anaerobic biodegradation of organic matter. Modified from Meslé et al. (2013).

Phyla	Genera	Metabolic activity	Functions
Actinobacteria	*Propionella* *Rhodococcus*	Cellulolytic, metabolism of poorly water-soluble organic compounds, degradation of ligninocellulosic compounds	Fermenters
Bacteroidetes	*Bacteroidetes* *Bacteroides* *Cytophaga*	Fermentation of organic acids and polymers, hydrocarbon degradation	Fermenters
Firmicutes	*Clostridium*	Hydrolytic digestion of macromolecular compounds, acetate production/ oxidation, hydrocarbon degradation	Fermenters Syntrophs
	Sedimentibacter,	Fatty acids fermentation	Fermenters Syntrophs
	Acetobacterium	Homoacetogens	Syntrophs
	Syntrophomonas	Fatty acid oxidation	Syntrophs
α-proteobacteria	*Sphingomonas* *Rhodobacter* *Roseobacter*	Metabolism of PAHs; oil-degradation; primary fermentation	Fermenters
β-proteobacteria	*Azoarcus* *Azovibrio* *Thauera* *Acidovorax* *Hydrogenophaga*	Saturated and aromatic hydrocarbon degradation, H_2-metabolism, NO_3^--reduction	Fermenters
γ-proteobacteria	*Pseudomonas* *Thalassolituus* *Acinetobacter*	Hydrocarbon degradation, primary fermentation, NO_3^- reduction, iron oxidation, sulphur oxidation, hydrogen oxidation	Putative fermenters
δ-proteobacteria	*Desulfuromonas* *Desulfovibrio*	SO_4^{2-} reduction, hydrocarbon degradation, organic acids fermentation	Fermenters Syntrophs
	Desulfomicrobium	Secondary fermentation of fatty acids to acetate and hydrogen	Syntrophs
ε-proteobacteria	*Syntrophus* *Smithella*	Fatty acids degradation, alkanes fermentation	Syntrophs
	Campylobacter *Arcobacter* *Sulfospirillum*	NO_3^--reduction, HS^--oxidation, fermentation of organic compounds, hydrocarbon degradation, acetate oxidation	Fermenters Syntrophs

The results obtained in this Chapter highlighted the need to find an active microbial population that can be used to test the four strategies for the stimulation of biogenic methane production in unconventional gas systems (*i*) biostimulation, (*ii*) bioaugmentation, (*iii*) increase the contact surface area of microorganisms to coal/shale and (*iv*) increase the bioavailability of organic matter (Colosimo et al., 2016). However, to test these four strategies, there is the need to find an active biodegradative microbial consortium that can be adapted to the extreme conditions encountered in unconventional gas systems. Moreover, molecular tools need to be adapted for the analyses of microbial community dynamics. To carry on these new experiments, a natural analogue of the bacterial and archaeal communities of shale/coal formations was identified in man-made bioreactor such as anaerobic digester that use as primary substrate petrochemical waste products. The rationale of this choice, as well as the molecular characterisation of this analogue bacterial inoculum, are described in the next Chapter.

The extensive review of Colosimo et al., (2016) pointed out that existing engineering technologies such as hydraulic fracturing could be adapted to favour positive microbial processes in unconventional gas systems. However, fundamental questions remain regarding the bioavailability of organic matter, the specific microbial communities and the metabolic pathways that lead to methane production. The answers to these questions have implications for recovery practices and sustainability of unconventional resources. The preliminary work carried described in Chapter 4a and the review of Colosimo et al., (2016) helped to develop and verify a protocol to handle and process shale and coal core samples for microbial community analyses. However, molecular and bioinformatics tools for the microbial community analysis need to be developed and adapted to the specific aim of this Ph.D. project. The investigation on the microbial community structure, using high-throughput 16S rRNA gene sequencing, is described in the next Chapter, as well as the optimisation of bioinformatics tools for the analysis and interpretation of complex data.

Chapter 5

Paper II: 454 pyrosequencing of biodegradative bacteria from thermal hydrolysis processes

5.1. Introduction

The preliminary experiments carried out during this Ph.D. project (described in Chapter 3, Section 3.1) lead to development of standard operating procedures to handle shale core rocks for microbial analyses. The protocol, validated through molecular methods, was effective to avoid contamination and to analyse the inner part of four shale cores obtained from two different sites in UK (Rosecote and Duffield, England). The molecular analyses showed no bacterial populations in any of the analysed cores, indicating that there were no active indigenous populations.

The absence of microbial activity was also confirmed by enrichment experiments, carried out in the attempt to multiply the number of cells in small samples. Further molecular methods applied to the enrichments, also showed no microbial activity, suggesting that no indigenous microbes were present and that the samples were not subject to recharge of water through weathering processes. Although this was a negative result the complete absence of microbial activity in the enrichments indicates no contaminations of any kind, confirming the validity of the developed protocol.

Developing an efficient method for the detection and monitoring of microbial consortia involved in the production of methane from organic-rich shale and coal

cores is a crucial step to evaluate the potential of microbial methane generation. Most of the studies concerning the microbial characterisation of shale gas plays (Martini et al., 1998; Jones et al., 2010) or coal beds (Green et al., 2008; Harris et al., 2008; Jones et al., 2010; Papendick et al., 2011; Penner et al., 2010; Ritter et al., 2015; Singh et al., 2012) focus on water and produced water within the formation, but none of them attempted to perform a microbial characterisation of the rock itself. Bacteria and Archaea in this case can rarely be quantified directly, as a result of low initial numbers. A culture-based approach, such as biostimulation, could be a method to increase the bacterial and archaeal counts above the detection levels, as well as increase the methanogenic activity of indigenous microorganisms. As the number of Archaea and Bacteria in the source rocks is too low to allow direct enumerations by the most widely accepted methods such as self-fluorescence observations or qPCR assays; a culture-based approach is likely to be the only possible way to overcome this limit, despite the numerous biases that it could bring.

The experiments carried out during this Ph.D. project consisted a combination of microbial culturing techniques and molecular methods. These two methodology were used in order to reduce the bias of a single approach. By carrying out these experiments, the effectiveness of the protocols has been confirmed by the “blank” results obtained, since there was no contamination, as shown in the PCR results and in the MPN experiments (Chapter 3, Section 3.1). Although the preliminary experiments have been proved effective for the analyses of shale/coal cores, the absence of autochthonous microbial communities moved the attention to the search for other degradative bacteria and Archaea.

Numerous oil and gas companies were contacted all across the UK, USA and Australia in order to obtain samples, but none of them was keen to provide samples. WSP Parsons Brinckerhoff, the industrial partner that supported this Ph.D. project, attempted several times to obtain samples from unconventional gas companies; despite the continuous effort, the current regulations and legislation discouraged gas companies from providing samples.

WSP Parsons Brinckerhoff also contacted AGL Energy (The Australian Gas Light Company) in order to obtain coal-bed methane samples from an active coal-bed methane drilling site. The agreement was nearly approved and, in preparation of this work, a research proposal and sampling procedures were developed (Appendix of Chapter 5).

During the second year of the Ph.D. I have also visited a site where a drilling project was carried out by Raeburn Drilling & Geotechnical Ltd, a company that owns and operates a fleet of boring and drilling rigs in Scotland. The site, located south of the Central Belt of Scotland, host a coal-bearing strata that was drilled for a third company. Thanks to an agreement between WSP Parsons Brinckerhoff, the University of Strathclyde and Raeburn Drilling, I managed to visit the site in order to take samples of coal cores and produced water. Unfortunately, the third company prevented sampling via a last minute communication, even though it was a small amount required (a minimum of 200 g of core and 2 L of produced water). These samples were necessary to analyse the microbial community, with culturing and molecular approaches and to set up microcosm experiments to assess methanogenic rates.

The research plan approved by the supervisory team reflected a particular interest of the Industrial Partner of the Ph.D. The overriding criterion of the research plan was to link the novel unconventional gas industry with well-established bioremediation technologies. The investigation of biodegradation processes in shale and coal is a key issue in determining the pathways that lead to methane production, and should provide valuable information for the design and optimization of stimulation strategies to increase production. Studying the biodegradation processes in unconventional gas system could also provide guidance for risk assessment and the effective planning of decontamination treatments.

The two overlapping objectives of the research were: (*i*) the development of strategies for the enhancement of unconventional gas production and (*ii*) the development of effective decontamination treatments for shale and coal produced

water. To achieve the objectives of the research, a combined approach of organic geochemistry analyses and molecular community assessments was planned. Further information regarding this research plan as well as the sampling protocol developed for this research can be found in the Appendices of Chapter 5.

The difficulties encountered in obtaining fresh samples such as shale/coal cores, formation water and flowback water led to the search for allochthonous biodegradative communities. When searching for an active bacterial and archaeal community, further considerations need to be taken regarding the ecology and physiology of the target microorganisms. There is no doubt that the anaerobic degradation of hydrocarbons can be accomplished axenically by microbial pure cultures under a variety of electron accepting conditions as well as syntrophically by nutritionally diverse microbial consortia. However, the environmental consequences of anaerobic hydrocarbon transformation in shale gas basins and coal beds depend on the geochemical settings and other factors described in detail in Colosimo et al., 2016 (Chapter 4) of this Dissertation.

Despite there being several difficulties in adapting an allochthonous microbial consortium to the conditions encountered in the deep subsurface, a natural analogue of the bacterial and archaeal communities of shale/coal formations could be found in man-made bioreactor such as anaerobic digester that use as primary substrate petrochemical waste products. The bacterial and archaeal populations commonly found in such anaerobic digesters are well adapted to high concentration of heavy hydrocarbons and are capable of mineralizing these contaminants to CH_4, CO_2 and H_2O.

The Bran Sands Advanced Digestion Facility, (Middleborough, UK), is the largest plant in the UK that uses thermal hydrolysis processes for the digestion of petrochemical waste. For this Ph.D. project, Bran Sands Teesside represented a great opportunity to provide a biodegradative microbial consortia that could serve as a source of adapted microorganisms to be used in this study. The analyses carried out on a digestate sample from Bran Sands were useful to assess the similarities between

the bacterial populations of the digester (Chapter 5, Paper II) and those commonly found in shale/coal formations described in Colosimo et al., 2016 (Chapter 4).

To characterise the biodegradative bacteria of the digestate samples, novel molecular tools were applied. The composition of biogas-producing microbial communities is commonly determined via construction of 16S-rDNA clone libraries and subsequent sequencing of 16S-rDNA amplicons (Klocke et al., 2007; McHugh et al., 2003; Mladenovska et al., 2003). Development of second-generation ultrafast sequencing technologies such as 454-pyrosequencing has led to the realisation of cost-effective large-scale environmental shotgun sequencing projects. High-throughput 16S rRNA gene sequencing is a versatile approach for exploration of different habitats for the structure, gene content and function of the respective autochthonous microbial communities (Edwards et al., 2006; Gill et al., 2006; Turnbaugh et al., 2006). Bioinformatics for the interpretation of metagenomic data has constantly been improved (Raes et al., 2007). In this work we carried out a deep molecular characterization of the digestate of the Bran Sands Anaerobic Digester. The results, obtained with 454 pyrosequencing revealed a complex bacteria population, that reflect the conditions used for biogas production. Moreover, the results highlighted significant similarities with the biodegradative bacterial population of shale/coal formations, as shown in the review paper presented in Chapter 4 (Colosimo et al., 2016).

5.2. 454 pyrosequencing of biodegradative bacteria from thermal hydrolysis processes

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Abstract

Anaerobic treatment process is a cost-effective method for treating organic wastes, since the biogas formed can be used for heat/electricity production and the digester residues can be recycled for other applications. An innovative use of the digestate could be as biodegradative and methanogenic inoculum for the stimulation of methane production in gas-producing or depleted wells. The microbial communities involved in the biodegradation of petrochemical waste are similar to the indigenous microorganisms typically found in unconventional basins. These communities also follow the same cascade of reactions: from the initial breakdown of complex molecules to the production of intermediate compounds used by methanogens. This study carried out a culture-independent assessments of the bacterial community composition of a digestate from the Bran Sands Advanced Digestion Facility (Middleborough, UK) and compared the results with the microbial populations found in unconventional gas basins. The 454 pyrosequencing analyses revealed a bacterial community dominated by *Thermotogae*, *Bacteroidia*, *Clostridia* and *Synergistia*, which are typically found in unconventional gas systems. The classification of

nucleotide sequence reads and assembled contigs revealed a genetic profile characteristic for an anaerobic microbial consortium running fermentative metabolic pathways. The assignment of numerous sequences was related to hydrocarbon decomposition and digestion of cellulosic material, which indicates that the bacterial community is engaged in hydrolysis of plant-derived material. The bacterial community composition suggest that the effluent of the digester can be used as a biodegradative inoculum for the stimulation of methane generation in unconventional wells, where events of microbial methanogenesis have been previously observed.

Keywords: Pyrosequencing, anaerobic degradation, petrochemical waste.

Index:

1. Introduction

Organic residues accumulate in large amounts as left-overs of industrial activities, food processing and as household refuse; these waste products are therefore available as a substrate for biogas production (methane (CH_4)) in anaerobic reactors (Wiesenthal et al., 2007). Petrochemical waste has a considerable advantage compared with renewable primary products (maize silage, fodder beat, green rye, etc.) as there is no competition between the use of the substrate for the production of biogas and the use of the substrate as food.

In general, the biogas-forming process is characterized by three phases which proceed simultaneously in a continually fed reactor. Initially, bacteria attack and utilize polymers (complex hydrocarbons such as carbohydrates, proteins, and lipids) by excreting hydrolytic enzymes (e.g. cellulases, cellobiases, xylanases, amylases, lipases, and proteases) (Weiland, 2010). This process is called (*i*) hydrolysis or primary fermentation (Schink, 1997). The major products released by these bacteria are carbon dioxide (CO_2), hydrogen (H_2), volatile fatty acids such as acetate, and a wide range of intermediates. Those intermediates are then further transformed to H_2, CO_2, acetate, and other acids during a process called (*ii*) acidogenesis/acetogenesis or secondary fermentation (Schink, 1997). H_2 and CO_2 or acetate is used by methanogenic archaea for the production of methane, in a process called (*iii*) methanogenesis or methanation (Weiland, 2010). Finally, the methane is converted to electricity by non-bacterial processes. To close the cycle, in most cases, the digestate of the biogas-forming process can be recycled as fertilizer or soil amendments (Bogner et al., 2008).

The influence of the composition and diversity of the microbial community on the stability of the biogas-forming process and on biogas yield is of great interest (Weiland, 2010). So far, several studies have focused on the microbial diversity in biogas plants supplied with renewable primary products and liquid manure as substrates (Schnürer et al., 1999; Cirne et al., 2007; Schlüter et al., 2008; Weiss et al., 2008; Kröber et al., 2009; Liu et al., 2009; Nettmann et al., 2010). Whilst most of these studies focused on the *Archaea* community structure, more research efforts

should address the complex interplay within the bacterial community that ferment complex organic material.

Research into anaerobic processes is currently undergoing a reawakening due to the development of techniques suitable for mechanistic linking of whole community function and phylogeny (Vanwonterghem et al., 2014), and as a platform to investigate phenomena such as direct interspecies microbial electron transfer (Morita et al., 2011). At the same time as these discoveries are enhancing our understanding of the process, the scope of applications as biotechnological processes is also expanding. As a waste valorisation process, anaerobic digestion is important, as it allows almost complete recovery of inherent chemical energy during relatively low cost conversion to methane, a transportable, vehicle and natural gas network compatible energy source (McCarty et al., 2011). Anaerobic digestion has been traditionally applied to either slurries (2-6% solids) in mixed reactors (Batstone and Jensen, 2011), or to low solids, concentrated industrial or domestic waste-waters in high-rate processes such as the Upflow Anaerobic Sludge Blanket (UASB) (Smith et al., 2012). Anaerobic processes have not been widely studied and applied to treat petrochemical residues or for treatment of industrial waste-products with high concentration of organics, largely due to treatment quality or process sensitivity.

This work analyses the composition and the diversity of the bacterial community of digestate sludge from the Bran Sands Advanced Digestion Facility. The anaerobic digester operates using a range of biowaste, such as sewage, sludge cake (Class A biosolid) as well as petrochemical waste. The recent development of 'next-generation sequencing' such as 454 pyrosequencing has made it possible to deep-sequence microbial communities in complex biological samples without the time-consuming cloning procedure. The technique has so far been used for the sequencing of metagenomes from a number of biogas reactors (Schluter et al., 2008). Schluter et al. (2008) described the bacterial community from a full-scale, completely stirred tank reactor (CSTR) digesting maize silage (63%) and green rye (35%) together with small amounts of chicken manure. Bacterial members of the taxonomic classes *Clostridia* and *Bacteroidetes* were most abundant. Among the *Archaea*, the hydrogenotrophic *Methanoculleus* sp. dominated, but the acetoclastic *Methanosarcina* sp. were also detected. Lee et al. (2012) used 454 pyrosequencing of

the V1, V2, and V3 regions of the 16S rRNA gene to assess the microbial community in seven full-scale reactors over time. Six of the reactors treated waste-activated sludge (one of these in combination with smaller amounts of food waste), and one reactor treated night soil. Sequences belonging to *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Chloroflexi* were found to be the most abundant, and the bacterial population was influenced by the digestion temperature. Werner et al. (2011) characterized bacterial communities in nine full-scale granulated sludge reactors treating brewery waste water by targeting a part of the rRNA gene. These bacterial communities were dominated by *Syntrophobacterales*, *Desulfuromonales*, *Bacteroidetes*, *Spirochetes*, *Clostridia*, *Chloroflexi*, and *Synergistia*. The present study carried out a characterisation of the bacterial communities in the effluent of a full-scale digester to identify bacteria related to hydrolysis and acetogenesis processes. High-throughput 16S rRNA gene sequencing of the V1-V5 hypervariable region was carried out using 454 technologies to investigate the bacterial communities, and the set of sequences obtained were further analysed using bioinformatics tools. These bacteria could potentially be used for application in unconventional gas systems to achieve a continuous generation of biogenic CH_4 from existing producing wells or depleted wells. In unconventional gas systems, such inoculum could help to achieve a continuous generation of biogenic CH_4, since the bacterial and archaeal consortium are already adapted to high temperatures and high concentration of organic compounds.

2. Material and Methods

2.1. Samples.

The samples used for this study were collected fresh from the effluent of the Bran Sands Advanced Digestion Facility, which employs thermal hydrolysis processes for the conversion of liquid, solid sewage waste and petrochemical waste into biogas. The samples were collected in sterile plastic PTFE containers and stored at -80°C until analysed.

2.2. Plant description

The Bran Sands Advanced Digestion Facility was commissioned in 2009 and designed to treat up to 40,000 tds/y of sewage sludge comprising:

- 14,500 tds/y of indigenous sludge from the adjacent effluent treatment works;
- 1,500 tds/y of liquid imports;
- 24,000 tds/y of cake imports from satellite works.

Liquid imports and indigenous sludges are pumped into a storage facility before being screened and transferred to a pre-dewatering plant. Cake transfer pumps move the dewatered cake to reception silos before being pumped to the thermal hydrolysis buffer tanks and two parallel thermal hydrolysis streams (provided by Cambi, www.cambi.com, CAMBI GROUP AS Skysstasjon 11A, 1383 Asker). Each stream consists of a pulper, four reactors and a flash tank. The header is supplied with steam from a combination of waste heat recovery modules, which recover high grade energy from the gas engine exhaust streams and triple fuelled boiler plant. The output from the second thermal hydrolysis process streams is combined before being split into three separate digesters. The three digesters provide 18 days retention time, based on 90% Effective Digester Volume (EDV) at design throughput. The mesophilic temperature within each digester is controlled by air blast sludge coolers installed on each feed line. Digested sludge is dewatered using centrifuges and transferred to cake storage. This cake store has been provided with separate bays to enable loading and unloading of cake by front loaders whilst also allowing cake to be stored for longer periods before transfer off-site (to increase de-waterability). Odourous air which is generated within the facility is ducted to the existing odour control plants, which make use of biofilter technology. Liquors generated by the pre and post-digestion plant operation are transferred to the main effluent treatment works for processing. Northumbrian Water's existing advanced digestion plant at Bran Sands on Teesside is shown in Fig. 1.

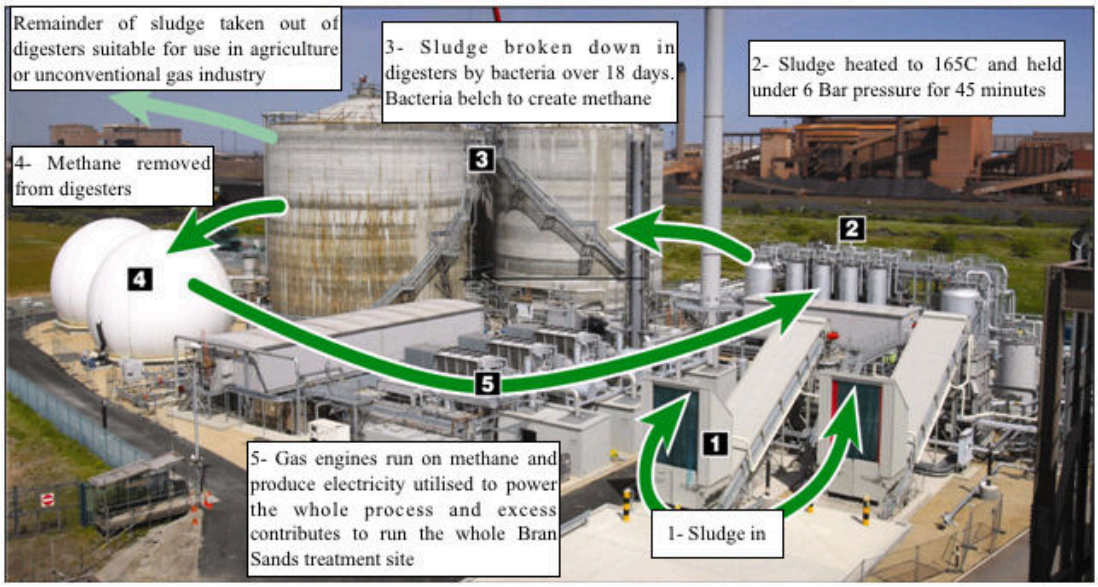

Figure 1. Schematic diagram of advanced digestion plant. Modified from North East Bioresources & Renewables (NEBR) (http://www.nebr.co.uk/).

2.3. 16S rRNA gene extraction

Prior to DNA extraction, all samples were shaken by hand before transferring 0.25 g to the bead-beating tube (PowerSoil DNA Kit PowerBead Tubes, MOBIO Laboratories, Carlsbad, CA, USA). The extraction was performed with few modifications of the manufacturer's protocol, and the DNA obtained was stored at -20°C until further analysis. Concentrations of double stranded DNA in the extracts were determined using the NanoDrop ND-1000 (Thermo Scientific).

2.4. 16S rRNA gene amplification and pyrosequencing

PCR of the V1-V5 hypervariable region of the bacterial 16S rRNA gene was performed using amplicon fusion universal bacterial primers 27F (Lane, 1991) and 907R, (Lane, 1995) synthesised by IDTdna (Integrated DNA Technologies, BVBA, Leuven, Belgium). The forward primer (5′ CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGAGAGTTTGAT CMTGGCTCAG 3 ′) consisted of a fusion containing the 454 Life Sciences 'Lib-L Primer A', a four-base 'key' sequence (TCAG), a unique ten-base barcode 'MID' (MID 5, Multiplex Identifier Adaptors, Roche. ATCAGACACG) sequence, and bacterial primer 27F.

The reverse fusion primer (5′ CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCGTCAATTCMTTTRAGTTT 3′) contained the 454 Life Sciences 'Lib-L Primer B', a 4 base 'key' sequence (TCAG), and bacterial primer 907R. The PCR amplification was performed in 75 µL volume reactions using 47.1 µL Roche PCR Grade Water, 7.5 µL Reaction Buffer (without $MgCl_2$), 9 µL 25 mM $MgCl_2$, 1.5 µL Nucleotide Mix, 3.75 µL DMSO, 1.2 µL 25M 907R Primer, 0.75 µL High Fidelity Enzyme Blend, 3 µL of sample DNA and 1.2 µL 25M The MID5 (Roche) was used to label the Forward Primer 27F. A negative and a positive control were also prepared for the PCR reaction, containing respectively 2 µl sterile H_2O and DNA extracted from *Geobacter sulfurreducens* (from the collection of The University of Manchester, School of Earth, Atmospheric and Environmental Sciences). The PCR conditions included an initial denaturing step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a final elongation step at 72°C for 5 min. At the end of the run, the whole PCR product was mixed with 12.5 µl of 5x gel-loading dye, and 35 µl of the mixture was loaded on a 2% Tris-Acetate-EDTA/agarose gel. A 2000-100 bp ladder was also loaded on the gel that was run at 80 mV for ~ 2 h. At the end of the run, the DNA bands were observed on a Gel Doc 2000 Gel Imaging System (Bio-Rad Laboratories). Following gel electrophoresis, bands of the correct fragment size (~ 410 bp) were excised, purified using a QIAquick Gel Extraction Kit (Quiagen, Limburg, Netherlands) according to the manufacturer's protocol, and eluted in 30 µL of DNAse free H_2O. The purified PCR products were quantified using the NanoDrop ND-1000 (Thermo Scientific). The DNA products were then stored at 4°C until it was sequenced. The emulsion PCR was performed at The University of Manchester, School of Earth, Atmospheric and Environmental Sciences, the pyrosequencing run was performed at the sequencing facilities of Faculty of Life Science of The University of Manchester using a Roche 454 Life Sciences GS Junior.

2.5. Pyrosequencing data analysis

The 454 pyrosequencing reads were analysed using the Quantitative Insights Into Microbial Ecology pipeline (Qiime 1.8.0 release) (Caporaso et al., 2010b). Raw sequences were first assigned to the different samples by using the barcode

sequences provided: sequences outside the 300–500 bp range were removed along with the reverse primer sequence, using the *split_libraries.py* script. The usearch 6.1 programme (Edgar, 2010) was used to perform filtering of noisy sequences and chimera checking. Operational taxonomic units (OTUs) were picked from and compared at 97% similarity with the May 2013 release (13_5) of greengenes OTU reference using the usearch 6.1 programme through the *pick_otus.py* script. The most abundant OTU sequence was chosen as a representative using the *pick_rep_set.py* script. Taxonomy assignment was based on the greengenes reference database (McDonald et al., 2012) using the Ribosomal Database Project Naive Bayes (RDP) classifier v 2.2 (Wang et al., 2007), with the confidence level set at 80% through the *assign_taxonomy.py* script. An OTU table was built using the *make_otu.py* script and a biological observation matrix file (BIOM) was built using *convert_biom.py* script. A OTU heatmap file was generated with the *make_otu_heatmap.py* script. The sequences were then aligned to the greengenes core reference alignment (De Santis et al., 2006) using PyNAST (Caporaso et al., 2010a) through the *align_seqs.py* script. Aligned sequences were then filtered using the *filter_alignement.py* script, and a phylogenetic tree was built through the *make_phylogeny.py* script (Price et al., 2009). Alpha diversity, alpha rarefraction and beta diversity were calculated using respectively the *alpha_diversity.py*, *alpha_rarefraction.py* and *beta_diversity_through_plots.py* scripts. Jackknifing was performed with the *jackkinfed_betadiversity.py* script to directly measure the robustness of individual Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clusters and build jackknifed 2D and 3D PCoA plots. Sequences (average length of 400 bp) were analysed against the NCBI (USA) database using BLASTn program packages and matched to known 16S rRNA gene sequences to retrieve the closest relatives.

3. Results and Discussion

3.1. Phylogenetic analysis

The total number of sequences obtained was 3090, with an average length of ~ 400 bp. In total, 8 phyla, 9 classes, and 15 genera were found among Bacteria. Estimates of phylotype richness were calculated according to the Chao1 non-parametric

estimator that takes into account the number of different OTUs in a sample. The Chao1 index of the sample, at 3% distance and with 96% confidence intervals together with the α-diversity (Fig. 2), indicates a trend of higher microbial richness when correlated with the number of OTUs (3090).

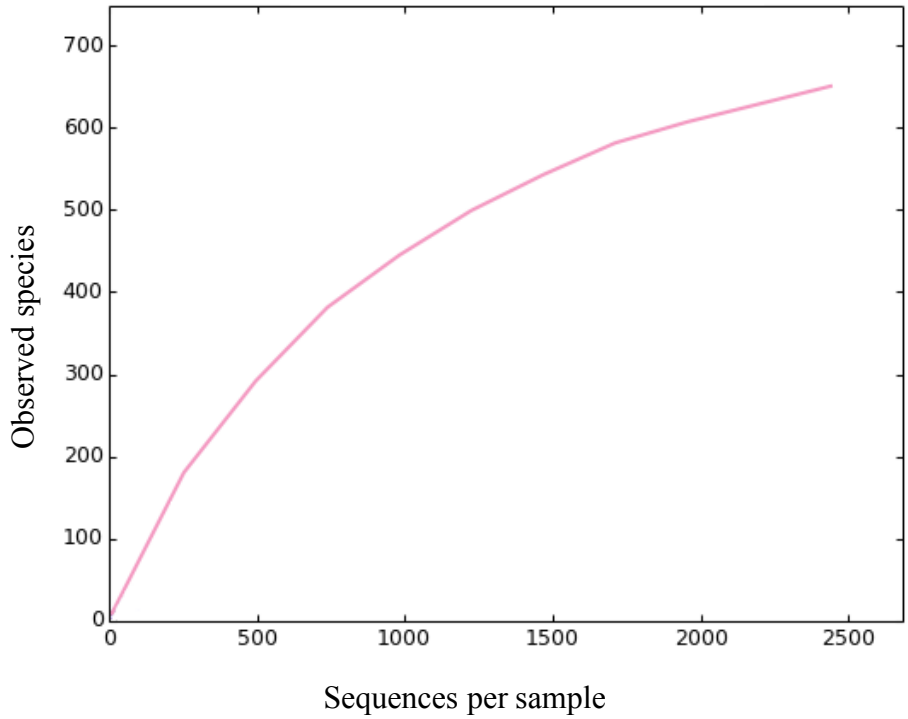

Figure 2. α-Diversity plot, showing the number of leaves in taxonomy.

The phylogenetic analysis showed a dominance of *Thermotogae* at the class level (Fig. 3), that accounted for the 60.71% of the sequence reads analysed. Microorganisms of this class have the genetic potential to oxidize butyrate to CO_2/H_2 and acetate. The second most abundant class is the *Bacteroidia* (15.28%), followed by *Clostridia* (10.42%) and *Synergistia* (7.18%). These observations suggest that the degrading consortium in the bioreactor consists of additional syntrophic interactions beyond the standard H_2-producing syntroph–methanogen partnership that may serve to improve community stability. Members of the *Thermotogae* in the bioreactor community may participate in the syntrophic interactions by producing acetate from an intermediate molecule. This intermediate molecule may be the butyrate produced by the *Clostridia* population. Lastly, a small presence of *Cloacamonae* belonging to

the phylum WWE1 was also detected (3.33%), microorganisms in the bacterial phylum WWE1 have been implicated in cellulose degradation in anaerobic sludge digesters (Limam et al., 2014) and were also found in the coal-bearing strata of the Cherokee Basin, USA (Kirk et al., 2015). The presence of this of these bacterial classes suggest a putative role in the degradation of complex macromolecules.

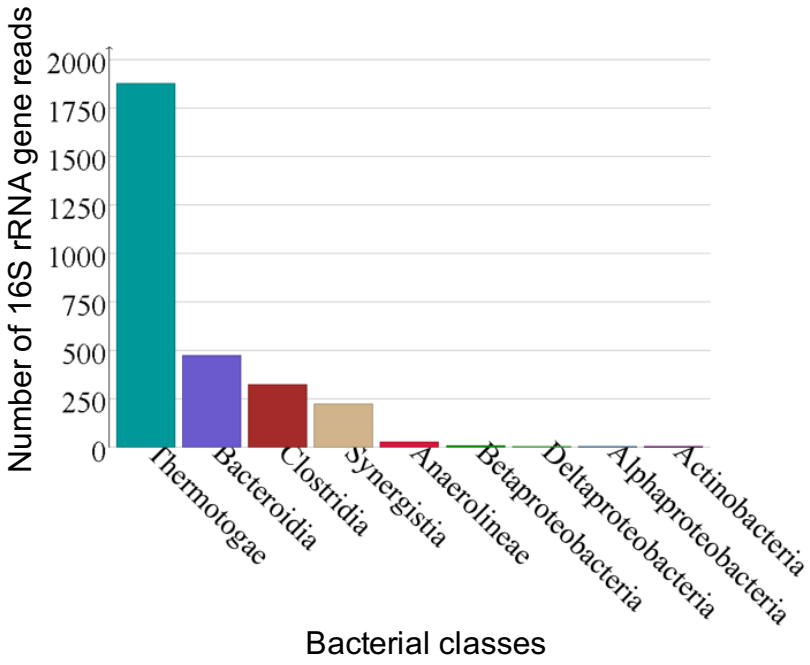

Figure 3. Bar chart showing the bacterial class in each sample that were tested.

At the genus level, the digestate sample was characterized by a significant dominance of unclassified members of the *Thermotogaceae* family (60.71%). Members of *Thermotogaceae* are moderately thermophilic to hyperthermophilic, fermentative bacteria, typically isolated from hydrothermal systems (Dahle et al., 2011). The cells of *Themotogaceae* members are wrapped in an outer membrane (toga), which confer resistance to high temperature. They typically metabolize carbohydrates and have varying amounts of salt and oxygen tolerance. (Huber et al., 1986) Members of these family were isolated from a number of subsurface environments: *Thermotoga subterranea* strain SL1 was found in a 70°C deep continental oil reservoir in the East Paris Basin, France (Li et al., 2007), *Petrotoga halophile* was isolated from an oil well in Congo and can grow in the presence of 5-

9% of NaCl (Miranda-Tello et al., 2009). The genus *Thermotoga* is anaerobic and reduces cysteine and thiosulphate to hydrogen sulphide, while the genus *Petrotoga* has moderate halophilic species. Other genera of the *Thermotogaceae* include *Kosmotoga* (Dipippo et al., 2009), *Marinitoga* (Wery et al., 2001) and *Thermosipho* (Huber et al., 1989). The second most abundant genera revealed by the taxonomy analyses were unclassified members of the Porphyromonadaceae family, which is composed of two genera of environmental bacteria, *Porphyromonas* and *Dysgonomonas*. Within the classified genera (Fig. 4), the taxonomy classification analyses performed by 454 pyrosequencing identified as the most abundant genera, bacteria such as *Paludibacter*, Clostridium, Thermoacetogenium and Syntrophomonas (Respectively 12.62%, 6.08%, 3.33% and 3.01% abundance) (Fig. 4).

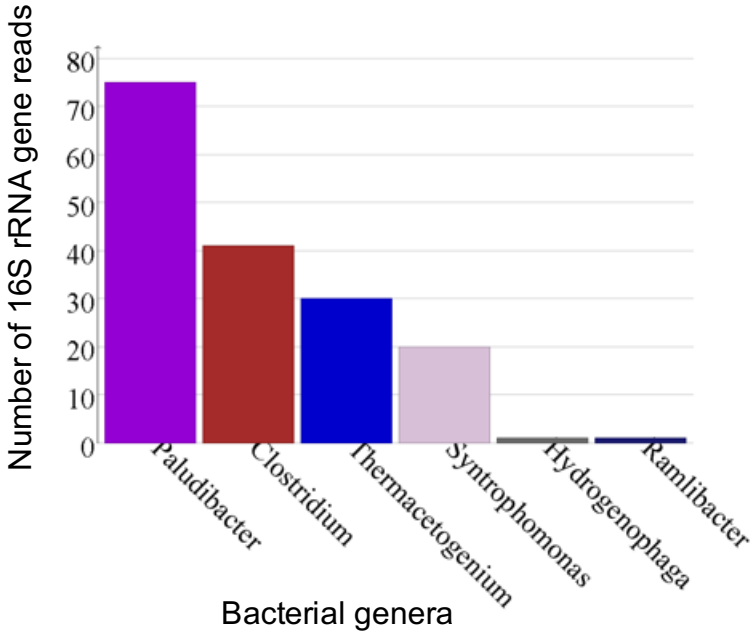

Figure 4. Bar charts showing the bacterial genera in each sample that were tested.

Further analyses were carried out using the BLAST programme package that matched the pyrosequencing data to known 16S rRNA gene sequences to find the closest known relatives to the OTUs identified in the sample (Table 1). This analysis showed that the dominant OTU was an uncultured *Thermotogae bacterium*, with 58.11% abundance and 97% identity. Other important bacteria retrieved using the

BLAST search platform are two uncultured bacteria belonging to *Bacteroidia* (6.96% and 3.84% abundance respectively), both at 99% identity and *Aminobacterium colombiense* (3.57 % abundance and 99% identity), belonging to the *Synergistia* phylogenetic class. Also significant is the presence of the Uncultured WWE1 bacterium clone (3.53% abundance, 99% identity) belonging to *Cloacamonae, isolated from a mesophilic anaerobic digester* and of the *Defluviitoga tunisiensis* strain SulfLac1 (3.46% abundance, 99% identity), isolated from a thermophilic anaerobic digester.

Table 1. OTU's Sequence Reads Analysed Filtered by Counts.

OTU	Abundance (%)	Closest Match	Accession	Match (Identities)	Phylogenetic Class	Isolation source
1	58.11	*Uncultured Thermotogae bacterium clone QEEA3DA10*	CU918794	97%	*Thermotogae*	Mesophilic anaerobic digester
11	6.96	*Uncultured bacterium clone B045*	HG530299	99%	*Bacteroidia*	Mesophilic agricultural biogas reactor
1080	3.84	*Uncultured bacterium clone: 3MP-B-1HY-65*	AB731261	99%	*Bacteroidia*	Methane Production Process. Food waste bioreactor
7	3.57	*Aminobacterium colombiense strain DSM 12261*	CP001997	99%	*Synergistia*	Anaerobic lagoon of dairy wastewater treatment plant in Colombia
10	3.53	*Uncultured WWE1 bacterium from clone QEDN11CH09*	CU925933	99%	*Cloacamonae*	Mesophilic anaerobic digester
1000	3.46	*Defluviitoga tunisiensis strain SulfLac1*	NR_122085	99%	*Thermotogae*	Thermophilic and anaerobic whey digester
16	2.50	*Uncultured Firmicutes bacterium from clone QEDV1BH08*	CU919563	99%	*Clorstridia*	Mesophilic anaerobic digester
14	1.99	*Uncultured Bacteroidetes bacterium from clone QEDP2BA06*	CU924061	99%	*Bacteroidia*	Mesophilic anaerobic digester
20	1.85	*Uncultured bacterium clone:BSA2B-03*	AB175375	97%	*Synergistia*	Mesophilic anaerobic digester
25	1.47	*Tepidanaerobacter syntrophicus strain JL*	NR_040966	99%	*Clostridia*	Anaerobic sludge
403	1.30	*Uncultured bacterium clone ATB_CM_534_02*	KP151393	99%	*Thermotogae*	Thermophilic chicken dung-cow slurry fermentation
67	1.13	Bacterium enrichment culture clone 4-44	KF460362	99%	*Clostridia*	Paddy soil
1135	1.03	*Uncultured bacterium clone S1_3_440*	JQ106154	99%	*Thermotogae*	Anaerobic sludge digester
36	0.99	*Tepidanaerobacter acetatoxydans Re1*	NC_019954	99%	*Clostridia*	Unkn.
40	0.93	*Uncultured Chloroflexi bacterium clone QEEA1DG07*	CU918614	99%	*Anaeroloneae*	Mesophilic anaerobic digester

4. Conclusions

In this work, the biodegrading bacterial community of the Bran Sands Teesside anaerobic digester was investigated using 454 pyrosequencing technology followed by sequence data interpretation using bioinformatics tools. The main goal of this study was to investigate the bacterial community composition in an anaerobic digester that include petrochemical waste as a substrate for biogas production. The bacteria population found in the digestate samples is comparable in term of composition with the bacteria found in unconventional gas systems. DNA-based assessment of the microbial community structure in unconventional gas basins have shown that bacterial diversity is higher than archaeal diversity (Barnhart et al., 2013; Penner et al., 2010; Green et al., 2008). Bacteria related to Proteobacteria (mostly Beta, Delta and Gamma-proteobacteria), Actinobacteria, Bacteroidetes and Firmicutes seem to be widespread in unconventional systems such as coal-bed methane (Green et al., 2008; Jones et al., 2008; Jones et al., 2010; Li et al., 2008; Strąpoć and Picardal, 2008; Warwick et al., 2008) and shale gas (Meslé et al., 2015; M Meslé et al., 2013; Struchtemeyer and Elshahed, 2012). These taxonomic groups are known for their versatile metabolic activity and hydrocarbon degrading capabilities. The classification of nucleotide sequence reads and assembled contigs carried out in this study revealed a genetic profile characteristic for an anaerobic microbial consortium running fermentative metabolic pathways. Moreover, the assignment of numerous sequences was related to hydrocarbon decomposition and digestion of cellulosic material, which indicates that many species in the samples are engaged in hydrolysis of plant-derived material. Since hydrolysis is the rate limiting step in degradation of plant biomass (Noike et al., 1985) and within the biogeochemistry of coal-bed methane (Strąpoć et al., 2011) it would be worthwhile to learn more about microorganisms and their metabolic features involved in this process. Some genetic traces of organisms dominating the hydrolysis step were already identified in the metagenome data set. Future work will concentrate on isolation of corresponding bacteria and analysis of their genomic properties with the objective to optimise initial steps in the decomposition of substrates for biogas production. Putative key organisms involved in intermediate steps of methanogenesis

were also identified. As a general conclusion, the bacterial community is comparable with results obtained from other mesophilic and slightly thermophilic digesters. Leven et al., (2007) studied the effect of process temperature on the anaerobic digestion of organic household waste and reported a dominance of *Thermotogae* and *Clostridia* in their slightly thermophilic reactors, while *Bacteroidetes* and *Chloroflexi* were the main phyla in the mesophilic reactors. Their results are in agreement with our findings for the mesophilic reactor in Bran Sands, but their mesophilic community seems less diverse than in our digestate samples. In a study investigating the microbial community of seven anaerobic sludge digesters, Riviere et al., (2009) identified organisms from the *Betaproteobacteria* (class level), *Anaerolineales* (order level), *Bacteroidetes* (phylum level), and *Synergistetes* (phylum level) as 'core' organisms. These results are also in accordance, with the results obtained for the bioreactor investigated in the present study. However, while sequences belonging to *Anaerolineae* (*Chloroflexi*) and sequences from classes of *Proteobacteria* (Alpha, Beta, Delta, and Gamma) are characteristic of the reactor, sequences of the class *Bacteroidetes* were less present in our samples. Moreover, our results are comparable, to some extent, with the bacterial profile characterized in unconventional gas systems. More than 10 different bacterial phyla, among which Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria dominate, have been identified in coal and oil.

The bacterial diversity in low temperature unconventional reservoirs is essentially dominated by Proteobacteria from the alpha, beta, gamma and delta classes. Alpha-proteobacteria dominate in the mesophilic Enermark oil field in Canada where they represent around 50% of the bacterial diversity (Kryachko et al., 2012). Alpha - proteobacteria represent circa 29% in sandstone and coal samples of the Eastern Ordos Basin in China. Gamma-proteobacteria dominate in the Bokor oil field in Malaysia (Guo et al., 2012), Beta- and Gamma-proteobacteria are also present in the Alberta coal beds in Canada (Penner et al., 2010). In these settings, Proteobacteria represent over 70% of the total diversity. Members of the Alpha-proteobacteria are known degraders of water-insoluble compounds such as PAHs, and Gamma-proteobacteria include primary hydrocarbon degraders (Guo et al., 2012; Kryachko et al., 2012; Penner et al., 2010). A large number of Delta-proteobacteria are syntrophic

bacteria, acetogens or secondary fermenters known to be associated with methanogenic Archaea, or sulphate-reducing bacteria, which can degrade hydrocarbons and organic acids with sulphate as the terminal electron acceptor (Zengler et al., 1999). Besides proteobacteria, Firmicutes are frequently observed, but sometimes in numbers close or below the detection levels of the studies, e.g. they can only be detected after cultivation. Firmicutes, which include fermenters and syntrophs able to hydrolyse water-soluble macromolecular compounds, homoacetogens, fatty acids- oxidizers, and acetogens are associated with oil formation waters (Yamane et al., 2011) and coal production waters (Guo et al., 2012; Wawrik et al., 2012). Conversely, Actinobacteria, which include cellulolytic organisms degrading poorly water-soluble organic compounds (Deng and Fong, 2011), are associated with bulk oil, coal, and sandstones. It is unclear why these organisms, many of which require oxygen to degrade cellulose, are present. Further elucidation is also required to establish whether these microorganisms are able to degrade the cellulose-like organic compounds in oil and coal. Nevertheless, there is a strong metabolic convergence towards the microorganisms typically found in anaerobic digesters and in unconventional systems.

Acknowledgements

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5.3. Final considerations

The experiments described in this Chapter showed that molecular tools could be adapted to analyse the bacterial population in extreme environment such as high temperature bioreactors fed with petrochemical waste. Moreover, taxonomic analyses of the OTU DNA sequence information could also be used to inform media conditions for the isolation and culture of these high-performing organisms. Previous research has shown that the addition of exogenous high- performing organisms in bioreactors, selected based on input composition, can boost methane production by 50% (Akila and Chandra, 2010). The availability of a custom inoculum would provide a system recovery tool that could decrease lag time and boost performance of an anaerobic digester. However, the use of such inoculum in unconventional gas systems is expected to behave differently than the most studied biodegradation in man-made anaerobic digester.

Additional direct comparisons of our findings with previous studies on anaerobic digester microbiota are difficult because of the differences in the broad-range PCR primers used. Depending on the primer design, each study may have different biases for or against certain phylogenetic groups. Furthermore, the differences in the 16S rDNA regions amplified prohibit sequence alignment to find corresponding OTUs across different studies. The comparison of these results with other bioaugmentation studies in unconventional gas systems is complicated by the policies of oil and gas companies, since their processes are often covered by confidentiality agreements.

Despite no further experiments were carried out using the bacterial population isolated from the Bran Sands Digester, the results of the high-throughput 16S rRNA gene sequencing show an active biodegradative bacterial community. The similarities between the bacterial community structure of the digestate from Bran Sands and the bacterial populations found in coal produced water (Green et al., 2008; Jones et al., 2008; Jones et al., 2010; Li et al., 2008; Strąpoć and Picardal, 2008; Warwick et al., 2008) and shale gas (Meslé et al., 2015; Meslé et al., 2013; Struchtemeyer and Elshahed, 2012) suggest that such inoculum could stimulate

methane production in depleted wells. The application of these bacteria in unconventional gas systems has also implications for the disposal of the digestate, which is typically used as a fertiliser. However, the digestate from the Bran Sand bioreactor, is not suitable for agricultural applications, since the digester is fed with petrochemical waste. Hence, the use of the effluent from Bran Sands as bioaugmentation inoculum to improve methane production in depleted well, provide a disposal route for the digestate with minimal risk to humans and the environment.

The results obtained in this Chapter discuss state-of-the-art molecular and bioinformatics analyses of high-throughput 16S rRNA gene sequencing data from 454 pyrosequencing. Moreover, this study promotes the applications of such data in exploring microbial diversity in extreme environments. The difficulties encountered in obtaining fresh samples such as shale/coal cores, formation water and flowback water (discussed in Chapter 5, Section 5.1), spurred the investigation of another extreme environment such as highly-contaminated soils and groundwater. Thanks to the industrial partner of this Ph.D. project (WSP Parsons Brinckerhoff) and National Grid Property, a number of coal tar samples were obtained to further the research on microbial communities in extreme environment. The molecular and bioinformatic tools developed in this Chapter were used to investigate microbial processes in coal tar contaminated soils and groundwaters. Moreover, the next Chapter, presents new analytical methodologies for the analysis of the microbial community structure in concentrated organic-phase coal tar DNAPLs.

Chapter 6

Paper III: Bacterial Community Structure and Multidimensional Isotope Signature of PAHs in Coal Tar

6.1. Introduction

The second extreme environment analysed during this Ph.D. project consisted of concentrated organic-phase coal tar DNAPLs. Coal tar samples were kindly provided by WSP Parsons Brinckerhoff and National Grid Property from different location across the UK and USA. From a bioremediation perspective, coal tar contamination poses several practical challenges. The main difficulties are quantification of single compounds and to establish an accurate mass balance of contaminants, electron donors and final products in heterogeneous groundwater systems. In light of the above considerations, coal tar contaminated sites represent an extreme environment for microorganisms. The presence and activity of biodegradative bacteria is limited by the bioavailability of water within the concentrated organic-phase, high concentration of toxic compounds, pH and availability of electron donors and acceptors.

The coal tar samples were not chosen as an analogue of shale and CBM subsurface environments, they were rather analysed to validate the molecular methodologies developed and to check if multidimensional CSIA can be coupled with high-throughput 16S rRNA gene sequencing to trace microbial activity in extreme settings. However, the chemical analyses carried out during this Ph.D. project highlighted a similar chemical composition, especially when analysing the aromatic

fraction of shale and coal tar. Despite these similarities, the organic composition of coal tar is more complex that the shale core analysed, with thousands of different compounds detected. The constrains to microbial life and activity in the shale and coal tar are also different, but the mechanisms by which microorganisms activate organic compounds could be remarkably similar.

Microorganisms require a source of carbon to proliferate in soil environments, as well as nitrogen, phosphorus, sulphur and a variety of trace nutrients. Given the competition for carbon in soil environment, certain natural microbes have developed the ability to utilise different molecules. Microorganisms have developed a wide variety of respiration systems. In all cases of aerobic respiration, the electron acceptor is molecular oxygen. Anaerobic respiration uses an oxidised inorganic or organic compound other than oxygen as an electron acceptor. Biodegradation of PAHs depends on the complexity of the chemical structure and the extent of enzymatic adaption. In general, PAHs which contain two or three rings such as naphthalene, anthracene and phenanthrene are degraded at reasonable rates both under aerobic and anaerobic conditions. Compounds with four rings such as chrysene and pyrene and pentacyclic compounds, in contrast, are highly persistent and are considered recalcitrant.

The biodegradation pathways of PAHs under anaerobic conditions are not well understood, although a number of studies for bioremediation have investigated the mechanisms by which anaerobic microorganisms activate and degrade aromatic hydrocarbons. The anaerobic degradation of PAHs in shale however, is expected to follow different pathways than the most studied biodegradation for bioremediation purposes. The vast body of scientific knowledge on contaminated land bioremediation can help to shed light on the complex degradation pathways of organic matter in unconventional gas systems

This Chapter aim to investigate the possibilities of coupling metabolomic multidimensional CSIA with high-throughput 16S rRNA gene sequencing to detect biological changes in PAH composition and isotope signature. This could be

particularly relevant for bioremediation studies, but also to detect any biological shift in the organic matter composition and isotope signature of organic matter in the deep subsurface.

6.2. Bacterial Community Structure and Multidimensional Isotope Signature of PAHs in Coal Tar

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Keywords. Pyrosequencing; DNAPL; CSIA; Natural Attenuation; Coal Tar.

Table of Contents Graphic and Synopsis

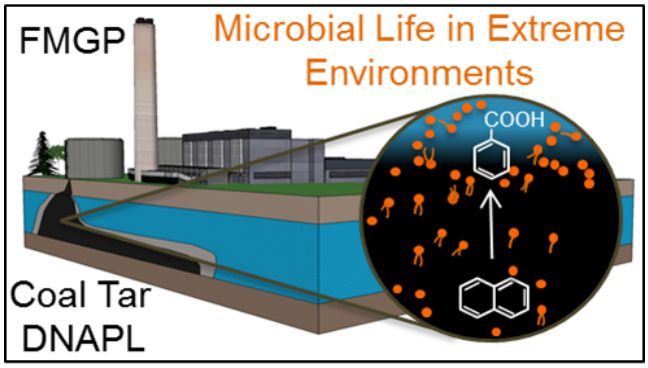

Fig. a. Schematic representation of a coal tar contaminated aquifer at FMGP, showing for the first time the presence of microorganisms in concentrated DNAPL.

KEYWORDS. Pyrosequencing, DNAPL, CSIA, Natural Attenuation, Coal Tar.

Index

Abstract

Coal tars are dense non-aqueous phase liquid (DNAPL), they are primarily composed of polycyclic aromatic hydrocarbons (PAHs). Due to high hydrophobicity, the compounds present within the DNAPLs are potentially unavailable to microbial degradation, although the presence of bacteria has been described in DNAPL-contaminated soils, sediments and aquifers. Whereas several reviews focused on bacterial communities of these environments, this study developed and applied, for the first time, molecular characterization tools for the study of concentrated DNAPLs. Particular emphasis was given on the physico-chemical factors that influence the analyses of this complex substrate and identification of indigenous biodegradative bacteria. The bacterial community structure and carbon/hydrogen isotope signature of PAHs in coal tar are readily distinguishable and can be used for source apportionment, being strictly related to the gasification processes. The bacterial activity in the DNAPLs do not introduce a shift in the carbon isotope signature, which reflects the source and gasification processes employed. In contrast, the hydrogen isotope signature is significantly different, indicating a contribution of different processes. The high hydrogen fractionation detected was also able to discriminate between parent and alkylated PAHs. Thus, high-throughput 16S rRNA gene sequencing and multidimensional compound specific isotope analysis (CSIA) suggest that, although multiple reactions may be involved, microbial activity contributes to the isotope shift.

1 Introduction

Prior to the discovery of large subsurface reservoirs of natural gas, former manufactured gas plants (FMGPs) supplied gaseous fuel derived from coal, coke and oil (Birak and Miller, 2009). A major by-product of manufactured gas and coke production processes was coal tar DNAPL, primarily composed of PAHs (Groshal et al., 1996). PAHs are also product of natural and anthropogenic processes involving the incomplete combustion of organic material (Keith and Telliard, 1979). Due to their ubiquitous distribution in the environment, and also their carcinogenic and toxic properties, PAHs pose serious environmental risks (Menzie et al., 1992). Coal tar

represents a long-term subsurface source of contamination (Luthy et al., 1994) that poses many practical challenges for its effective remediation. Due to the lack of efficient, cost-effective active remediation technologies, passive risk management strategies such as monitored natural attenuation (MNA) have emerged as promising strategies (Bahr et al., 2014). The intrinsic biodegradation potential of contaminants by indigenous microbial populations is a key component of MNA (Anneser et al., 2008; Bouchard et al., 2008). Typical approaches to establish the biodegradation potential include: (*i*) the molecular characterization of soil or groundwater samples, to verify the presence of indigenous biodegradative microorganisms; (*ii*) metabolite tracing and quantification of the concentration of key contaminants during biodegradation; and (*iii*) CSIA for the direct assessment of biodegradation rates. Quantification of contaminants in coal tar-contaminated sites is complicated by various geochemical, hydrogeological and biological factors. The main difficulties are also to establish an accurate mass balance of contaminants, electron donors and final products in heterogeneous groundwater systems (Steinbach et al., 2004). Despite the large number of sites around the world affected by hydrocarbon pollution and an increasing attention to bioremediation technologies, no universal technique exists to measure biodegradation rates (Morasch et al., 2001). In this study, a combination of high-throughput pyrosequencing-based assessment of the microbial community structure and metabolomics multidimensional carbon/hydrogen CSIA were used as two powerful and combined methods to evaluate the intrinsic biodegradation potential and for source apportionment of PAHs in coal tar.

1.1 Biodegradation at hydrocarbon-contaminated sites

Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which hydrocarbons can be removed from the environment (Ulrici, 2001). Bioremediation may be more cost effective than other remediation technologies (Leahy and Colwell, 1990) usually constrained by mass transfer limitations (Harms and Bosma, 1997), but for PAHs with low aqueous solubility, this results in low bioavailability and biodegradation rates (Bosma et al., 1997). Coal tar, when released into the subsurface, migrates downward as a result of gravity until it encounters a low permeability layer: contact with groundwater results in the

dissolution of some tar constituents following Raoult's law and generation of contaminated groundwater plumes (Hauswirth et al., 2012). Inside contamination plumes, the biodegradation of PAHs is limited or absent due to depletion of dissolved electron acceptors and toxicity of contaminants (Takahata et al., 2006; Thornton et al., 2001). Recently, the *plume fringe concept* is gaining consensus in the scientific community: the plume fringe is a hotspot for microbial activity, thanks to overlapping countergradients of electron donors and acceptors (Winderl et al., 2008). At the plume fringe, some hydrocarbons dissolve from the DNAPL to the ambient water and mix with electron acceptors (Tuxen et al., 2006; Watson et al., 2005) reducing toxic concentrations of contaminants in the plume fringe (Bauer et al., 2008). Moreover, the plume fringe is characterized by high microbial activities and biomass (Berkowitz et al., 2004; Bouchard et al., 2008). The microbial degradation of PAHs, is mediated by a range of microorganisms, the most studied are bacteria (Yanik et al., 2003). Common bacterial genera typically encountered during biodegradation of PAHs are *Alicaligens*, *Pseudomonas*, *Geobacter*, *Sphingomonas*, *Bacillus*, *Burkholderia*, *Cyanobacter*, *Vibrio* and *Mycobacterium* (Johnsen et al., 2005; Mueller et al., 1997). Low molecular weight PAHs are usually biodegraded first, although 3 and 4 rings aromatic compounds can be biodegraded too (Heitkamp et al., 1988; Weissenfels et al., 1990). To date, hydrocarbon-degrading bacteria have been isolated and described from a number of polluted environments such as soils, sediments, aquifers, fresh and marine waters. Not surprisingly, the presence of PAH-degrading microorganisms has been investigated at several FMGP sites (Ehrlich et al., 1982; Madsen, 1991; Wilson et al., 1985).

1.2 Compound specific isotope analysis

CSIA is a powerful tool to assess biodegradation rates in laboratory and in-situ studies (Barth et al., 2002; Meckenstock et al., 2004). carbon and hydrogen elements in PAHs consist of various combinations of their stable isotopes, the ratio of heavy to light isotopes provides information that can improve our understanding of biodegradation processes. Microorganisms tend to degrade molecules containing lighter ^{12}C and ^{1}H isotopes, causing an enrichment of the heavier isotopes in the residual, non-degraded substrate, while the product is depleted in the heavy isotopes

(Anneser et al., 2008). This shift in the isotope ratios is known as isotope fractionation. Early studies have suggested that microbial degradation may lead to the $^{13}C/^{12}C$ isotope fractionation of compounds with high molecular masses such as alkanes (Stahl, 1980). Although less studied, isotope fractionation was also observed during biodegradation of aromatic compounds under different redox conditions (Ahad et al., 2000; Dempster et al., 1997; Meckenstock et al., 1999). Only recently, naphthalene was used as a model for low molecular weight PAHs, showing that fractionation was significant for hydrogen but not for carbon (Bergmann et al., 2011). The identification of biodegradation pathways based on isotopic investigation of single elements is not always reliable, since enrichment factors for a specific reaction can differ significantly due to the influence of non-fractionating rate-limiting steps preceding the bond cleavage (Elsner et al., 2005). Since carbon and hydrogen fractionation of a biochemical reaction is similarly affected by rate limitations, two-dimensional plots of carbon versus hydrogen isotopes data have been suggested for characterizing specific degradation pathways of hydrocarbons (Feisthauer et al., 2012; Fischer et al., 2007; Vogt et al., 2008).

2 Experimental

2.1 Samples

A total of 7 coal tar samples, hereafter referred to as S-I to S-VII, were analysed in this study (Table 1). Coal tars were provided by WSP Parsons Brinckerhoff from a range of FMGPs in UK (S-I to S-VI) and USA (S-VII). Tars were obtained as free phase DNAPLs, except S-V (tar mixed with soil used for comparison with concentrated DNAPLs), these were sealed in amber glass containers and stored at 4°C prior to analysis. Additional information is summarised in the SI (Table S1).

Table 1. Summary and description of coal tar DNAPL samples investigated in this study[a].

Samples	MGP process(es)[b]	Sampling location	Viscosity	Additional comments
S-I	VR, potential traces of CWG and HTHR	Downstream from the tar tank	Very low	Dark brown. Fluid at top, but sludge-like at the base of the vial
S-II	VR, potential traces of CWG and HTHR	Downstream from the tar tank	Very low	Dark brown/black. Fluid at top, but sludge-like at the base of the vial
S-III	HTHR	Within tar tank	Very high	Dark brown/black. Easily poured liquid
S-IV	CWG	Sump, containing tar/creosote oil	Very low	Dark red/brown. Easily poured liquid
S-V	LTHR	Unkn.	Low	Black. Tar mixed with soil and fine gravel
S-VI	ICO	Tar tank at 60°C	Very high	Black. Very sticky and dense
S-VII	CWG	Landfill	Low	Dark/brown. Easily poured liquid

[a]FMGP sites have been anonymized for confidentiality reasons. [b]VR, Vertical Retort; HTHR, High Temperature Horizontal Retort; LTHR, Low Temperature Horizontal Retort; CWG, Carburated Water Gas; ICO, Inclined Chamber Oven.

2.2 16S rRNA extraction procedure

Heterogeneity of viscosity and density of the DNAPLs, did not allow the samples to be processed with conventional DNA extraction techniques. Therefore, due to the physico-chemical properties of DNAPLs, an *ad hoc* protocol was developed in order to make DNA extraction feasible. Prior the isolation of environmental DNA, the DNAPLs were mixed with pre-furnaced (at 450°C for 8h) sand (30-70 mesh) (Table S1) to decrease viscosity of the samples and simulate the physico-chemical properties of a polluted soil. Tools and glassware were autoclaved 3 times and/or furnaced at 450°C for 8h. To avoid bacterial contamination, individual glass vials (20

mL) were filled with 5 g of sand each and autoclaved three times. The vials were then furnaced at 450°C for 8 h to eliminate any trace of contaminant DNA. After sterilization, the vials containing sand were transferred directly to a Microbiological Safety Cabinet Class II (CAS Ltd., Manchester, UK) and exposed to UV light until they reached room temperature of 20°C. DNAPLs were then mixed with sand in sterile conditions. DNA was isolated from DNAPL/sand mixture (0.25 g) using the PowerSoil DNA Extraction Kit (MOBIO Laboratories, Carlsbad, CA, USA) with few modifications of the manufacturing protocol. Sterilised sand was also included in the DNA extraction to verify the presence of contaminant DNA. The quality of the DNA extracts was verified by gel electrophoresis.

2.3 16S rRNA gene amplification and pyrosequencing

Pyrosequencing PCR of the V1-V5 hypervariable region of the bacterial 16S rRNA gene was performed using the FastStart High Fidelity PCR System (Roche, Basel, Switzerland) using amplicon fusion universal bacterial primers 27F (Lane, 1991) and 907R (Lane, D. J., Shah, J., Buharin, A., & Weisburg, 1995) synthesized by IDTdna (Leuven, Belgium). Details on the forward and reverse fusion primers are given in the SI . The PCR amplification was performed in 75 µL volume reactions using 69.6 µL of PCR reaction mixture, 3 µL of sample DNA and 1.2 µL of each fusion primer. A negative and a positive control were also prepared for the PCR reaction, containing respectively 2 µL of sterile H_2O and DNA extracted from *Geobacter sulfurreducens*. A blank DNA extract from furnaced sand was prepared for PCR as an additional negative control. The PCR conditions included an initial denaturing step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a final elongation step at 72°C for 5 min. At the end of the run, the whole PCR product was mixed with 12.5 µL of 5x gel-loading dye, and 35 µL of the mixture was loaded on a 2% Tris-Acetate-EDTA/agarose gel that was run at 80 mV for ~2 h. Following gel electrophoresis, bands were excised, purified using a QIAquick Gel Extraction Kit (Quiagen, Limburg, Netherlands), and eluted in 30 µL of DNAse free H_2O. The purified PCR products were quantified using the NanoDrop ND-1000 (Thermo Scientific). The pyrosequencing run was carried out at the sequencing facilities of the Faculty of Life Science of the University of

Manchester using a Roche 454 Life Sciences GS junior. The 454 pyrosequencing reads were analysed using the last release of the QIIME pipeline (Caporaso et al., 2010).

2.4 CSIA and metabolomic analysis: samples preparation and fractionation

To isolate the aromatic fraction of the tar samples, an automated fractionation method was used for the accelerated solvent extraction system ASE 350 (Dionex, Camberley, UK) equipped with 10 mL stainless steel cells. The extraction cells were lined with two glass-fibre filters and packed with 3 g of silica gel 60 (10% deactivated w/w using deionized water) to provide simultaneous sample extraction and clean up. For all extractions, ground mixtures of coal tar, diatomaceous earth and Na_2SO_4 were prepared with 1:1:1 ratio. Approximately 1.5 g of the ground mixture was added to the extraction cell and the remaining cell volume was packed with diatomaceous earth. To allow sample fractionation, two separate ASE methods were employed to sequentially extract the same cell. Hexane (50% cell volume) was used to obtain the first fraction: the oven and static times were switched off to encourage elution of aliphatic compounds. The second fraction was eluted with hexane:toluene (9:1 ratio; 70% cell volume): the oven temperature was maintained at 50°C for 5 min prior to extraction. The extracts were concentrated to approximately 1 mL using a rotatory poly-evaporator and then transferred into autosampler vials. Hexane was added to reach a final volume of 1.5 mL for each sample.

2.5 CSIA and metabolomic analysis

Prior to CSIA, the analytes of interest were identified and quantified using Thermo Scientific (Hertfordshire, UK) Trace Ultra GC fitted with a 30 m ZB-SemiVolatiles capillary column (0.25 mm ID, 0.25 μm Film thickness) and a DSQII mass spectrometer. The helium flow was kept constant at 2 mL/min. The initial oven temperature was set at 65°C and held for 2 min before ramped at 5°C/min to 320°C, with a final hold time of 2 min. Injections of 0.5 μL were carried out using a Triplus (Thermo Scientific) autosampler. The ion source and transfer line were maintained at 200°C and 320°C, respectively. The electron ionization voltage was set at 70 eV. Target analytes in the DNAPL extracts were identified and quantified using a

calibration mixture containing the 16 US EPA priority PAHs. Alkylated PAHs were identified by comparison against the National Institute of Standards and Technology (NIST) Mass Spectral Library (Gaithersburg, MD, USA). GC-MS data processing was performed using the Xcalibur® software (Thermo Scientific, MA, USA). CSIA were carried out using a GC-IRMS system comprising a Trace GC, GC Isolink, ConfloIV interface and a Delta V advantage isotope ratio mass spectrometer (Thermo Fischer Scientific, MA, USA). The gas chromatograph was fitted with the same column used for GC-MS analysis and injections were carried out using the same oven conditions and He flow. The combustion reactor for carbon and the thermal-conversion reactor for hydrogen analyses were maintained at 1050°C and 1440°C, respectively. The same calibration standards used for GC-MS analysis were injected for CSIA; alkylated PAHs were recognized by comparison with GC-MS chromatograms. Further details are reported in the SI. The ratios, expressed in δ notation, are in units per mille (‰), relative to the isotopic composition of the internationally agreed standards VPDB (Vienna Pee Dee Belemnite) and VSMOW (Vienna Standard Mean Ocean Water), respectively for carbon and hydrogen isotope analyses. The isotopic values were calculated relative to standard gases with known isotopic composition: CO_2 ($\delta^{13}C$ = -37.0 [‰] vs. VPDB) and H_2 ($\delta^{2}H$ = -261.8 [‰] vs. VSMOW) (Agroisolab GmbH, Germany). Data processing for CSIA was performed using the Isodat® 3.0 software (Thermo Scientific).

3 Results and discussion

3.1 Bacterial community structure

PCR-based 16S rRNA gene analyses of the microbial communities in these samples using 454 pyrosequencing showed that all coal tars have a significantly diverse bacterial community structure. This is evident from the α-diversity plot (Fig. 1a), where a complex community structure is observed especially for S-V, VI and VII. A lower number of observed species was found for S-I and II, obtained from the same site but different sampling location. A total of 15205 operational taxonomy units (OTUs) were analysed and the average sequence length was around 400 bp. At the phylum level (Fig. 1b), all samples were dominated by Gram-negative

Proteobacteria; S-V, VI and VII showed a significant presence of Gram-positive bacteria such as Actinobacteria (14.16%, 21.99%, 19.42%, respectively) and a low number of Firmicutes (11.87%, 7.54%, and 9.23%, respectively). At the genus level (Fig. 1c), S-I was characterized by a significant dominance of aerobic *Acidovorax* (23.91%), a genus that was proposed by reclassification of *Pseudomonas* species (Willems et al., 1990) and other members of the family Comamonadaceae (64.34%). The bacterial genera that dominated S-II were *Pseudoxanthomonas* (48.54%), *Pseudomonas* (13.25%) and other members of the family Comamonadaceae (25.50%). The genus *Pseudoxanthomonas* is phylogenetically related to the genera *Xanthomonas*, *Xylella* and *Stenotrophomonas* (Finkmann et al., 2000). However, members of the genus *Pseudoxanthomonas* differ from members of these related genera because they are capable of nitrate reduction but not nitrite (Yang et al., 1993). A homogeneous bacterial community structure was detected in S-III, with *Pseudomonas* being the dominant genera (83.29%). S-IV was composed by a large majority of members of the Comamonadaceae (58.45%), and the Fe(III)-reducing anaerobe *Geobacter* (7.44%). The community structures for S-V and VI were more complex at the genus level, where the high bacterial diversity masked dominance of any particular bacterial genus in these samples. Furthermore, very few OTUs were identified down to the genus level; however, these samples contained common soil bacteria such as *Azovibrio* (S-V, 8.09%) and *Thermomonas* (S-VI, 4.03%). Of note is the presence of the slightly thermophilic *Thermomonas* in S-VI, which was obtained from a tar tank maintained at 60°C constantly. Due to its different geographical origin, S-VII showed a more diverse community structure when compared to the other samples analysed in this study, with the metabolically versatile *Geobacter* being the dominant genus (44.95%). Further analyses were carried out using the BLAST programme package that matched the pyrosequencing data to known 16S rRNA gene sequences to find the closest known relatives to the OTUs identified in each sample (Table S2-S8). This analysis showed that the OTU dominating S-I (23.51%) and the second more abundant in S-II was a close relative to the uncultured *Acidovorax sp. clone 5_12_D11_b* (at 99% identity), isolated from the vadose zone of a hydrocarbon-contaminated aquifer (Tischer et al., 2013). The dominant OTU in S-II (48.54%) matched (at 97% identity) with *Pseudoxanthomonas sp. BZ60*, isolated

from a soil contaminated with high amount of heavy oil and heavy metals. A clear dominance of Gammaproteobacteria was observed in S-III, where the most abundant affiliation (83.29%) was a close relative (at 99% identity) of *Pseudomonas sp. DK2009-17* and *Pseudomonas poae strain Pier1*, both isolated from contaminated soils (Colinon et al., 2013). The Blast search of the more abundant OTU's sequence reads analysed in S-IV (58.45%) found that the closest relative (at 98% identity) was the *Uncultured bacterium clone N-122*, isolated from a Spanish PAH-contaminated soil (Guazzaroni et al., 2013). In S-V the most abundant OTU (8.09%) was a close relative (99% identity) of the *Uncultured Rhodocyclaceae bacterium clone D12_22*, obtained from a tar oil-contaminated aquifer (Takahata et al., 2006), while in S-VI the closest relative of the most abundant OTU was the *Uncultured bacterium clone N1DA011*, isolated from a PAH-contaminated soil. S-VII, due to its geographical origin, was characterized by a very different bacterial community composition, the largest contribution (44.95%) had as closest relative the anaerobe, Gram-negative *Geobacter sulfurreducens strain PCA* and *Geobacter sulfurreducens KN400* (at 99% and 98% identity, respectively). Of note is that the genus *Geobacter* contains the only organisms known to oxidise both acetate and PAHs to CO_2 with Fe(III) as the sole electron acceptor (Lovley, 1993), as observed in sediments of the Bemidji aquifer (Anderson and Lovley, 1999). S-VII was produced in the USA using carbureted water gas process which produces tar from an oil feedstock, which likely had an impact on the microbial communities, due to the different chemical composition.

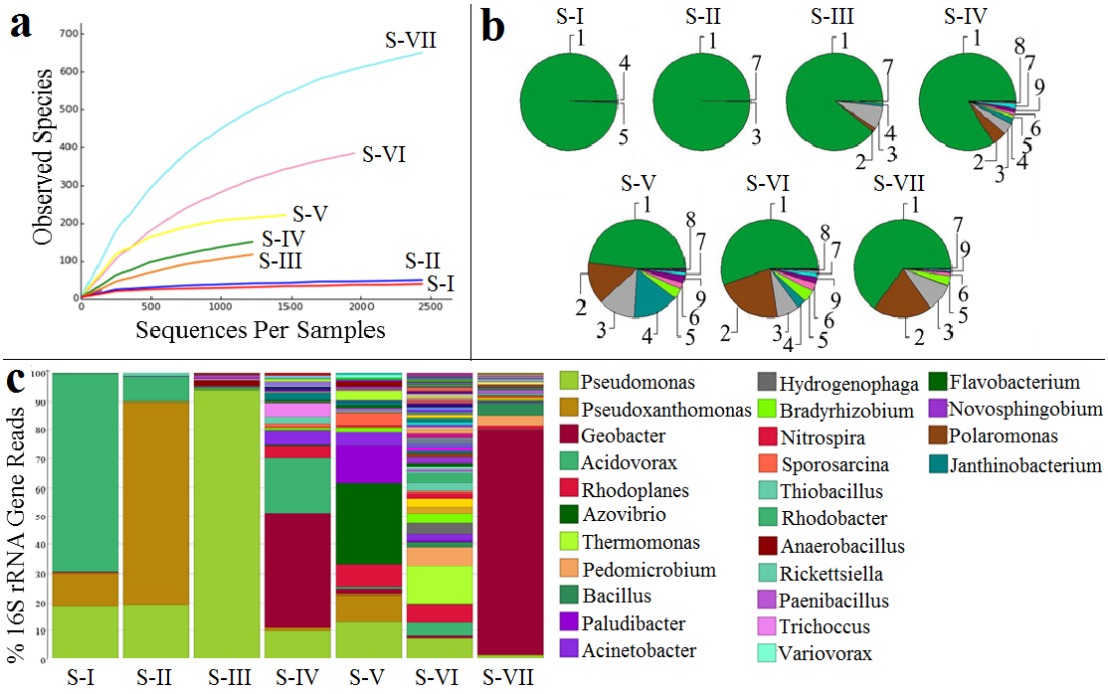

Figure 1. Microbial diversity from 454 pyrosequencing analyses of the seven coal tar samples analysed in this study. (a) The α-diversity plot, showing the number of OTUs in each sample. (b) Bacterial phyla detected, numbers correspond to: 1, *Proteobacteria*; 2, *Actinobacteria*; 3, *Firmicutes*; 4, *Bacteroidetes*; 5, *Cloroflexi*; 6, *Planctomycetes*; 7, *Acidobacteria*; 8, *Synergistetes*; 9, *Gemmatimonadetes*. (c) Bacterial genera in each sample that were tested. Taxa that show >2% expression are shown in the graph but not in the legend.

3.2 Carbon and hydrogen isotopes signature of polyaromatic hydrocarbons

Compound-specific stable carbon and hydrogen isotopes were used to identify possible shifts in the isotope signature and as a biomarker for tracing biological reactions. Extracts from all samples were analysed for their stable carbon ($\delta^{13}C$) and hydrogen ($\delta^{2}H$) isotope ratios. Typically, the variation in the $\delta^{13}C$ values for PAHs from different sources cover a range of only a few ‰, with those from coal conversion being close to -24 to -25 ‰ (Sun et al., 2003). The $\delta^{13}C$ values ranged between -27 and -23‰ (Table S9). Due to this narrow range of $\delta^{13}C$, no substantial variation was observed for aromatics of increasing molecular weight or among samples, however, a slightly more negative $\delta^{13}C$ values were found in S-VII, which

perhaps reflect a contribution of the source of coal/oil employed during the gasification process(es). Of note is also the variation of the $\delta^{13}C$ between S-I and II (with an average $\delta^{13}C$ of -26.1‰ and -25.3‰, respectively). The 454 pyrosequencing analyses highlighted a similar microbial community structure in these samples. Overall, the $\delta^{13}C$ values of aromatic compounds measured in the present study are consistent with the data obtained from: UK coal tars (Sun et al., 2003), soils from a domestic coal-burning village near Glasgow (McRae et al., 2000), coal-derived PAHs (McRae et al., 1999) and forest soils from Czech Republic (Bosch et al., 2015). In contrast with carbon isotope analysis, the aromatic extracts exhibited wider variations of $\delta^{2}H$ among samples, with values in the range of -303.6 to +40.7‰ (Table S10). Due to both the high variability of $\delta^{2}H$ values in these samples and the lack of hydrogen isotope data in the literature, no strong correlation was found among samples. However, a significant variation between unsubstituted PAHs and alkylated PAHs was found for all samples, in particular we observed an enrichment in ^{2}H for all unsubstituted PAHs, while metabolomic "daughter" alkyl-substituted PAHs were characterized by a more negative $\delta^{2}H$ value. S-I and II exhibit a different composition, with S-I being more enriched in ^{2}H (average $\delta^{2}H$ value of -14.9‰) than S-II (-51.6‰). Particularly interesting is the tar-contaminated soil S-V, where a large fractionation is observed especially between naphthalene and alkylated-naphthalenes. Very little variations were found for S-IV (creosote tar oil) and S-VI (tar from coke oven). The little fractionation observed for S-IV and VI are probably linked with their origin (SI) and chemical composition, usually rich in phenolic compounds (Mueller et al., 1989) which have a biocidal activity and might limit bacterial survival. Coal tar creosote is typically composed of ~85% polycyclic aromatic hydrocarbons (PAH's), ~10% phenolic compounds and ~5% N-, S- and O-heterocyclic compounds (McRae et al., 2000).

3.3 Multivariate statistical analyses

To observe relationships among the tar samples and to determine the degree of differentiations between the bacterial community structure and the carbon/hydrogen isotope signature of the selected PAHs, multivariate statistics using principal component analysis (PCA) and hierarchical cluster analysis were carried out using

the most recent release of Statistical Package for Social Sciences (SPSS) software (IBM Corp, NY). PCA of $\delta^{13}C$ could explain 91% of variability of carbon isotope in the coal tar samples (PC2 = 21% and PC1 67%) (Fig. 2a). PCA highlighted substantial relationships between the carbon isotope signature and the bacterial community structure (Fig. 1a and 1e, respectively), which clustered S-I and II in one group, while S-VII is clearly separated from the others. Furthermore, the creosote oil S-IV appears to be separated probably due to its different chemical composition. This is consistent with the geographical origin and manufacturing processes for each DNAPL which shaped the chemical composition of these samples (McGregor et al., 2011). However, the variations of $\delta^{13}C$ ranged over only few ‰, and were consistent with previous data on source apportionment of PAHs. Due to little variation, PCA and cluster analysis of $\delta^{13}C$ (Fig. 2a and b, respectively) was able to elucidate little information. In contrast, the results of $\delta^{2}H$ analyses showed a wide range of isotope ratios, which was highlighted by the multivariate statistical analyses that clustered unsubstituted and alkylated PAHs in two different branches (Fig. 2c and 2d). Furthermore, PCA of $\delta^{2}H$ could explain 84% of variability in the coal tar samples (PC2 = 12%; PC1 = 72%) (Fig. 2c). The higher hydrogen isotope fractionations observed could not be resolved by the source apportionment, suggesting the contribution of different metabolic reactions that determined a shift in the hydrogen isotope ratios. Cluster analysis of the selected PAHs confirmed the difference between the hydrogen isotope signature of unsubstituted- and alkylated-PAHs, where all unsubstituted PAHs (excluding anthracene) are clearly separated from alkylated PAHs and trimethyl-naphthalene. Although individual differences where noted on the microbial communities of the samples, a multivariate statistical approach was undertaken on pyrosequencing data to further distinguish the bacterial communities. PCA of pyrosequencing clearly separate the samples in three groups characterized by a dominance of *Pseudoxanthomonas* (S-I and II), *Pseudomonas* (S-III) and *Geobacter* (S-IV to VII) (Fig. 2e). This separation was also confirmed by cluster analysis of pyrosequencing data (Fig. 2f). The score plot and cluster confirmed similarities of S-I and II due to their geographical origin and similar chemical composition (Table S11).

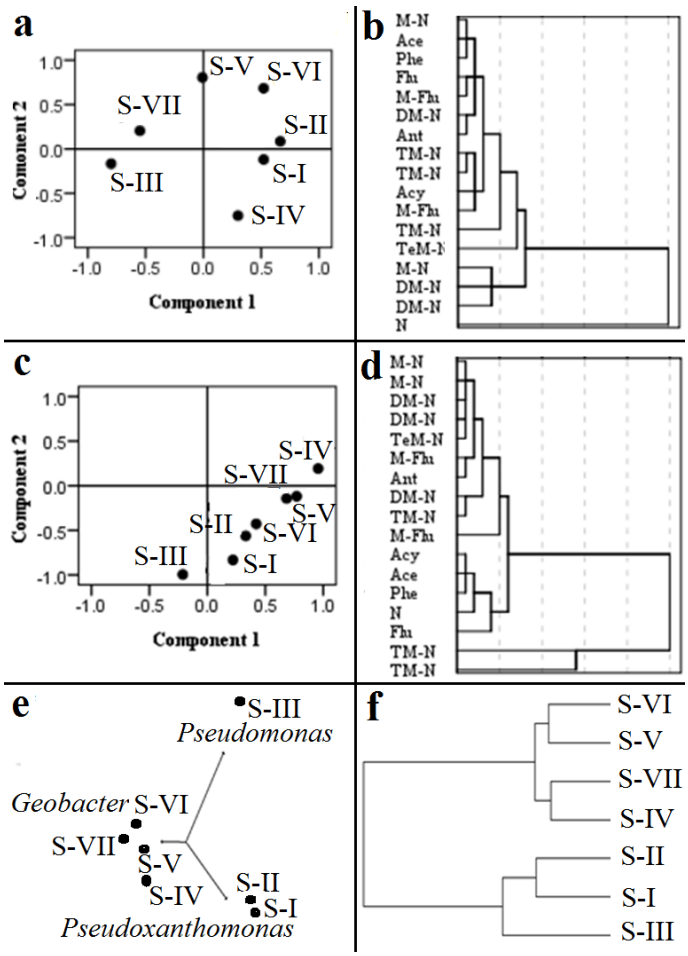

Figure 2. Multivariate statistics of the tar samples analysed in this study. (**a**) PCA plot of the $\delta^{13}C$ values. (**b**) Dendogram of the $\delta^{13}C$ values. (**c**) PCA plot of the $\delta^{2}H$ values. (**d**) Dendogram of the $\delta^{2}H$ values. (**e**) PCA plot of the bacterial communities and (**f**) Dendogram based on 454 pyrosequencing data.

To help support much needed studies on the natural attenuation and bioremediation of manufactured gas and coking works, this study developed and applied, for the first time, molecular characterization tools for the study of concentrated coal tar DNAPLs. The methodologies developed for the DNA extraction represent a simple and cost-effective step toward the molecular characterization of complex substrates such as DNAPLs. Further considerations should be made on interstitial water that may be present in the tar samples. In general, it is rather hard to collect ideal DNAPL samples and assure that the community is not contaminated by organisms present in interstitial water or sediment in proximity to the organic phase. For CWG tars, oil in

water and water in oil emulsions can readily form, which could trap water and microorganisms within the concentrated organic-phase. Nevertheless, microorganisms in coal tar environments are constrained by several physico-chemical factors and even their survival in small droplets of toxic medium show remarkable adaptation capabilities. The presence of bacteria in coal tar represents a significant expansion to the *plume fringe concept*, where it is assumed that biogeochemical gradients at the fringes of a contaminant plume facilitate hotspots of microbial activity. This concept has been demonstrated via geochemical analyses (Takahata et al., 2006), molecular biomarkers (Anneser et al., 2010) and two-dimensional aquifer models using naphthalene (Watson et al., 2005). According to the *plume fringe concept*, biodegradation in groundwater systems may be limited by the depletion of essential nutrients or by a low number of degradative bacteria. We described the presence of bacteria in DNAPLs, suggesting that the presence of bacteria might not be a limitation to biodegradation within the concentrated organic-phase. Our findings also suggest the main reason for a low microbial activity is the insufficient mixing of electron donors and acceptors necessary for bacterial activity. We believe that the complex bacterial community structures found in concentrated coal tar could potentially be active (as evidenced by δ^2H of alkylated PAH metabolites), and could therefore be further stimulated with nutrients to enhance biodegradation. The results of CSIA also show that multidimensional CSIA provides useful information on biochemical processes even in such complex settings. The application of CSIA for biodegradation quantification in the field is subject to uncertainty, due to a number of factors that impact on isotope fractionation and the complexity of biological transformation in the field. Therefore, it is critical to ensure a conservative assessment of the in-situ biodegradation and couple the application of multidimensional CSIA with molecular biological methods for the identification of dominant microorganisms. The combined approach of deep sequencing methods with metabolomic multidimensional CSIA was confirmed by statistical analysis. Thus, when coupling high-throughput 16S rRNA gene sequencing and metabolomic carbon/hydrogen isotope signature, the advantages for assessing the biodegradation potential become evident. The multidimensional CSIA approach is more sensitive than single-element CSIA, and is useful when the degradation conditions are

ambiguous or heterogeneous, or several different pathways potentially play a role under given conditions (Bergmann et al., 2011; Kuder et al., 2005).

The present study (*i*) developed and applied molecular characterization techniques for the analysis of coal tar and (*ii*) described for the first time the bacterial community structure in these extreme settings. This study also provided (*iii*) reference data for the compound specific hydrogen analysis of aromatic hydrocarbons in coal tar and demonstrated that (*iv*) coupling deep sequencing methods with metabolomics multidimensional CSIA can implement the assessment of the biodegradation potential in field environments.

Associated content

Samples description. Preparation of high-throughput 16S rRNA gene sequence analysis. Tables with OTU's Sequence Reads Analysed Filtered by Counts for each sample. Tables with results of metabolomic multidimensional CSIA.

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Supporting information for:

The bacterial community structure and multidimensional isotope signature of PAHs in coal tar

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22 pages, 11 tables, 7 figures

Sample description and history of the manufacturing processes employed.

S-I and II. The FMGP of this site (UK) employed a variety of manufacturing processes, encompassing almost all possible production methods throughout the years of operation (1836 until 1971). Initially, the site operated simple horizontal retorts, but the gasworks site was redeveloped in 1878 after a review by a gas engineer stated it was unsuitable for operation. After redevelopment, the works were expanded in 1912 to include five continuous vertical retort (CVR) beds. In 1914, annual gas production had reached 434 million cubic feet (cf). This was extended in the 1920s by addition of a further five CVR beds. In 1942, the site was affected by a World War II air raid, which destroyed a large oil tank containing heavy oil and an ammoniacal liquor plant. From the early 1930s until 1952 the plant remained at capacity, with various improvements to increase efficiency, including the addition of two water gas plants. The site was further expanded in 1952 by construction of a purification plant, additional vertical retorts and the development of storage areas for primary flash distillate (a light petroleum distillate). It follows that circa 1959 the site began production of gas from oil (mainly primary flash distillate). S-I was collected from a borehole slightly down gradient of the tar well structure and near the boundary of a suspected coal tar DNAPL plume. S-II was obtained from a borehole within a former tar well. Both samples are likely to be of vertical retort in origin, but in theory may have been influenced by any of the processes used on site throughout the years of operation.

S-III. Coal tar S-III was acquired from a former tar tank during a remediation project. This site (UK) was constructed in 1856 and by 1971 it was used solely as a gasholder station. Site plans dated 1932 and 1950 indicate that horizontal retorts and CWG were the major manufacturing processes on site. Coal tar S-III was taken from a former 250,000 gallon tank associated with the horizontal retort house.

S-IV. Very little is known of this site (UK) other than the fact it was a former creosote works associated with a timber yard. The surrounding area has a long history of industrial processes dating back to the late nineteenth century, including a naphthalene oxidation plant, tar distillery, a bitumen roadstone coating plant and a chemical fertiliser plant. Coal tar S-IV is likely to be a distilled fraction of coal tar, such as creosote oil, used for preserving timber (biocidal activity against wood rotting bacteria and fungi).

S-V. Coal tar S-V was acquired from a typical, small country gasworks (UK) in operation from 1854 until 1946. The site only every employed horizontal retorts for gas production. Site plans, dated 1961, indicate that a single gasholder with a maximum capacity of 21,000

cf was employed at this site. The tar sample was taken from a borehole (at a depth of 1.9 m) in the location of a former tar tank, associated with the simple, low temperature, horizontal retorts. The site has since been remediated and redeveloped for commercial and residential purposes.

S-VI. This sample was obtained from a gaswork site in UK which operated an inclined chamber oven (early 19^{th} century). S-VI was removed from a high temperature tar tank (permanently kept at 60 ºC).

S-VII. This sample is the only non UK tar analysed in this study. S-VII was obtained from a US landfill site associated with a former MGP which operated a CWG plant.

Supplemental information for the preparation of high-throughput 16S rRNA gene sequence analysis. Tables with OTU's sequence reads analysed with 454 pyrosequencing.

Fusion primers.

The forward primer, (5´ CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNAGAGTTTGATCMTG GCTCAG 3´) consisted of a fusion containing 454 Life Sciences 'Lib-L Primer A', a four-base 'key' sequence (TCAG), a unique ten-base barcode sequence (MID, Roche) for each sample (S5 Table no. 11), and bacterial primer 27F. The reverse fusion primer (5´CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCGTCAATTCMTTTRAGTTT 3´) contained the 454 Life Sciences 'Lib-L Primer B', a four-base 'key' sequence (TCAG) and bacterial primer 907R.

Table S1. Barcode sequences and percentage of coal tar DNAPL mixed with sand for each sample.

Samples	Barcode (Multiplex Identifier, MID) (Roche)	DNAPL/Sand mixture (wt. %)
S-I	ACGAGTGCGT	46,2
S-II	ACGCTCGACA	102,6
S-III	AGACGCACTC	50,2
S-IV	AGCACTGTAG	46,6
S-V	ATATCGCGAG	-
S-VI	CGTGTCTCTA	57
S-VII	CTCGCGTGTC	29,2

Table S2. S-I: OTU's sequence reads analysed filtered by counts.

OTU	Abundance	Closest match	Accession	Match (identities)	Phylogenetic class	Isolation source
0	58.62%	*Uncultured Acidovorax sp. clone 5_12_D11_b*	JQ087034	99%	Betaproteobacteria	Push core sediment sample from the vadose zone of a hydrocarbon contaminated aquifer
1085	10.12%	*Uncultured Acidovorax sp. clone W18i3*	AM948962	97%	Betaproteobacteria	BTEX contaminated groundwater
821	6.56%	*Uncultured Comamonadaceae bacterium clone D25_14*	EU266893	99%	Betaproteobacteria	Tar-oil contaminated aquifer sediments
892	4.22%	*Uncultured Acidovorax sp. clone LC-9-22*	KC481245	100%	Betaproteobacteria	Oilfield pipeline
1117	4.09%	*Acidovorax sp. clone Van23*	HQ222268	99%	Betaproteobacteria	Gasoline-polluted soil
4	3.23%	*Pseudoxanthomonas sp. BZ60*	HQ588838	99%	Gammaproteobacteria	Soil sample from an industrial site containing high amounts of heavy oil and heavy metals
5	3.2%	*Pseudomonas sp. DK2009-17*	FN600408	99%	Gammaproteobacteria	Soil
252	1.65%	*Uncultured gamma proteobacterium clone FTLpost107*	AF529130	96%	Betaproteobacteria	Trichloroethene-contaminated site
73	1.42%	*Pseudomonas sp. DRE-2009-B3*	FM956661	99%	Gammaproteobacteria	Phenol-degrading microcosm (borehole 59 inoculum); 4 weeks, position 3
45	1.22%	*Acidovorax sp. NA3*	EU910093	98%	Betaproteobacteria	Unkn.

Table S3. S-II: OTU's sequence reads analysed filtered by counts.

OTU	Abundance	Closest match	Accession	Match (identities)	Phylogenetic class	Isolation source
4	42.79%	*Pseudoxanthomonas sp. BZ60*	HQ588838	99%	Gammaproteobacteria	Soil sample from an industrial site containing high amounts of heavy oil and heavy metals
0	22.61%	*Uncultured Acidovorax sp. clone 5_12_D11_b*	JQ087034	99%	Betaproteobacteria	Push core sediment sample from the vadose zone of a hydrocarbon contaminated aquifer
5	6.50%	*Pseudomonas sp. DK2009-17*	FN600408	99%	Gammaproteobacteria	Soil
425	5.55%	*Uncultured bacterium clone N-204*	HQ218645	97%	Gammaproteobacteria	PAH-contaminated soil from Lugones, Asturias, Spain
64	3.12%	*Uncultured Xanthomonadales bacterium clone SLO-120*	JF262949	99%	Gammaproteobacteria	Contaminated soil
73	2.87%	*Pseudomonas sp. DRE-2009-B3*	FM956661	99%	Gammaproteobacteria	Phenol-degrading microcosm (borehole 59 inoculum); 4 weeks
45	2.25%	*Acidovorax sp. NA3*	EU910093	98%	Betaproteobacteria	Unkn.
804	1.89%	*Uncultured Acidovorax sp. clone 5_0_G4_b*	JQ087027	99%	Betaproteobacteria	Push core sediment sample from the vadose zone of a hydrocarbon contaminated aquifer
879	1.74%	*Uncultured bacterium clone RDX 5*	EU907869	99%	Gammaproteobacteria	RDX-contaminated soil
892	1.42%	*Uncultured Acidovorax sp. clone LC-9-22*	KC481245	100%	Betaproteobacteria	Oilfield pipeline

Table S4. S-III: OTU's sequence reads analysed filtered by counts.

OTU	Abundance	Closest match	Accession	Match (identities)	Phylogenetic class	Isolation source
5	38.16%	*Pseudomonas sp. DK2009, isolate 17*	FN600408	99%	Gammaproteo bacteria	Soil
2	37.09%	*Pseudomonas poae strain Pier1*	KC195894	99%	Gammaproteo bacteria	Heavy metal contaminated soil
6	7.95%	*Pseudomonas stutzeri strain 24a80*	AJ312170	99%	Gammaproteo bacteria	Soil beneath pavement of filling station
1122	2.40%	*Uncultured bacterium clone BVESI8F6*	KJ809441	99%	Gammaproteo bacteria	Intraradical propagules of arbuscular mycorrhizal fungi in hydrocarbon contaminated soil
42	2.07%	*Uncultured bacterium clone NS034*	JX391750	98%	Bacilli	Marine sediments
38	1.90%	*Uncultured bacterium clone N-151*	HQ218593	99%	Gammaproteo bacteria	PAH-contaminated soil from Lugones, Asturias, Spain
99	1.90%	*Uncultured bacterium clone N-119*	HQ218562	99%	Gammaproteo bacteria	PAH-contaminated soil from Lugones, Asturias, Spain
1160	1.82%	*Uncultured bacterium clone BANW560*	DQ264529	99%	Gammaproteo bacteria	Subsurface groundwater during polylactate stimulated chromate bioremediation
1163	0.99%	*Pseudomonas sp. 2B29*	JX177694	98%	Gammaproteo bacteria	Baltic Sea surface water
1114	0.99%	*Pseudomonas sp. DJ22*	KM054653	97%	Gammaproteo bacteria	Tank bottom sludge

Table S5. S-IV: OTU's sequence reads analysed filtered by counts.

OTU	Abundance	Closest match	Accession	Match (identities)	Phylogenetic class	Isolation source
655	58.94%	*Uncultured bacterium clone N-122*	HQ218565	98%	Betaproteobacteria	PAH-contaminated soil from Lugones, Asturias, Spain
0	11.98%	*Uncultured Acidovorax sp. clone 5_12_D11_b*	JQ087034	99%	Betaproteobacteria	Push core sediment sample from the vadose zone of a hydrocarbon contaminated aquifer
13	8.94%	*Uncultured Geobacter sp. clone 3_16_F6_b*	JQ086879	99%	Deltaproteobacteria	Push core sediment sample from the vadose zone of a hydrocarbon contaminated aquifer
1085	3.23%	*Uncultured Acidovorax sp. clone W18i3*	AM948962	97%	Betaproteobacteria	BTEX contaminated groundwater
30	2.76%	*Contamination: Uncultured bacterium clone nbw02f12c1*	GQ060516	99%	Alphaproteobacteria	Skin microbiome
15	1.24%	*Uncultured Shigella sp. clone C254*	JF833728	99%	Gammaproteobacteria	Potassium mine soil
94	1.14%	*Uncultured bacterium clone BST22-80*	HQ436556	99%	Alphaproteobacteria	Oligosaline lake water
106	1.05%	*Uncultured Carnobacteriaceae bacterium clone BLUC-K*	DQ196615	95%	Bacilli	Acidic Louisiana groundwater contaminated by DNAPL
343	0.86%	*Uncultured soil bacterium clone SalCon01*	EF101781	98%	Gammaproteobacteria	PAHs contaminated soil
128	0.76%	*Uncultured bacterium clone kua078*	HM150244	98%	Gammaproteobacteria	Sediment sample, thermal spring

Table S6. S-V: OTU's sequence reads analysed filtered by counts.

OTU	Abundance	Closest match	Accession	Match (identities)	Phylogenetic class	Isolation source
9	12.88%	*Uncultured Rhodocyclaceae bacterium clone D12_22*	EU266828	99%	Betaproteobacteria	Tar-oil contaminated aquifer sediments
15	6.44%	*Uncultured Shigella sp. clone C254*	JF833728	99%	Gammaproteobacteria	Potassium mine soil
17	5.61%	*Paludibacter sp. clone A9*	EU377679	99%	Bacteroidia	Anoxic BTEX-contaminated aquifier
4	4.05%	*Pseudoxanthomonas sp. BZ60*	HQ588838	99%	Gammaproteobacteria	Soil sample from an industrial site containing high amounts of heavy oil and heavy metals
23	3.74%	*Uncultured Proteiniphilum sp. clone 3_28_A4_b*	JQ086804	99%	Gammaproteobacteria	Push core sediment sample from the vadose zone of a hydrocarbon contaminated aquifer
128	3.74%	*Uncultured bacterium clone kua078*	HM150244	98%	Gammaproteobacteria	Sediment sample, thermal spring
28	3.12%	*Uncultured bacterium clone nbw1228c11c1*	GQ059516	99%	Actinobacteria	Skin microbiome
19	3.01%	*Uncultured Gemmatimonadetes bacterium clone BB-1-H6*	AY214733	99%	Gemm-1	Soil
34	2.91%	*Uncultured alpha proteobacterium clone GC0AA5ZF09PP1*	JQ919489	96%	Alphaproteobacteria	Gasoline-polluted soil
6	2.80%	*Pseudomonas stutzeri strain 24a80*	AJ312170	99%	Gammaproteobacteria	Soil beneath pavement of filling station

Table S7. S-VI: OTU's sequence reads analysed filtered by counts.

OTU	Abundance	Closest match	Accession	Match (identities)	Phylogenetic class	Isolation source
8	8.17%	*Uncultured bacterium clone N1DA011*	FQ658732	99%	Gammaproteobacteria	PAH-contaminated soil; retention systems which treat road runoffs
0	6.15%	*Uncultured Acidovorax sp. clone 5_12_D11_b*	JQ087034	99%	Betaproteobacteria	Push core sediment sample from the vadose zone of a hydrocarbon contaminated aquifer
22	4.35%	*Uncultured Tissierella sp. clone Fe61*	KF362103	96%	Clostridia	Alkaline chromium ore processing residue disposal site
15	3.45%	*Uncultured Shigella sp. clone C254*	JF833728	99%	Gammaproteobacteria	Potassium mine soil
27	3.30%	*Uncultured Ectothiorhodospiraceae bacterium clone CM41G12*	AM936552	99%	Gammaproteobacteria	Hydrocarbon contaminated soil
21	3.30%	*Rhodococcus sp. HA73*	KF011657	98%	Actinobacteria	Tundra soil
6	3.15%	*Pseudomonas stutzeri partial 16S rRNA gene, strain 24a80*	AJ312170	99%	Gammaproteobacteria	Soil beneath pavement of filling station
26	2.92%	*Uncultured alpha proteobacterium clone B09L-1*	HE614737	98%	Alphaproteobacteria	Arsenic and gold mine
655	2.70%	*Uncultured bacterium clone N-122*	HQ218565	98%	Betaproteobacteria	PAH-contaminated soil from Lugones, Spain
12	2.55%	*Uncultured alpha proteobacterium clone D08-1*	FM253597	99%	Alphaproteobacteria	Biofilm in arsenic and gold mine

Table S8. S-VII: OTU's sequence reads analysed filtered by counts.

OTU	Abundance	Closest match	Accession	Match (identities)	Phylogenetic class	Isolation source
3	54.34%	*Geobacter sulfurreducens strain PCA*	NR075009	99%	Deltaproteobacteria	
1166	13.01%	*Geobacter sulfurreducens* KN400	CP002031	98%	Deltaproteobacteria	
12	4.79%	*Uncultured alpha proteobacterium clone D08-1*	FM253597	99%	Alphaproteobacteria	Biofilm in arsenic and gold mine
1102	4.19%	*Geobacter sp. R-HL6*	KP059287	99%	Deltaproteobacteria	Wastewater
39	1.67%	*Uncultured bacterium, clone WE21_87*	HE985058	99%	Alphaproteobacteria	Soil
54	1.52%	*Uncultured bacterium clone A3-127*	KC554818	99%	Thermoleophilia	Soil of Yanshan Mountain (China)
24	1.29%	*Uncultured bacterium clone Biotrap_608_G11*	KJ670583	98%	Actinobacteria	BioTrap samplers in groundwater monitoring well
29	1.22%	*Uncultured bacterium clone Baren07SC34*	FR871563	99%	Thermoleophilia	Greenhouse soil
196	1.07%	*Uncultured gold mine bacterium D35*	AF337888	99%	Alphaproteobacteria	Gold mine
80	0.84%	*Uncultured bacterium clone ncd2267g06c1*	JF188106	98%	Thermoleophilia	Skin microbiome

Tables and chromatograms for CSIA and metabolomic analysis.

Numbers after the sign '±' represent standard deviations. Final $\delta^{13}C$, $\delta^{2}H$ and concentration values are the results of triplicate injections of two different extractions, for a total of six values.

Abbreviations

N, naphthalene; M-N, methyl-naphthalene; DM-N, dimethyl-naphthalene; Acy, acenaphthylene; Ace, acenaphthene; TM-N, trimethyl-naphthalene; Flu, fluorene; TeM-N, tetramethyl-naphthalene; M-Flu, methyl-fluorene; Phe, phenanthrene; Ant, anthracene; Flt, fluoranthene; Pyr, pyrene; BaA, benzo(a)anthracene; Chr, chrysene; BbF, benzo(b)fluoranthene; BaP, benzo(a)pyrene; SD, standard deviation; Bd, below detection.

Table S9. Mean $\delta^{13}C$ values (‰ vs. VPDB) of the selected PAHs analysed in this study. Numbers after the sign "±" represent the Standard Deviation of triplicate injections of two different extractions, for a total of two values. *Bd = below detection.

PAHs	S-I	S-II	S-III	S-IV	S-V	S-VI	S-VII
N	-26.2 ± 0.03	-24.2 ± 0.96	-25.6 ± 0.06	-25.9 ± 0.04	-26.5 ± 0.04	-26.7 ± 0.39	-28 ± 0.07
M-N	-26.2 ± 0.33	-26.2 ± 0.09	-24.9 ± 0.13	-25.1 ± 0.02	-25.6 ± 0.08	-24.9 ± 0.11	-27.8 ± 0.09
M-N	-25.8 ± 0.07	-25.7 ± 0.88	-23.1 ± 0.04	-24.7 ± 0.13	-25.2 ± 0.14	-25.4 ± 0.06	-33.2 ± 0.46
DM-N	-29.4 ± 0.47	-31.6 ± 0.76	-22.5 ± 0.00	-24.1 ± 0.38	-25.3 ± 0.06	-24.5 ± 0.27	-28.3 ± 0.35
DM-N	-25.4 ± 0.11	-26.4 ± 0.20	-25.7 ± 0.32	-24.4 ± 0.23	-25.5 ± 0.21	-24.4 ± 0.04	-28.3 ± 0.19
DM-N	-26.2 ± 0.39	-27 ± 0.16	-19.8 ± 0.10	-23.5 ± 0.35	-25.6 ± 0.08	-25.9 ± 0.10	Bd*
Acy	-25.4 ± 0.12	-26.7 ± 0.11	-24.1 ± 0.01	-24 ± 0.28	-23.7 ± 0.09	-23.5 ± 0.05	-26.7 ± 0.09
Ace	-26.2 ± 0.28	-25.3 ± 0.95	-25.7 ± 0.05	-24.6 ± 0.17	-24.9 ± 0.12	-25.1 ± 0.13	-27.3 ± 0.09
TM-N	-23.2 ± 0.48	-24.3 ± 1.38	-26.8 ± 0.12	-23.2 ± 0.29	-26.2 ± 0.17	-23.2 ± 0.23	-28.5 ± 0.14
TM-N	-24.6 ± 0.37	-24.9 ± 0.30	-25.5 ± 0.10	-23.9 ± 0.30	-24.1 ± 0.63	-23.3 ± 0.22	-28.3 ± 0.06
Flu	-26.8 ± 0.03	-26.3 ± 0.89	-26.2 ± 0.00	-25 ± 0.12	-23.8 ± 0.19	-25.6 ± 0.03	-27.9 ± 0.05
TeM-N	-26.8 ± 0.14	-21.8 ± 0.62	-35.8 ± 0.15	-23.1 ± 0.15	-24.8 ± 0.21	-21. ± 0.04	-24.6 ± 0.37
TeM-N	-27.7 ± 0.16	-25.6 ± 0.28	Bd	Bd	Bd	-22.5 ± 0.52	Bd
M-Flu	-27 ± 0.32	-25.7 ± 0.91	-27.2 ± 0.31	-25.4 ± 0.16	-23.2 ± 0.16	-24.1 ± 0.04	-27.9 ± 0.48
M-Flu	-27 ± 0.36	-24.3 ± 0.92	Bd	-23.6 ± 0.60	-24.1 ± 0.22	-23.2 ± 0.13	-26.1 ± 0.17
Phe	-27 ± 0.05	-26.2 ± 0.90	-25.9 ± 0.38	-25.4 ± 0.15	-26 ± 0.09	-25.4 ± 0.11	-28.6 ± 0.26
Ant	-26.5 ± 0.13	-25.8 ± 0.91	-26.2 ± 0.01	-24.5 ± 0.16	-25.2 ± 0.11	-25.1 ± 0.10	-26 ± 0.17
Flt	-26.2 ± 0.02	-25.7 ± 0.11	-25.5 ± 0.07	-24.4 ± 0.71	-26 ± 0.15	-25.4 ± 0.07	-26.5 ± 0.53
Pyr	-27.4 ± 0.13	-26.2 ± 0.87	-25.3 ± 0.07	-24.5 ± 0.03	-25.7 ± 0.11	-25.3 ± 0.05	-24.8 ± 0.06
BaA	-26.7 ± 0.18	-25.2 ± 0.91	-25 ± 0.26	-24.7 ± 0.18	-25.2 ± 0.06	-25 ± 0.28	-27 ± 0.37
Chr	-26.7 ± 0.13	Bd	-25.2 ± 0.29	-25.1 ± 0.03	-25.4 ± 0.16	-24.7 ± 0.11	-26.7 ± 0.35
BbF	Bd	Bd	-24.8 ± 0.05	-25.1 ± 0.66	-25.3 ± 0.32	-24.7 ± 0.99	-27.3 ± 0.35
BaP	-26 ± 0.54	Bd	-24.4 ± 0.78	-24.5 ± 0.55	-25.2 ± 0.55	-24.2 ± 0.14	-28.7 ± 0.37

Table S10. Mean δ^2H values (‰ vs. VSMOW) of the selected PAHs analysed in this study. Numbers after the sign "±" represent the Standard Deviation of triplicate injections of two different extractions, for a total of two values. *Bd = below detection.

PAHs	S-I	S-II	S-III	S-IV	S-V	S-VI	S-VII
N	13.1 ± 4.45	-48.4 ± 6.87	-50 ± 2.47	-49.4 ± 2.07	-62.4 ± 0.87	-70.2 ± 0.59	14.2 ± 0.47
M-N	-21.9± 0.54	-61.7 ± 2.90	-57.6 ± 2.75	-98.5 ± 0.53	-87.2 ± 0.04	-96.9 ± 0.73	-25.4± 3.11
M-N	-4.3 ±12.20	-50.7 ± 2.61	-48.4 ± 3.47	-105.6± 0.45	-102.3± 1.41	-99.2 ± 0.26	-34.5± 0.99
DM-N	-23.7± 9.15	-42.3 ± 0.66	-45.2 ± 2.46	-97.9 ± 1.83	-87.5 ± 0.10	-103.4± 2.31	-53.2± 0.12
DM-N	-19.4± 6.21	-35.2 ± 0.09	-79.9 ± 4.18	-97.9 ± 0.35	-73.7 ± 3.92	-110.6± 0.43	-49.7± 0.82
DM-N	-69.1± 8.42	-83.7 ± 0.86	-77.9 ±12.27	-96.2 ± 0.63	-115.5± 2.04	-106.1± 0.33	Bd*
Acy	20 ± 6.27	-21.2 ± 0.31	-24 ± 2.70	-85.8 ± 1.13	-78.6 ± 1.34	-97.7 ± 1.23	-40 ± 1.39
Ace	12.1 ± 7.74	-3.1 ± 3.60	-18.6 ± 5.47	-86.8 ± 0.04	-53.1 ± 1.41	-77.1 ± 0.56	-28 ± 2.03
TM-N	-64.2± 8.21	-179.9±15.67	-87.8 ± 7.33	-94.3 ± 1.67	-122.2± 2.59	-204.2± 5.03	-51.1± 1.23
TM-N	-62.4±13.09	-187.6±21.32	-90.5 ±14.40	-124.9± 2.59	-78.5 ± 2.13	-77.8 ± 1.26	-66.1± 2.25
Flu	12.8 ± 8.26	-8.9 ± 0.51	-35 ± 2.36	-71.1 ± 0.78	28.3 ± 0.13	-56.3 ± 2.18	-24.6± 1.68
TeM-N	-16.8± 9.89	-15.1 ± 3.22	-65.6 ±13.59	-96.3 ±81.72	-75.3 ± 0.82	-78.4 ± 8.60	-55.7± 3.08
TeM-N	-52 ± 6.34	-51 ± 2.49	Bd	Bd	Bd	-82.7 ± 2.47	Bd
M-Flu	-56.4±15.02	-80.8 ± 3.07	-147.2±14.73	-72.3 ± 2.02	-47.2 ± 4.71	-107.1± 5.36	-39.1± 1.77
M-Flu	-39.5± 2.24	-53.7 ± 1.97	Bd	-47.4 ± 2.84	-54 ± 3.43	-95.4 ± 5.95	-42.9± 1.75
Phe	18.3 ± 2.39	1.7 ± 0.96	-40.5 ± 2.95	-53.2 ± 0.58	-54.9 ± 0.79	-65.8 ± 0.54	-12.5± 2.45
Ant	-52 ± 9.51	-57.5 ± 4.49	-83.9 ± 7.78	-72.4 ± 7.38	-88.8 ± 1.33	-86.1 ± 2.84	-22.5± 1.81
Flt	0.7 ± 4.35	-3.4 ± 0.29	-46.4 ± 2.00	-59.1 ± 0.33	-50.2 ± 0.20	-58.5 ± 1.85	-12.6 10.79
Pyr	25.4 ± 0.35	7.8 ± 0.03	-34.2 ± 2.97	-55.8 ± 1.06	-39.2 ± 1.50	-62.2 ± 0.89	-11.2± 5.74
BaA	37.6 ± 7.27	15.4 ± 4.51	-19.4 ±16.72	-61 ± 1.41	18.1 ± 2.31	-38.1 ± 0.81	12.4 ± 6.27
Chr	13.1 ± 2.39	Bd	-11.5 ± 2.33	-73.7 ± 0.64	-12.2 ± 1.10	-54.4 ± 1.94	-7 ±11.28
BbF	Bd	Bd	-59.3 ± 9.49	-67.6 ± 0.90	-64.3 ±17.83	-64.8 ± 4.49	6 ± 7.95
BaP	-13.9±33.54	Bd	-51.7 ± 4.36	-46.1 ± 7.93	-33 ± 6.64	-64.3 ± 4.12	40.7 ± 1.79

Table S11. Concentration (μg/mL) of the selected PAHs in the coal tar DNAPLs investigated in this study. Numbers after the sign "±" represent the Standard Deviation of triplicate injections of two different extractions, for a total of two values.

PAHs	S-I	S-II	S-III	S-IV	S-V	S-VI	S-VII
N	20.2 ± 1.99	7.3 ± 0.16	155.9± 2.85	21.4 ± 0.57	105.8± 3.24	700.5±20.17	86 ± 5.19
M-N	41.9 ± 0.41	14.1 ± 1.16	139.5± 7.75	37.3 ± 0.47	123.2± 2.49	238.2± 7.50	867.3±10.84
M-N	27.3 ± 1.02	122.7± 2.77	83.6 ± 3.85	21.4 ± 0.41	63 ± 0.49	159.9± 6.47	426.6±12.62
DM-N	16.9 ± 0.72	60.1 ± 2.78	38.6 ± 1.82	23.1 ± 1.14	36.7 ± 0.98	103.7± 4.89	119.5± 4.99
DM-N	33.7 ± 1.65	196.2± 9.21	60.6 ± 3.45	35.5 ± 0.59	45.4 ± 3.46	162.6± 2.31	316 ±12.28
DM-N	18.6 ± 0.70	107.3± 5.44	33.5 ± 1.82	24.8 ± 1.36	32.4 ± 2.08	148.4± 1.52	184.7± 3.55
Ace	7.1 ± 0.22	18.8 ± 0.25	10.6 ± 0.30	30.2 ± 0.80	7.3 ± 0.03	27.8 ± 0.77	26.8 ± 0.98
Acy	23.3 ± 1.35	61.2 ± 1.14	64.3 ± 3.35	7.3 ± 0.03	28.7 ± 0.90	200 ± 6.83	8.5 ± 0.08
TM-N	69 ± 1.68	376.4± 3.03	84.5 ± 3.54	105.5± 1.17	74.8 ± 1.03	296 ± 9.46	398.4± 9.28
TM-N	49.5 ± 1.28	272.1± 5.65	61.9 ± 4.53	79.7 ± 2.81	57.1 ± 0.73	225.6± 1.61	320.6±11.06
Flu	28.9 ± 3.36	96.4 ± 8.02	54.7 ± 2.06	78.7 ± 0.99	23.9 ± 0.68	238.8± 6.10	32.2 ± 0.87
Te-MN	21.3 ± 0.76	97.8 ± 1.68	15.1 ± 0.88	50.8 ± 2.24	17 ± 0.36	70.2 ± 2.64	87.2 ± 3.45
Te-MN	13.5 ± 0.63	65.8 ± 0.80	9.1 ± 0.75	34.3 ± 0.19	11.6 ± 0.41	46.3 ± 0.31	56.5 ± 2.21
M-Flu	10 ± 0.67	30.7 ± 0.59	8.7 ± 0.42	13.3 ± 0.16	3.6 ± 0.25	13.6 ± 1.23	38.7 ± 0.97
M-Flu	5.8 ± 0.11	8 ± 0.10	7.1 ± 0.51	5.3 ± 0.07	4.9 ± 0.05	5.3 ± 0.09	4.9 ± 0.05
Phe	67.4 ± 0.86	174.9± 4.34	165.6±12.05	158.2± 1.00	100.9± 1.95	383.1±10.45	69 ± 1.70
Ant	24 ± 0.09	92.4 ± 1.51	56.1 ± 4.33	88.7 ± 1.90	39.3 ± 0.48	162.6± 7.47	17.4 ± 0.28
Flt	44.9 ± 1.45	158.4± 3.89	153 ±12.83	95.5 ± 2.19	46.9 ± 1.25	226.7± 8.04	23.9 ± 0.22
Pyr	39.1 ± 1.08	141.2± 6.63	117.6±10.24	45.7 ± 0.60	34.1 ± 0.93	166.1± 6.66	29.4 ± 0.23
BaA	25.9 ± 0.74	71.2 ± 1.31	65.1 ± 9.01	23.2 ± 0.47	13.3 ± 0.49	68.5 ± 4.89	12 ± 0.16
Chr	26.1 ± 0.21	62.3 ± 2.54	63.5 ±11.01	28.7 ± 0.82	15 ± 0.34	58.6 ± 3.04	13.5 ± 0.02
BbF	21 ± 5.42	49.6 ± 0.67	50.8 ± 7.49	12.7 ± 0.14	6.9 ± 0.04	24.3 ± 1.91	7.3 ± 0.21
BaP	21 ± 0.40	52.2 ± 2.28	57.5 ± 8.37	11.6 ± 0.19	7.1 ± 0.15	28.8 ± 2.42	8.2 ± 0.38

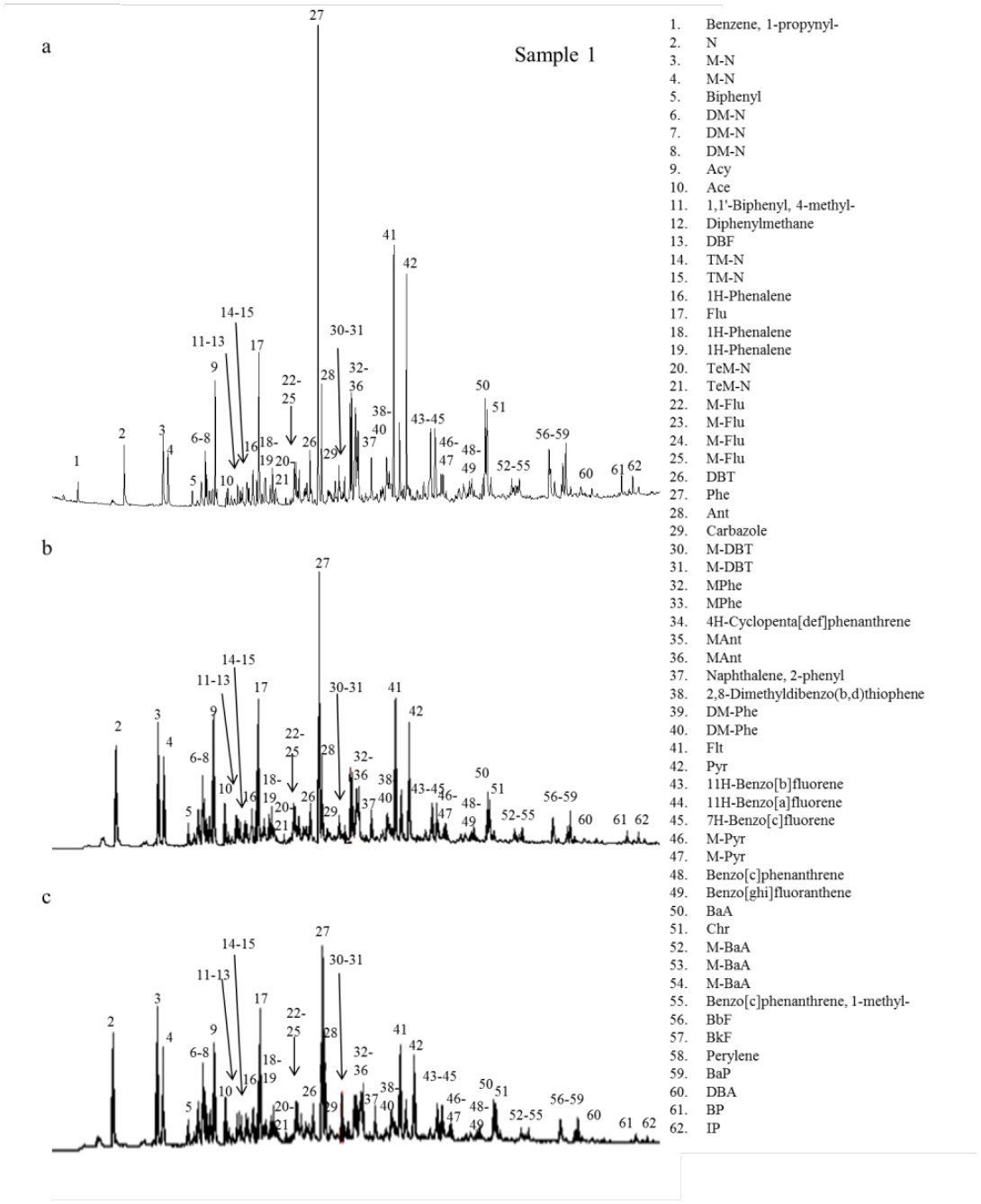

Figure S1. S-I: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

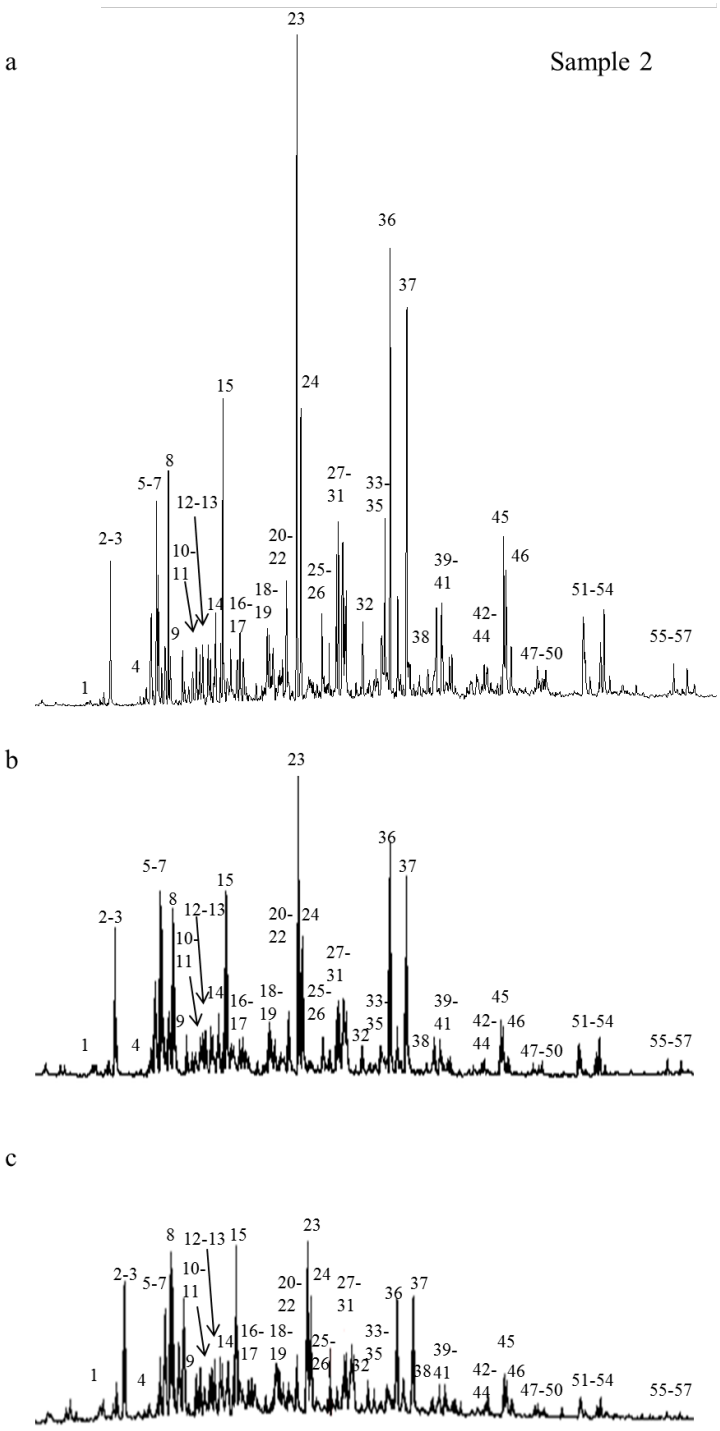

Figure S2. S-II: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

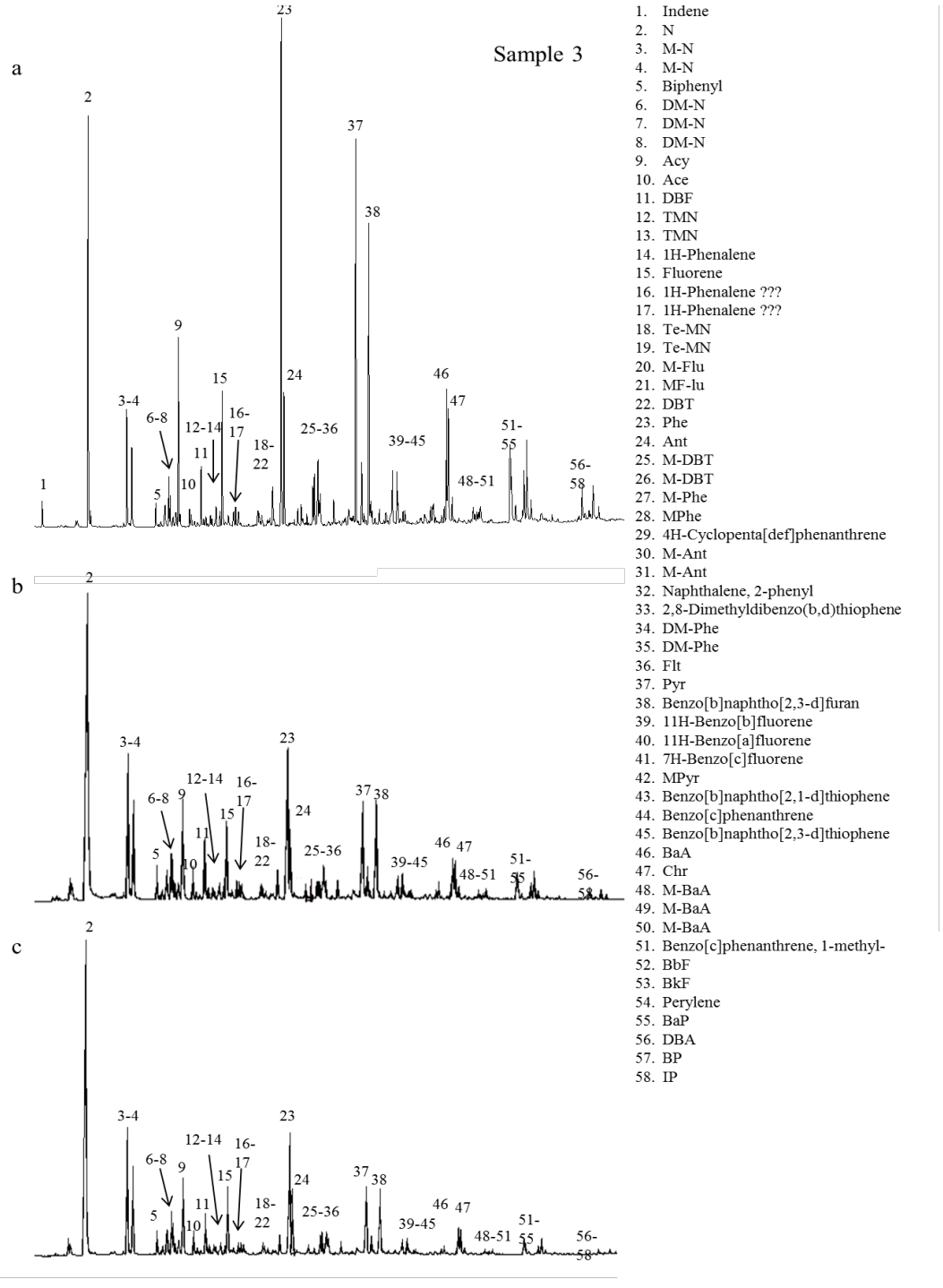

Figure S3. S-III: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

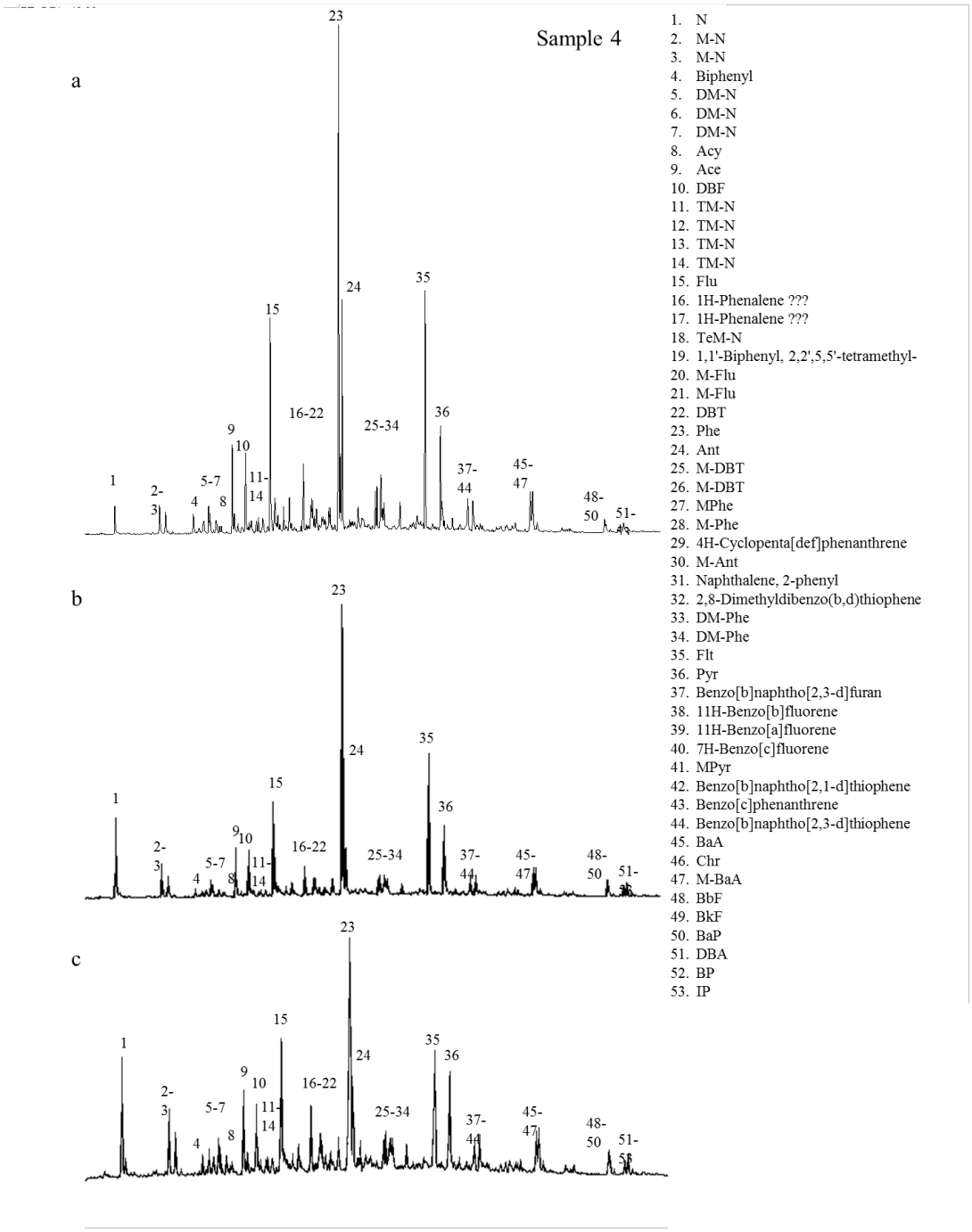

Figure S4. S-IV: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

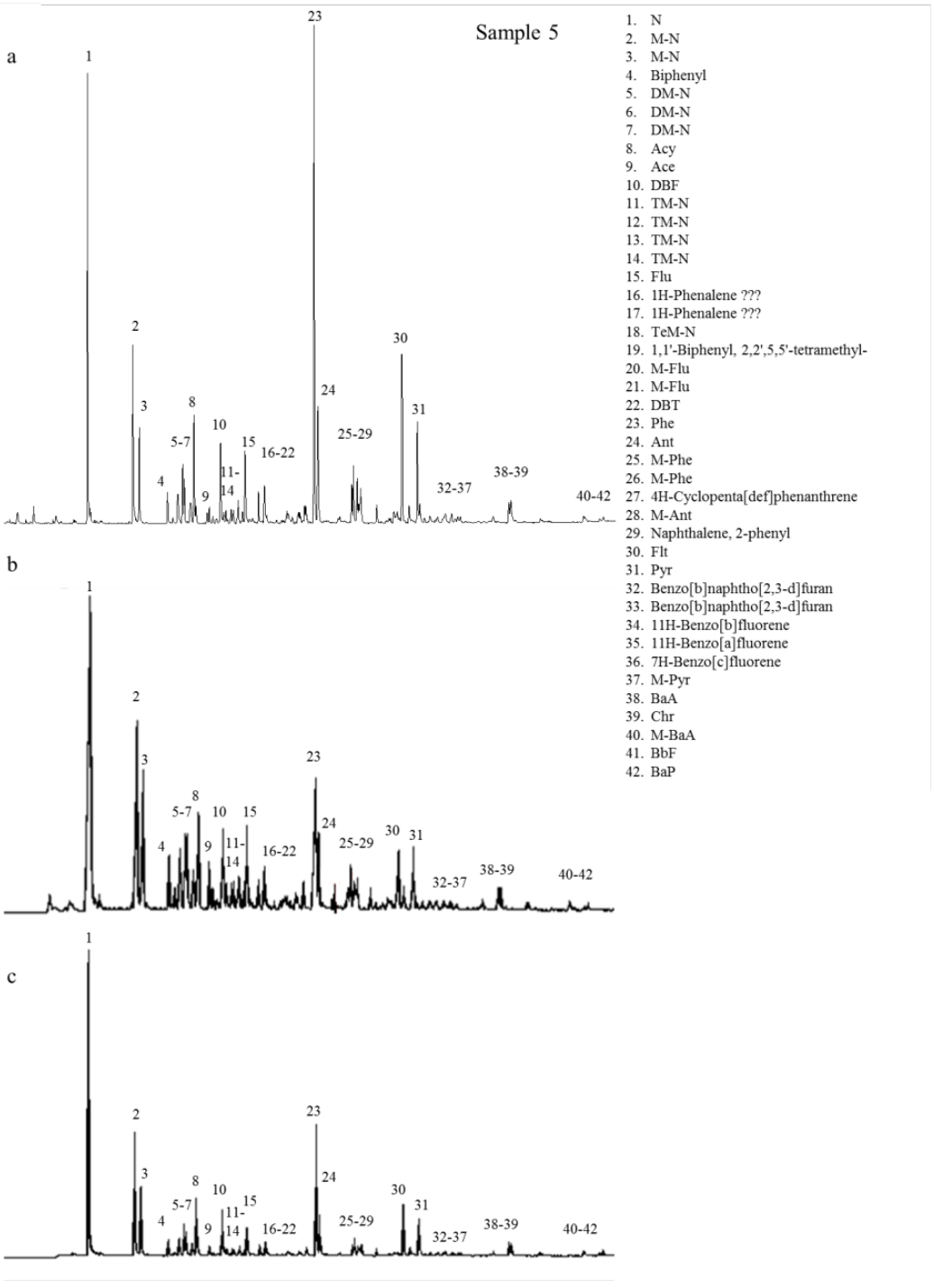

Figure S5. S-V: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

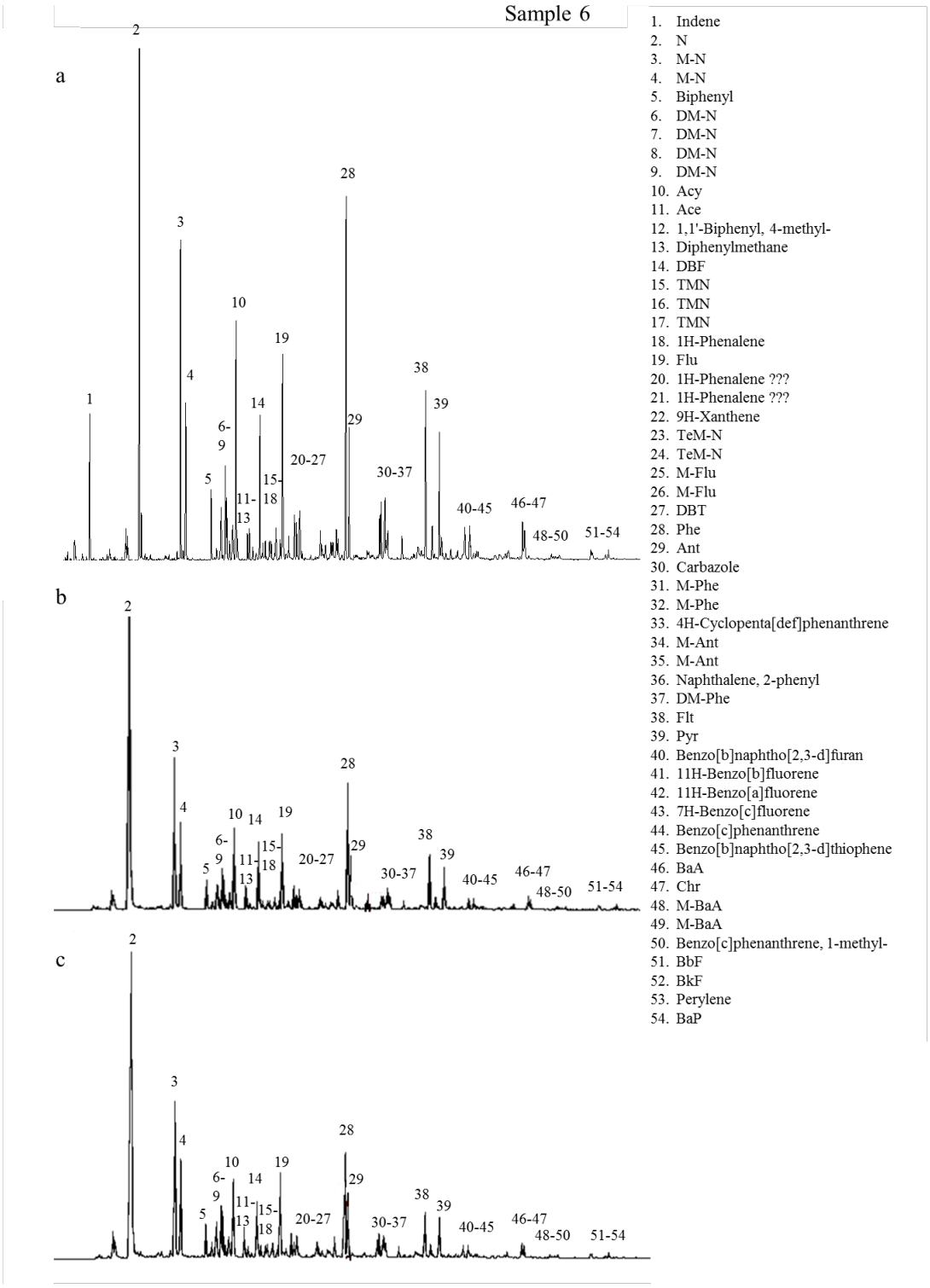

Figure S6. S-VI: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

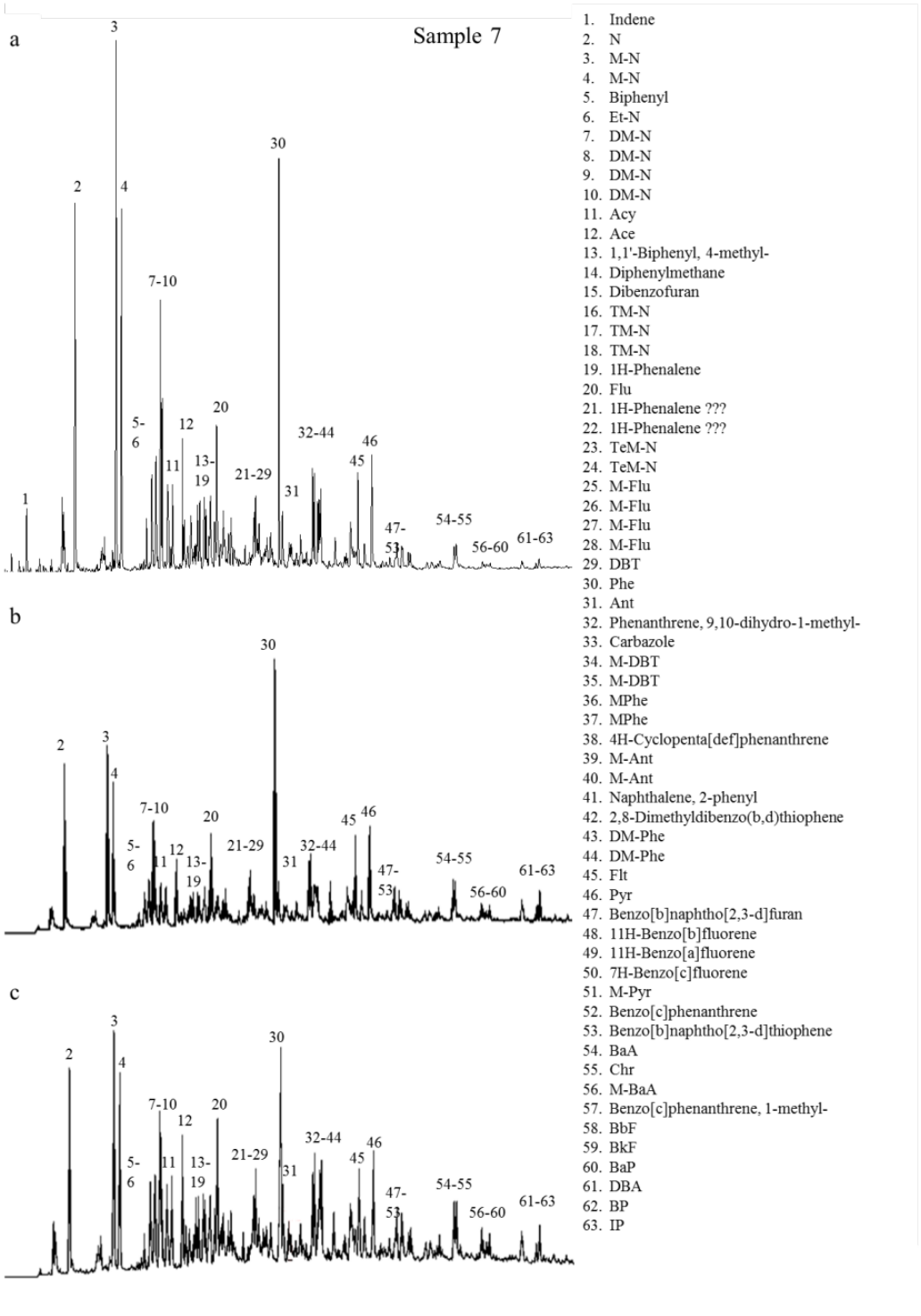

Figure S7. S-VII: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

6.3 Final considerations

The combined approach of deep sequencing methods with metabolomic multidimensional CSIA, confirmed by multivariate statistics, is a powerful tool to understand microbial community dynamics in extreme environments. When coupling high-throughput 16S rRNA gene sequencing and metabolomic carbon/hydrogen isotope signature, the advantages for assessing the biodegradation potential become evident. The methodologies developed in this Chapter could have several applications in the unconventional gas industry, where a major problem is to determine the presence of active methanogenic communities. In fact, the research in this field have been done with two main methodologies: (*i*) the first is using geochemical indicators such as carbon and deuterium isotopes found in the carbon source, produced waters, methane and carbon dioxide to establish whether the gas is biogenic or thermogenic in origin (Martini et al., 1998, Shurr and Ridgley, 2002). (*ii*) The second are methane generation experiments of biodegradation using samples of coal/shale in laboratory to determine methane production rates (Gieg et al., 2008; Jones et al., 2008). The methodologies developed during this Ph.D. project could be a cost effective approach that can be used alone or in combination with the two previously mentioned methods.

The paper presented in this Chapter (under review of the Journal of Contaminant Hydrology) also developed and applied for the first time methodologies for the molecular characterisation of concentrated coal tar DNAPLs. The presence of bacteria in these coal tar samples represents a significant expansion to the *plume fringe concept* (Winderl et al., 2008), where it is assumed that biogeochemical gradients at the fringes of a contaminant plume facilitate hotspots of microbial activity. The sequencing analyses of bacteria in the tar samples also represent the first description of biodegradative communities within concentrated organic-phase DNAPLs.

In this Chapter, the molecular tools previously developed during the Ph.D. project, were applied for the first time to allow a fast, accurate and precise extraction

procedure able to analyse concentrated organic-phase coal tars with CSIA. Furthermore, the output of high-throughput 16S rRNA gene sequencing was compared with CSIA results using statistical methods. Correlations between microbial processes and the isotope signature of PAHs were found for all of the samples investigated. This is especially evident when comparing dual plot of carbon and hydrogen CSIA with the microbial population obtained through 454-pyrosequencing (Appendix C). Samples characterised by a higher relative number of biodegradative classes of bacteria resulted to have all methylated PAHs heavily depleted in ^{2}H, while non-methylated "parent" PAHs were enriched of the heavier isotope ^{2}H. The data obtained from hydrogen CSIA represent a reference material that could be used for future work on biodegradation of both coal tar contaminated soils and sediments and organic matter in unconventional gas systems. In the light of the above considerations, a possible set of experiments that could help to elucidate the complex interactions between the microbial communities, could be the application of SIP (Stable Isotope Probing). A recommendation for future work that involve SIP is given in the Appendix D.

Chapter 7

Conclusion and recommendations

7.1. Restatement of the hypotheses

In the first part of this Ph.D. project, the relationships between microbial community composition, biogeochemical conditions and microbial methane generation in unconventional gas systems were investigated and the following primary hypotheses were identified:

1. The stimulation of microbial methane generation in unconventional gas systems is complicated by several biogeochemical factors such as the bioavailability of water and organic matter, redox conditions, pore size of the formation, temperature, pH, salinity, microbial competition, etc. However, biogenic methane is observed in several shallow, low temperature shale and coal formations.
2. The current state of research regarding the stimulation of microbial methane generation in extreme environment is poorly linked with commercial activities. Managing the microbial communities in unconventional gas systems might have implications for both recovery practices and a sustainable development of unconventional resources.
3. Culture-independent techniques could be developed and applied to analyse the microbial communities in shale and coal core samples.

In order to chemically and microbially characterise concentrated organic-phase coal tar, this dissertation addressed three primary hypotheses:

1. Microbial communities might be present in concentrated organic-phase coal tar.

2. If there is any microbial community in concentrated organic-phase coal tar, their structure could be analysed with culture-independent methods.
3. Microbial communities in concentrated organic-phase coal tar may slowly change the isotopic signature of hydrocarbons.

7.1.1. Summary of accepted and rejected hypotheses

The hypotheses identified for the chemical and microbial characterisation of shale gas and CBM were complicated by the difficulties in obtaining fresh shale/coal samples. However, preliminary experiments carried out on the Jurassic and Carboniferous shale samples provided by the BGS, as well as the extensive literature review of biogenic methane in unconventional formations partially accepted the three hypotheses identified.

1. The comprehensive review on microbial methane generation in shale gas and coal bed methane highlighted the occurrence of biogenic gas in shallow, low temperature shale/coal formations. Moreover, this work expanded our current knowledge on the biogeochemistry of the deep biosphere by providing other key parameters that need to be considered when looking for microbial life in such environments.
2. Our understanding of microbial processes in shale gas and CBM is still in its infancy. Consequently, the scientific community is poorly linked with industrial applications. The review carried out during this Ph.D. project highlighted and proposed new methodologies that could be applied to increase biogenic methane production, while promoting a sustainable development of unconventional resources.
3. The preliminary work carried out on the shale samples demonstrated that it is possible to use culture-independent techniques to analyse the indigenous microbial community in shale core samples without cross-contamination.

The hypotheses identified to investigate microbial processes in concentrated coal tar samples were accepted and, the methodologies developed during this work may be a significant catalyst for future studies in extreme environemts.

1. The new molecular techniques developed for the 16S rRNA gene extraction demonstrated that a very diverse microbial community is present in concentrated organic-phase coal tar.
2. The culture-independent analyses of the microbial communities in the tar samples were effectively analysed with culture-independent methods. The culturing approach is time consuming and fails to observe the complex structure of microbial communities.
3. The isogeochemical characterisation of the coal tar samples, carried out with multidimentional CSIA, demonstrated that the bacterial community is not capable to produce a shift in the carbon isotope signature. However, hydrogen CSIA showed a significant change in the isotope signature. Indeed, samples characterised by a higher relative number of biodegradative classes of bacteria resulted to have methylated PAHs heavily depleted in ^{2}H, while non-methylated "parent" PAHs were enriched of the heavier isotope ^{2}H. This isotope shift between methylated/parent compound is typical of microbial processes in the environment.

7.2. Restatement of the research objectives

The aim of this research is to investigate and expand experimental tools for the characterisation of extreme environments, using microbial, molecular, chemical and isotopic techniques. To achieve the aims of this project, the following research objectives were identified:

- To develop and verify a protocol to handle and process shale and coal core samples for microbial community analyses.

- To optimise bioinformatics tools for the analyses of high-throughput 16S rRNA gene sequencing.
- To develop analytical methodologies for the analysis of the microbial community structure in concentrated organic-phase coal tar (dense non-aqueous phase liquid, DNAPL).
- To develop a fast, accurate and precise extraction procedure to allow analysis of concentrated organic-phase coal tars by multidimensional CSIA without the need for complex fractionation processes.
- To develop a comprehensive method for the analyses of complex organic matter from coal tar using GC-MS and multidimensional CSIA, with focus on the 16 US priority PAHs;
- To compare the output of high-throughput 16S rRNA gene sequencing with CSIA results with statistical methods to identify potential correlations with microbial processes.

7.3. Summary details of key findings for Chapter 3 to 6

7.3.1. Biogeochemistry of shale gas and CBM

The extensive review of microbial processes in shale gas and CMB carried out during this research project highlighted several key factors that influence biogenic methane production in unconventional gas basins. Yet, our understanding of community dynamics in shale gas and CBM is far from complete, as well as the metabolic capabilities of syntrophs and methanogens. The review describes the occurrence of microbial methane generation in shales and coals, illustrating the analogies and differences of the two environments. Furthermore, the analysis of the biogeochemistry of the two environments was carried out, with a focus on biodegradation processes of complex organic matter. Lastly, the review describes the current knowledge about enhanced microbial methane generation in unconventional reservoirs and how existing engineering technologies can be applied to achieve optimum conditions for microbial methane generation.

The biogeochemical factors that affect microbial methane production in shale and coal can be summarised as follow:

- Availability of water.
- Redox conditions.
- Pore-size and pore-size distribution of the formation.
- Contact surface area of microorganisms to organic matter.
- The initial solubilisation of complex macromolecular compounds.
- Bioavailability of organic matter.
- Presence and bioavailability of trace elements.
- Community dynamics.

- Hydrogen production and consumption within the syntrophic bacterial community.
- Presence of sulphate and competition with sulphate-reducers or other microorganisms.
- Other factors such as pH, salinity, temperature, pressure etc.

Any shallow, low temperature shale gas basin and coal-bed represent an opportunity for microbial methane stimulation. Every shale and coal is different and unique for its geological, chemical and biological characteristics; hence any study for the *in-situ* stimulation of microbial methane production should consider the parameters listed above. Shale and coal are characterised by high content of aromatic and aliphatic hydrocarbon as well as many other heteroatoms, their chemical complexity requires the syntrophic association of several different types of bacteria and Archaea. The biodegradation of organic matter in shale and coal occurs independently of the rank of the coal or the type of kerogen, indicating that recalcitrance of higher complexity molecules is not a major limiting factor. Recent studies suggest that the limiting factor is to be found in the activation of complex molecules and in the biochemical interactions among the microbial communities involved in biodegradation of organic matter, rather than on environmental parameters. More efforts should be driven to the understanding of the complex interplay among the syntrophs and syntrophs/methanogens.

Moreover, the review of the biogeochemistry of unconventional gas systems individuate potential engineering technologies that can be used for the in situ stimulation of microbial methane production. Hydraulic fracturing is a well-established technology to increase the permeability of a formation: engineering approaches, including physical alteration of the shale/coal formation, should be applied *in-situ* to achieve or maintain favourable conditions for microbial activity.

7.3.2. Biogeochemical characterisation of shale gas

Despite the growing interest on microbial methane generation in organic rich sedimentary rocks, few studies focus on the biogeochemistry of shale. Microbial communities have been characterized for substrates such as coal formation waters or petroleum reservoirs production waters, but there is almost no data available for organic-rich shales. The research in this field has been done with two main methodologies: (1) the first is using geochemical indicators such as carbon and deuterium isotopes found in produced waters, methane and carbon dioxide to establish whether the gas is biogenic or thermogenic in origin. (2) The second is laboratory biodegradation enrichments using samples of coal to determine methane production rates.

All of these studies have examined the microbial population present in liquid samples, including fracking fluids, formation water and flowback, but none of these was specifically aimed to characterize the microbial population trapped in the solid matrix of the shale. From a microbiological point of view, it is very complicated and laborious to analyse a solid sample such as a shale rock/core, as the microbial population attached to the particles of shale and then trapped in the rock results difficult to be extracted and quantified. This is especially due to the small size of the pores and the nature of the rocks, which allow very low number of bacterial and archaeal cells, making the direct detection of methanogens nearly impossible. Also, the high concentration of organic compounds requires additional steps aiming to clean the samples from organic acids and inorganic particles.

Developing an efficient method for the detection and monitoring of the microbial consortia involved in the production of methane from organic-rich shales is a crucial step to evaluate methane generation potential. Bacteria and Archaea in this case can rarely be quantified directly, as a result of low initial numbers. Basically, the number of Archaea and Bacteria in the rocks is too low to allow direct enumerations by the most widely accepted methods such as self-fluorescence observations or qPCR assays.

During this Ph.D. project, a protocol ad-hoc to handle and process shale and coal core-samples was developed in order to minimize cross-contamination and preserve the microbial community. The protocol developed in this study was validated with culture independent methods and MPN experiments. The experimental procedure aimed to minimize cross contamination of the samples and was proved effective for the analysis of tight rocks. Due to the nature of the shale samples investigated, no indigenous microbial communities were found in the low porosity of the cores, demonstrating that these samples were not subjected to re-inoculum of microbial cells, that could happen through recharge of water or weathering processes. The complete absence of DNA, verified through molecular and culturing methods, demonstrate that the protocol used for the preparation of samples is effective in avoiding cross-contamination.

Moreover, the chemical analysis of the Carboniferous shales highlighted the presence of biodegradable organic compounds. The shale extracts contained biomarkers such as hopanes and terpenoids. Monomethylalkanes and alkylcyclohexanes, which are typical of microbial mats, have also been detected in this study. The biodegradation of aliphatic and cyclic hydrocarbons might be a source of metabolites such as fatty acids in coal and shale produced waters. All of these intermediates can potentially be further oxidized to methanogenic substrates. The organic composition of the Carboniferous samples can be considered a suitable substrate to study the dynamics of organic matter biodegradation. Although the absence of an indigenous microbial populations in the shale samples proved the effectiveness of the protocol developed, it represents a limit to study biodegradation rates. The addition of an external consortium might be a solution to overcome this limit, and can be used to evaluate bioaugmentation as a tool to enhance the microbial degradation of organic matter.

7.3.3. Culture independent methods for the analysis of the bacterial community structure in concentrated coal tar

A novel method for the analysis of bacterial community structure in concentrated organic-phase coal tar was developed and applied for the first time. To help support much needed studies on the natural attenuation and bioremediation of manufactured gas and coking works, this study developed and applied, for the first time, molecular characterization tools for the study of concentrated coal tar DNAPLs. The methodologies developed for the DNA extraction represent a simple and cost-effective step toward the molecular characterization of complex substrates such as DNAPLs and LNAPLs. The results of this study represent the first description of bacterial communities living in concentrated organic-phase coal tar. The microbial characterisation, carried out with high-throughput 16S rRNA gene sequencing, highlighted a very diverse bacterial community structure within the samples, suggesting that the main reason for a low microbial activity is the insufficient mixing of electron donors and acceptors necessary for bacterial activity.

The presence of bacteria in coal tar represents a significant expansion to the plume fringe concept, where it is assumed that biogeochemical gradients at the fringes of a contaminant plume facilitate hotspots of microbial activity. This concept has been demonstrated via geochemical analyses, molecular biomarkers and two-dimensional aquifer models using naphthalene. This study confirmed, through culture independent methods, that the presence of bacteria might not be a limitation to biodegradation within the concentrated organic-phase.

7.3.4. Isotopic analysis of coal tar

The carbon and hydrogen isotopic composition of PAHs in coal tar samples was investigated and used as a tool to trace microbial activity in extremely contaminated

environments. The majority of coal tar PAHs were found to carbon isotopic values in the narrow range of -24 to -28‰, thus it was difficult to establish whether or not the isotopic ratios were affected by biological activity. However, the variation of hydrogen isotopic values of PAHs metabolites suggests the potential contribution of biological reactions. A significant variation between unsubstituted PAHs and alkylated PAHs was found for all samples, in particular we observed an enrichment in ^{2}H for all unsubstituted PAHs, while metabolomic "daughter" alkyl-substituted PAHs were characterized by a more negative $\delta^{2}H$ value, typical of compounds subject to biodegradation.

The present study shows that coupling high-throughput 16S rRNA gene sequencing with metabolomic carbon/hydrogen isotope signature, is effective for assessing the biodegradation potential in contaminated environments. This study developed and applied molecular characterization techniques for the analysis of concentrated coal tar and described for the first time the bacterial community structure in these extreme settings. The CSIA carried out during this Ph.D. project also provided reference data for the compound specific hydrogen analysis of aromatic hydrocarbons in coal tar and demonstrated that coupling deep sequencing methods with metabolomics multidimensional CSIA is useful to trace microbial activity in highly contaminated soils and groundwater.

7.4. Conclusions

This thesis has presented the development and application of novel molecular tools for the analysis of extreme environments. The methodologies developed to isolate and analyse 16S rRNA from extreme environments (shale rock, DNAPL) provide a powerful tool that can be used to evaluate microbial mediation of gas production and wastes in unconventional gas systems. This microbial mediation approach can be applied to reduce environmental pollution encountered at former gas production sites and also to enhance the production of methane from shale gas and CBM deposits.

There are only few reports in the literature regarding shale reservoir geomicrobiology, highlighting the need for novel insight into guiding practical strategies for enhanced gas recovery and for mitigating undesirable microbial processes and environmental impact. The extensive review of the biogeochemistry of shale gas and CBM provide an improved level of information of these extreme environments. The investigation on methane extraction technologies also highlight potential use of hydraulic fracturing to support indigenous microbial communities and to favour a sustainable development of unconventional gas.

Culture-based and culture-independent techniques have been used to evaluate the microbial ecology of PAHs-degradation. However, culturing techniques greatly underestimates the diversity of the prokaryotic world and fails to account for the complex interactions of the members of microbial communities with each other and with their native environment. Culture-independent techniques can help us better estimate the prokaryotic diversity of complex systems, where it can be difficult to establish which organisms are responsible for the degradation of a particular contaminant.

The robust culture-independent methodologies investigated in this thesis for the analysis of extreme environments were evaluated also to assess the biodegradation potential of PAH-contaminated soils from FMGP sites in UK and USA. This thesis developed and applied, for the first time, molecular tools for the study of concentrated coal tar DNAPLs. We described the complex bacterial community structures adapted to extremely toxic substrates and demonstrated that the presence of bacteria in the concentrated organic-phase DNAPL does not represent a limit for bioremediation in-situ.

Furthermore, multidimensional CSIA of carbon and hydrogen elements of PAHs in coal tar DNAPLs were carried out, highlighting significant differences in the hydrogen isotope signature of unsubstituted- and alkylated-PAHs. This clear shift in the isotope signature is a strong indicator of biodegradation processes that can be

used as biomarker for monitoring in-situ bioremediation and also for source apportionment of coal tar.

However, the application of CSIA for biodegradation quantification in the field is subject to uncertainty, due to a number of factors that impact on isotope fractionation and the complexity of biological transformation in the field. It is critical, therefore, to ensure a conservative assessment of the in situ biodegradation and couple the application of multidimensional CSIA with molecular-biological methods for the identification of dominant microorganisms.

Multivariate statistical analyses using principal component analysis and hierarchical cluster analysis were also used to observe relationships among the coal tar DNAPLs and to determine the degree of differentiations between the bacterial community structure and the carbon/hydrogen isotope signature of the 16 US EPA priority PAHs. Metabolomic multidimentional CSIA and high-throughput 16S rRNA gene sequencing highlighted substantial relationships between the bacterial community structure and carbon/hydrogen isotope signature.

Furthermore, the combined approach has the potential to assess biodegradation rates even for complex contaminants such as coal tar DNAPLs in eterogenoeus groundwater systems.

7.5. Recommendations for future work

Drilling operations are spoiling many shale gas basins, causing a decrease in biogenic methane production due to the introduction of a negative microbial population that is capable to outcompete useful biodegradative bacteria and methanogenic Archaea. Besides affecting methanogenesis, negative microorganisms that are present in unconventional gas fields cause a number of other problems that lead to significant costs for the oil and gas industries. It is vital, therefore to understand community dynamics in extreme environments.

Microbial ecology offers a logical framework for analysis of the feedbacks and interconnections between the physical, chemical, and biological features of an environment. A detailed understanding will require documentation of the makeup of biological communities, determination of the ways in which community members interact at the biochemical level, the nature of system inputs (e.g., nutrients, electron donors/acceptors) and outputs (e.g., CO_2, CH_4), and the ways in which organisms modify and respond to changes in their environments. For example, microbial cells may compete for limited resources with the other members of their community; they may supply nutrients to the system by breaking down a substrate that then becomes available to other organisms; they may generate toxic metabolic by-products that restrict the growth of other members of their community; and they may influence the genetic program of their neighbours by sending out molecules that control gene expression. In modelling ecological networks, it is not only important to characterize these interactions, but it is essential to have a metric for monitoring the overall state of the system.

A promising approach for studying ecological complexity in extreme environments, might be to model a simple community in the laboratory, where species composition and environmental conditions may be strictly controlled. A starting point for such an analysis might be to study the interactions of a mixed community of biodegradative bacteria under different environmental conditions (e.g., varying electron acceptors, carbon limitation and composition of the surface).

This approach has obvious limitations: it is necessary to consider where, in the real world, we may encounter relatively simple microbial ecosystems that might be tractable. The extreme environments found within reasonably isolated, subsurface unconventional gas systems provide a target. Here, the biological complexity of communities is reduced to a limited number of organisms. A starting point for development of methods for field analyses is to use laboratory-scale bioreactors to create and test biogeochemical models for unconventional systems. In these systems, the geochemistry of the input and outflow solutions can be measured over time.

Changes in the community size, structure, and composition and levels of expression of genes (selected using community genomic information) as a result of system perturbation can be monitored with the use of oligonucleotide probes and microarrays.

Microbial communities in extreme environments are subject to a number of factors (e.g. nutrients, water/organic, pressure and temperature) that could negatively affect their performance and dynamics. Moreover, extraction engineering technologies (e.g. fracking, coal gasification, remediation) could disrupts the stasis of natural microbial systems. It is critical, therefore, to explore the impact of operations such as shale and coal gas extraction on community function and structure to ensure that new engineering infrastructure or enhancements support in-situ microbial communities, and in turn support the function we wish them to undertake (e.g. continued or enhanced methane production, or bioremediation).

To the author's knowledge, there are only few reports in the literature of hydrogen isotope composition of shale rocks and coal tars. This Ph.D. project provided reference data on the isotope signature of PAHs in coal tar DNAPLs. However, hydrogen isotope analysis is a less robust method if compared with carbon CSIA: the combination of carbon and hydrogen isotope data with culture independent methods (high-throughput 16S rRNA gene sequencing) is a powerful approach that can be used in situ to trace microbial activity over time. Consequently, field studies are required to provide further information that can help to understand community dynamics. The results of these analyses may generate new understanding of relations between substrate diversity, species diversity, partitioning of function, and energy and materials flow.

Stable-isotope probing (SIP), a new technique in microbial ecology, is used for tracing fluxes of nutrients in biogeochemistry. SIP consists in the enrichment of a substrate with a heavier stable isotope that will be consumed by the target microorganisms. SIP can then be coupled with the molecular tools developed during this Ph.D. and used to identify microorganisms involved in the metabolism of

complex substrates such as shale rocks and DNAPLs. With the findings of this Ph.D. project, a natural future plan of research could be to carry out SIP experiments in both shale gas and coal tar contaminated environments. Field experiments using SIP could identify populations of PAH-degraders and heterotrophs in the bacterial community and follow changes in their diversity as well as analyse the biodegradation activities of the bacterial community upon selected PAHs. Multidimensional CSIA could also be used to assess the biodegradation rates through changes of the carbon and hydrogen isotope signature of PAHs undergoing biodegradation. Further details regarding a proposed research on microbial ecology of contaminated plumes can be found in the Appendix D.

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Appendix A

Appendices to Chapter 3

A1. Section of a shale core

Figure A1. Section of a shale core.

A2. MPN experiments

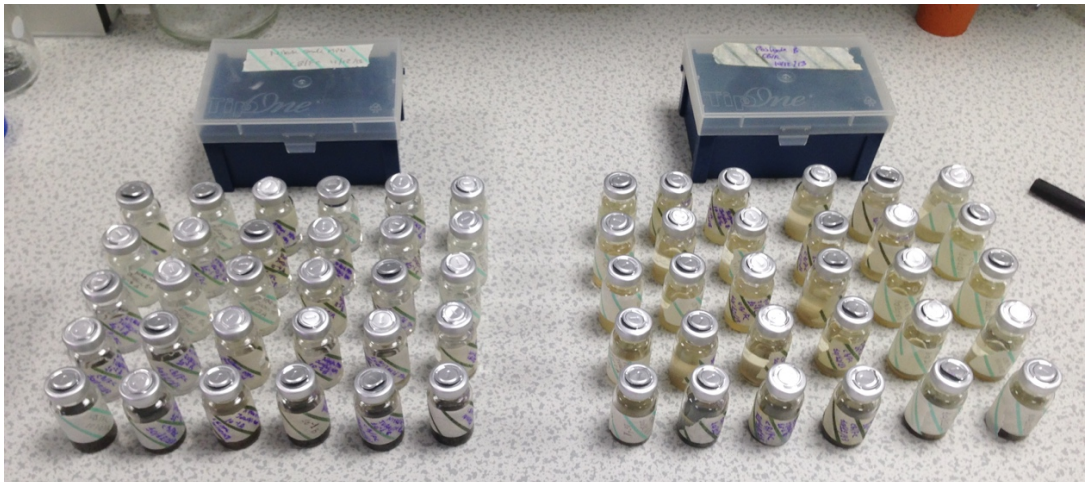

Figure A2. MPN experiments using media for NRB (left) and media for SRB (right).

A3. MPN experiments

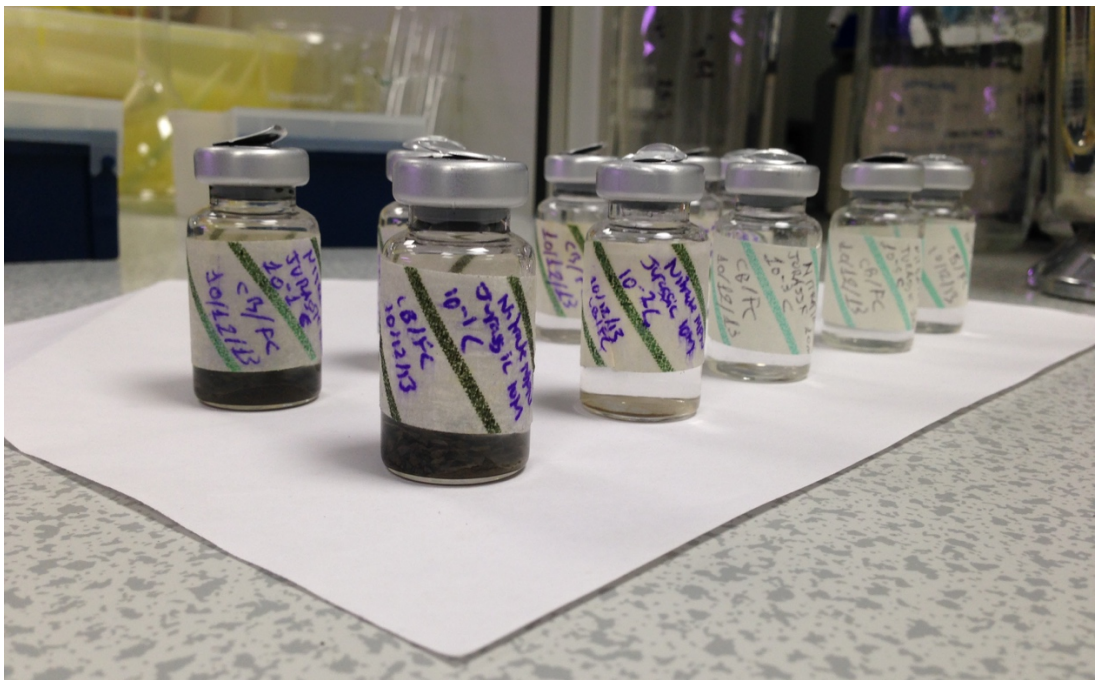

Figure A3. MPN vials showing the small chips of shale in the first fraction.

A4. Dionex ASE 350

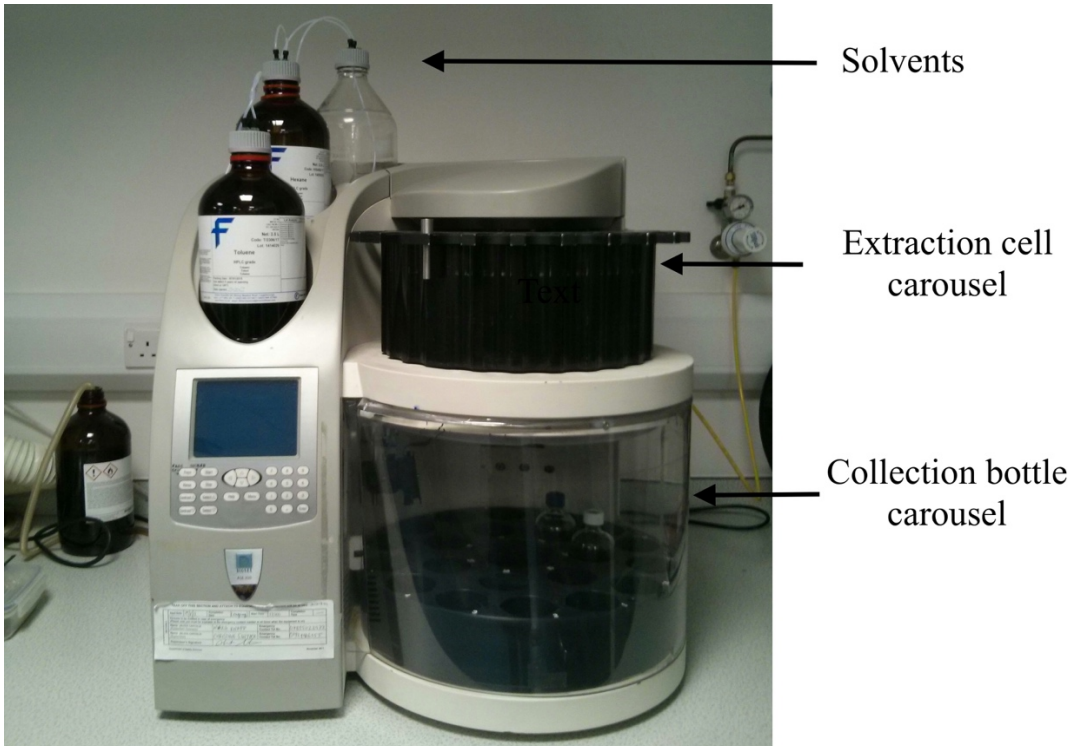

Figure A4. Dionex ASE 350 accelerated solvent extraction system.

A5. ASE extracts

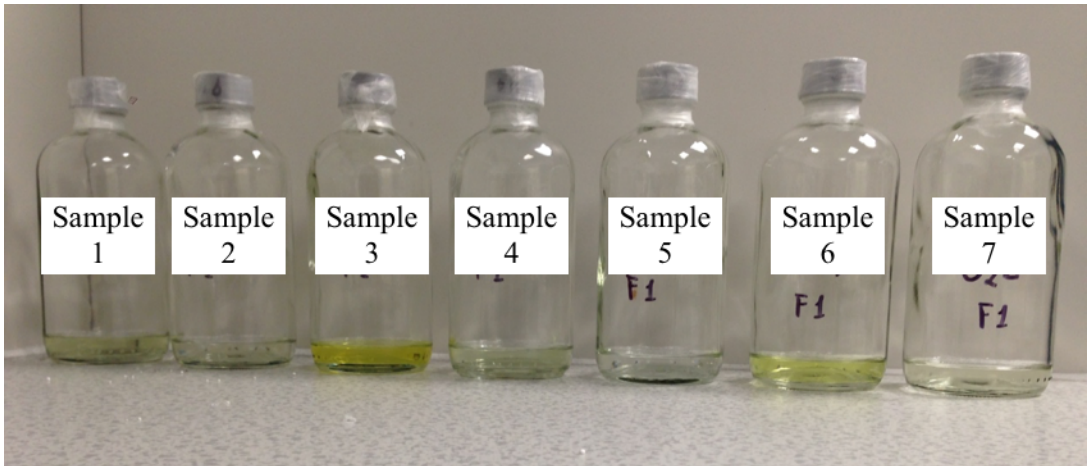

Figure A5. Example of coal tar ASE extracted samples.

A6. Büchi Syncore® Analyst

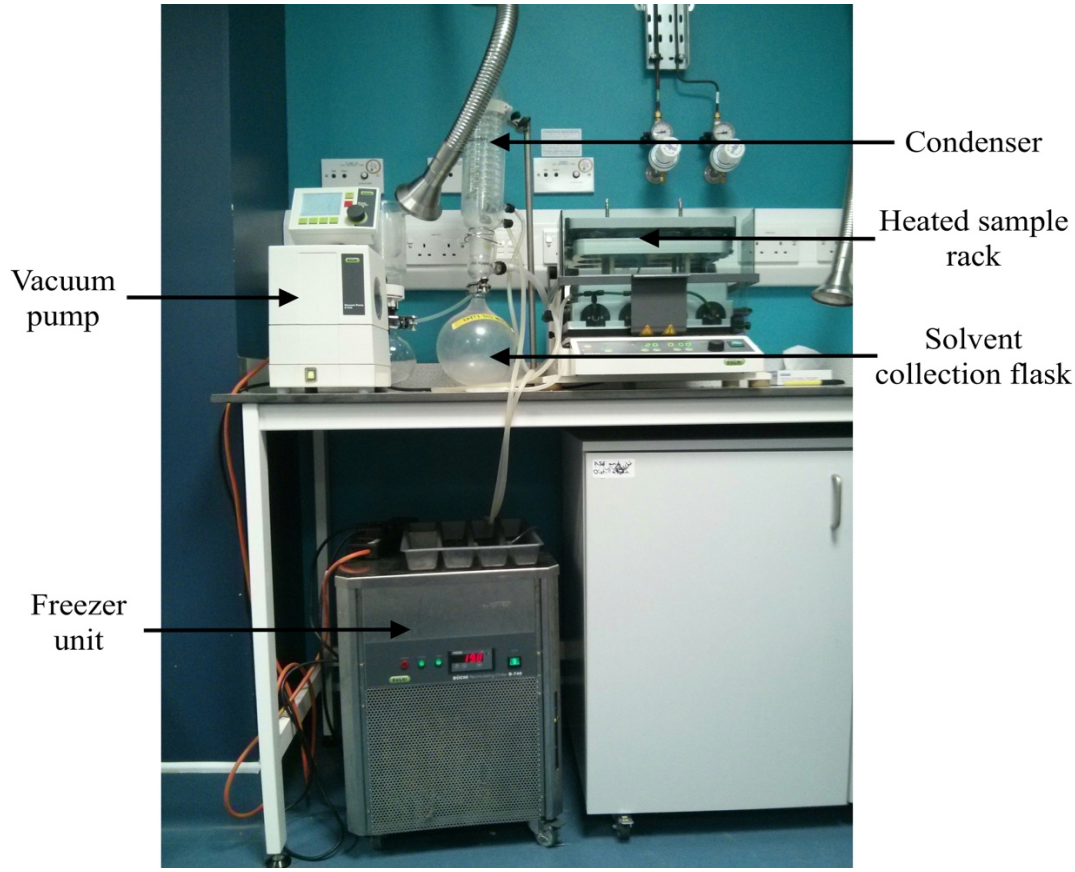

Figure A6. Büchi Syncore® Analyst polyvap system.

A7. Thermo Scientific Trace GC-MS

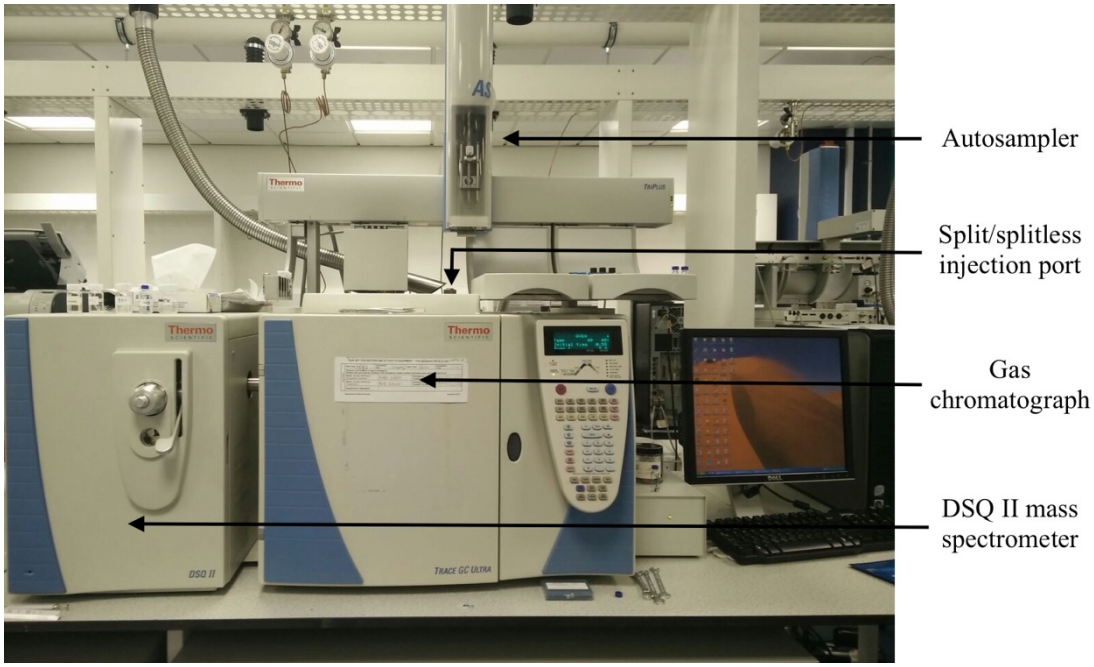

Figure A7. Thermo Scientific GC - DSQ II Quadrupole MS.

A8. Thermo Scientific GC Isolink Delta IV IRMS

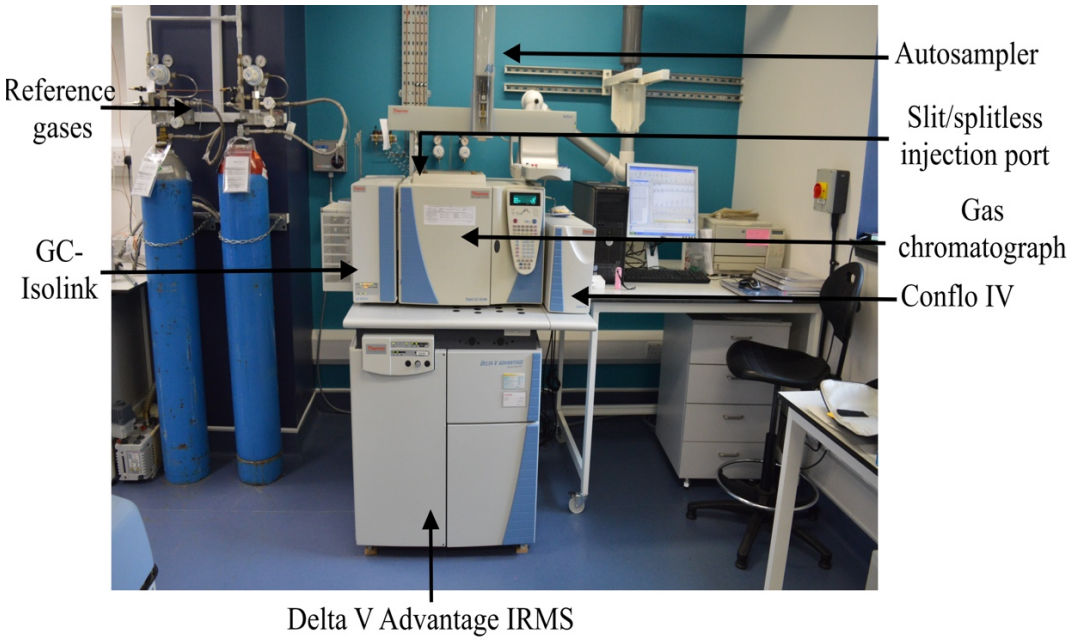

Figure A8. Thermo Scientific GC Isolink Delta IV IRMS equipped for GC-C-IRMS and GC-TC-IRMS analyses.

A9. Microbiological Safety Cabinet Class II

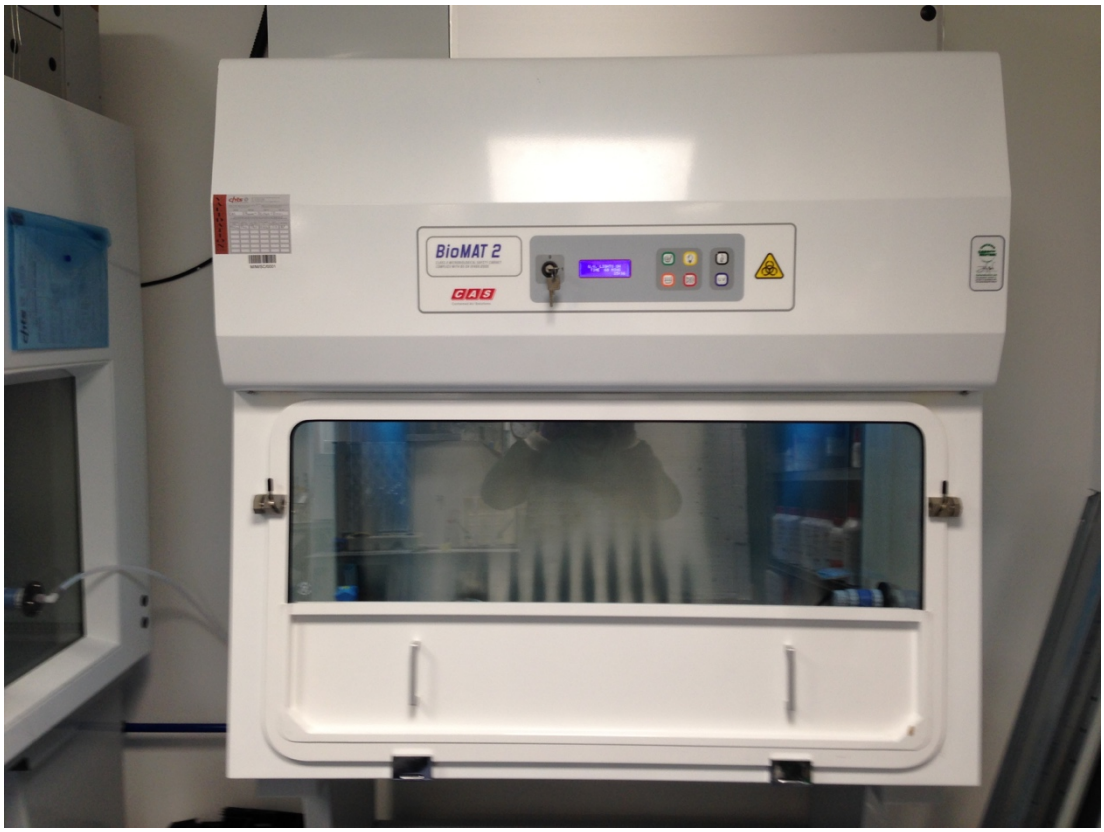

Figure A9. Microbiological Safety Cabinet Class II used for the preparation of coal tar samples.

A10. Electrophoretic gel of shale samples

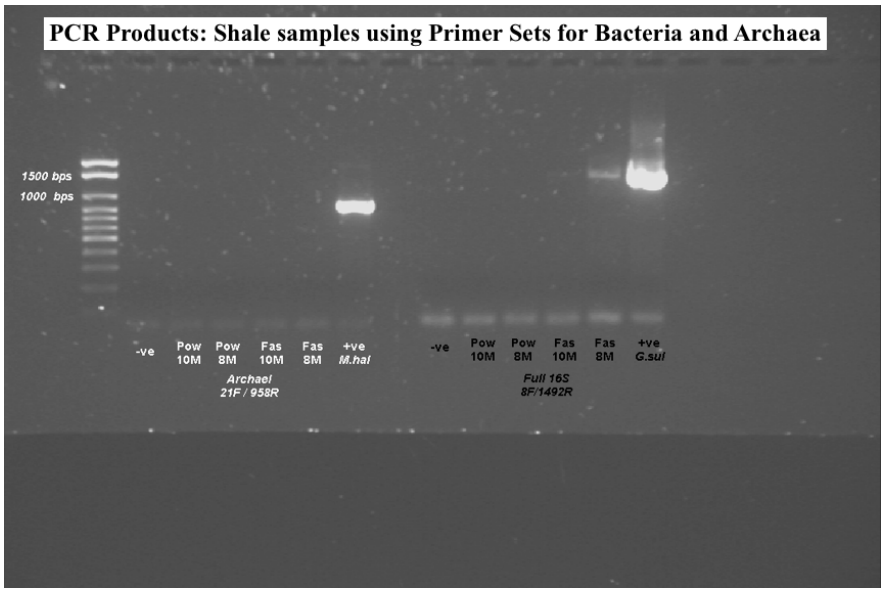

Figure A10. 16S rRNA profile of bacteria and archaea of the shale samples.

A11. Electrophoretic gel of coal tar samples

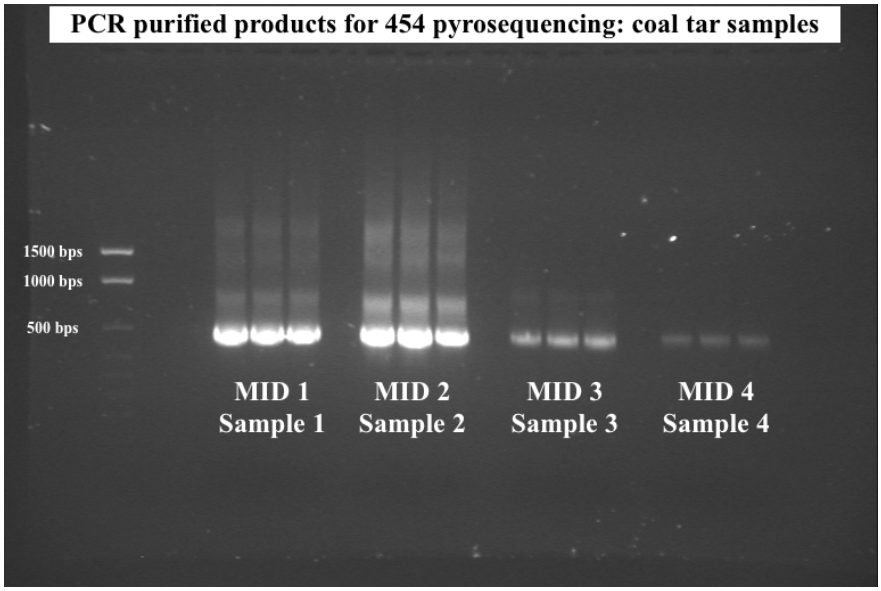

Figure A11. 16S rRNA bacterial profile of the coal tar samples for 454 pyrosequencing analysis: MID 1 to 4.

A12. Electrophoretic gel of coal tar samples

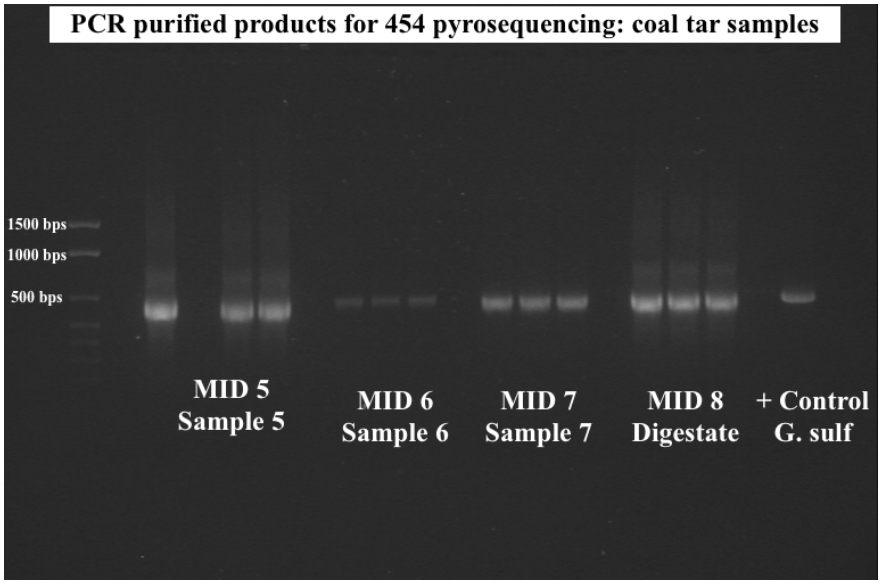

Figure A12. 16S rRNA bacterial profile of the coal tar samples for 454 pyrosequencing analysis: MID 5 to 8.

A13. Chloroform/Phenol rRNA extraction

Fabrizio Colosimo, 2014

This method can be used to extract and purify total nucleic acids from soil/sediment samples, plant parts, bacterial or yeast cultures and fungal mycelia. The method below details the approach for soil or sediment and is a modification of the method of Griffiths et al. (2000).

Materials

1.5 mL eppendorf tubes
Filter tips
0.1 mm glass beads & 0.5 mm zirconia/silica beads (0.5 g each) in 2 mL screw-top microcentrifuge tubes
Modified CTAB extraction buffer
Equal volumes of 10% CTAB in 0.7 M NaCl and 240 mM potassium phosphate buffer [pH8]
Phenol (pH>7.8):chloroform:isoamylalcohol (25:24:1)
Chloroform:isoamylalcohol (24:1)
PEG/NaCl solution
    1.6 M NaCl
    30% (wt/vol) polyethylene glycol 6000
Sterile MilliQ H_2O
70% EtOH
Tris-EDTA pH7.4

Preparation

* All vessels and reagents should be DNAse/RNAse free, or treated with DEPC to denature proteins.
* DEPC treatment:
    * Solutions: Add DEPC to a final conc. of 0.1% and incubate overnight at 37°C, then autoclave.
    * Equipment: Soak in a DEPC solution (0.1% in H_2O) overnight at 37°C and autoclave.
* Cool centrifuge to 4°C.

Methods

1. Add 0.5 g soil to 2 mL screw-capped microcentrifuge tubes containing 0.5 g each of 0.1 mm glass and 0.5 mm zirconia/silica beads. Add 0.5 mL of modified

CTAB (hexadecyltrimethylammonium bromide) extraction buffer and vortex briefly.

2. Add 0.5 mL phenol:chloroform:isoamylalcohol (25:24:1) and shake tubes in the FastPrep Instrument (Qbiogene) at 5.5 m/s for 30 s.
3. Following bead-beating, centrifuge tubes at 16000 x g for 5 min at 4°C.
4. Remove the aqueous top layer to a new tube and add an equal volume of chloroform:isoamylalcohol (24:1). Mix tubes well and centrifuge at 16000 x g for 5 min at room temperature. Remove the aqueous top layer into a clean 1.5 mL microcentrifuge tube.
5. Add 2 volumes PEG/NaCl precipitate solution and incubate at room temp for 2 hours.
6. Spin at 18,000xg at for 10 min at 4°C. Wash pelleted DNA/RNA in ice-cold 70% EtOH and spin again at 18,000xg at for 5 min at 4°C. Dry pellet in vacuum desiccator for about 5 minutes (do NOT totally dry). Re-suspend pellet in 50 µL DNAse/RNAse-free TE (pH 7.4 or 8.0) if using the Qiagen kit for further purification. Alternatively, re-suspend in DEPC treated water.

If a maximum yield of both DNA and RNA is required from the sample, or if the sample contains high amounts of organic matter (yellow to brown pellet) then proceed with a Qiagen purification. If sample is low in organic matter then treat re-suspended pellet with either DNase or RNase to remove unwanted fraction.

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Appendix B

Appendices to Chapter 5

B1. Coal-bed methane produced water: biodegradation experiments

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Thursday 9 October 2014

1. Introduction

Coal-bed methane (CBM) produced water differs from conventional oil and gas produced waters by the way it is generated, its composition, and its potential impact on the environment. In coal formations, methane is adsorbed onto the crystal surfaces of coal due to the hydrostatic pressure of the water contained in the coal beds: the reservoir pressure in the coal seam must be reduced in order to extract methane. CBM produced water is generated when the water that permeates the coal beds is removed. In contrast to conventional oil and gas production, the produced water from a CBM well usually comes in large volumes in the early stages of production.

Similarly to shale gas produced water, typical treatments of CBM produced water include re-injection or decontamination prior to the discharge.

Unconventional gas, such as CBM and shale gas, is becoming an important source of energy. Microbial methane generation has been observed in many shallow, low temperature coal seams and shale gas basins. The depletion of conventional resources and the increasing demand of natural gas for human consumption have spurred the development of unconventional gas resources in many parts of the world. Such unconventional systems represent the opportunity for the stimulation of microbial methane generation: hydrocarbons generation can be greatly enhanced, contributing to maximising production. Biogenic methane in coal and shale is produced by anaerobic biodegradation of organic matter: methanogenic *Archaea* represent only the final step of microbial methane generation. Several communities of microorganisms are involved in the biodegradation of organic matter: from the initial breakdown of high complex molecules to the production of intermediate compounds used by methanogens.

Three main methanogenic pathways have been reported to occur in coal and shale formations. These include *hydrogenotrophic* (CO_2-reduction, Eq1), *acetoclastic* (acetate utilisation, Eq2), and *methylotrophic* (methanol utilisation, Eq3) pathways, as represented by the following equations (Zinder, 1984 and 1993).

(1) $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ ΔG^o ¼ −131 kJ/mol

(2) $CH_3COOH \rightarrow CH_4 + CO_2$ ΔG^o ¼ −36 kJ/mol

(3) $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$ ΔG^o ¼ −106:5 kJ/mol

2. Objectives

The overriding criterion of the research project is to link the novel unconventional gas industry with the well-consolidated bioremediation technologies. The investigation of biodegradation processes in shale and coal is a key issue in determining the pathways that lead to methane production, and should provide valuable information for the design and optimization of stimulation strategies to increase production. Studying the biodegradation processes in unconventional gas

system also provide guidance for risk assessment and the effective planning of decontamination treatments.

The two overlapping objectives of the research are:

1. Development of strategies for the enhancement of unconventional gas production;
2. Development of effective decontamination treatments for shale and coal produced water.

A combined approach using organic geochemistry analyses and molecular community assessments will be used to achieve the objectives of the research.

3. Microcosms set up

Microcosm experiments provide a way to study microbial interactions with contaminants in a controlled and reproducible way, while retaining the complexity of the matrix and being representative of the processes occurring in the field. Laboratory microcosms allow the measuring of biodegradation as well as methanization (CH_4 production) and mineralization (CO_2 production) rates. Microcosm experiments are widely used to study the effect of bioremediation treatments, including biostimulation and bioaugmentation.

Microcosm experiments will be made in triplicate using 100 mL serum bottles, capped with Teflon faced butyl stoppers and aluminium crimps. The incubations will be made by adding 20 mL of produced water and 7-10 g of coal/shale to the serum bottles; headspace to solution ratio of 1:1, and a weight/weight ratio of solution to coal of about 10:1; gas phase with a mixture of N_2/CO_2 with a ratio of 80:20.

All activities, such as coal/shale and produced water handling, weighing, solution preparation, and sealing of microcosms will conducted within the anaerobic cabinet to minimize exposure to oxygen. All glassware, containers and tools to be used for microcosms preparation will be autoclaved to avoid microbial contamination.

Two sets of microcosms with four conditions each will be prepared in triplicate. (1) The first set will be prepared with coal sediments and coal produced water, (2) the second set will be prepared with shale sediments and coal produced water.

1st set: coal

1a *Untreated:* Coal and produced water only.

2a *Biostimulation:* Coal, produced water, and nutrient amendment.

3a *Bioaugmentation:* Coal, produced water and an external inoculum.

4a *Biostimulation/bioaugmentation:* Coal, produced water, nutrient amendment and an external inoculum*.

2nd set: shale

1b *Untreate*d: Shale and produced water only.

2b *Biostimulation:* Shale, produced water, and nutrient amendment.

3b *Bioaugmentation:* Shale, produced water and an external inoculum.

4b *Biostimulation/bioaugmentation:* Shale, produced water, nutrient amendment and an external inoculum*.

*The external consortium consists of a digestate sample from Howdon (New Castle, UK) anaerobic digester (CAMBI system).

An explanation of the different amendments chosen for this study is given below:

1. Treatments 1a and 1b are intended to investigate the natural processes occurring in the well, and weather the indigenous populations is able to grow with coal/shale organic matter as the only carbon source (respectively treatment 1a and 1b). These untreated microcosms are suitable to evaluate the natural attenuation potential of the indigenous microbial population.
2. Treatments 2a and 2b aim to assess if an additional carbon source can enhance methane production as well as the biodegradation of contaminants in the produced water.
3. Treatments 3a and 3b evaluate the possibility of enhance biodegradation and methanization by adding an external biodegrading inoculum.
4. Treatment 4a and 4b are a combined approach to evaluate the effect of biostimulation and bioaugmentation. By adding a supplementary carbon source and a microbial consortium, a synergistic effect on biodegradation and methane production should be observed.

The nutrient amendments have no carbonaceous constitutions but include basic chemical macronutrients (e.g., ammonium and phosphate) and chemical micronutrients (e.g., vitamin mix and trace elements) which are similar in composition as reported in Green et al., (2008) and Fallgreen et al., (2013). Each microcosm will be capped with Teflon faced butyl stoppers and aluminium crimps. The microcosms will be incubated (static) at 21°C in the dark to prevent any photochemical effects. Each microcosm headspace gas will be sampled to monitor CH_4 and CO_2 composition. Liquid phase will be sampled on a weekly base for chemical analyses. Microbial samples will be collected at the end of each experimental run.

4. Analytical methodology

4.1. Ionic-chromatography

The concentration of important ions in the liquid phase, such as SO_4^{2-} and NO_3^- will be measured using a Metrohm 850 Ion chromatography system. Data processing will be performed using the Chromeleon 6.8® chromatography data system.

4.2. TOC analyses

Total organic carbon (TOC) will be analysed using the Apollo 9000 TOC Analyzer, integrated with high throughput autosampler and equipped with combustion chamber (680°C to 1000°C) in total carbon (TC) and non-purgeable organic carbon (NPOC) mode. Water sample will be homogenized, decanted and filtrated with a Whatmann filter no.40 prior to carbon analysis in order to remove any samples containing visible particulate matter of non-dissolved matter. The filtered water will be then diluted to a 1/100th fraction using deionized water (DI). The filtered water will be added to glass vials (pre-cleaned and furnaced at 450°C).

4.3. Headspace analyses

Methane concentration in the headspace will be analysed on a weekly basis using a GC-FID.

4.4. Gas chromatography-mass spectrometry (GC/MS) analysis

Two aliquots (500 mL) will be withdrawn from the sample containers for GC/MS analysis. Before decanting of the two aliquot samples, the jar will be shaken to achieve homogeneity. The aliquots will be extracted four times with 25 mL volumes of chloroform. The organic phase will be filtered through anhydrous sodium sulphate. The chloroform extract will be transferred to an evaporation vessel. The vessel will be cooled to 0°C and the solvent concentrated under a stream of nitrogen over 24h. The concentrated extract will be re-diluted with chloroform and transferred to a GC-MS vials for analysis.

GC-MS analyses will be carried out using a Thermo Scientific (Hertfordshire, U.K.) Trace Ultra GC fitted with a DSQII quadrupole mass spectrometer and Triplus autosampler. Helium (BOC Ltd., 99.999% purity) as the carrier gas at a flow rate of 1 mL/min. A J&W Scientic DB-5 column with dimensions 30 m x 0.25 mm id x 0.25 μm, lm thickness will be used for all GC-MS analyses. The oven temperature will be programmed as follow: 60°C for 3 min, 60°C to 80°C at a rate of 1°C/min, then 80°C to 230°C at a rate of 5°/min, 230°C to 310 at 10°C/min, followed by an isothermal period of 8,50 at 310°C.

Data processing will be performed using the Xcalibur® software (Thermo Scientific Corporation, Massachussetts, USA). The mass spectra of unknown organic compounds will be compared against those within the National Institute of Standards and Technology (NIST) Mass Spectral Library (Gaithersburg, MD, USA).

4.5. Molecular analyses

The PowerWater® and PowerSoil®DNA Isolation Kits (Mo Bio Laboratories, USA) will be used to extract bacterial DNA from 0.1g of shale/coal samples. Universal bacterial primers 8F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3′) (full 16s rRNA) will be used to amplify the DNA extracts to quantify total bacterial population. The archaeal populations will be

screened using the 109F (5′-ACK GCT CAG TAA CAC GT-3′) and 915R (5'-GTG CTC CCC CGC CAA TTC CT-3') primers.

One microliter of each DNA preparation from the samples will be amplified in polymerase chain reaction (PCR) mixture (50 μL). Each PCR mixture contains 45 μL of PCR MegaMix, and 2 μL of each primer.

PCR conditions will be set as follow for the full 16S rRNA amplification of bacteria and *Archaea*: an initial denaturation step at 94°C for 4 minutes, followed by 30-35 cycles of melting (94°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 1.5 min). The final extension will be achieved with the last step (72°C for 5 min). 10 μL of PCR products will be visualized by electrophoresis in agarose gels (10 g/L agarose, add 0.2 mg/L ethylene bromide). Electrophoresis at 80 mV for 70 min. PCR products containing strong bands will be purified using a commercial kit and stored at -20°C. Cleaned products will be ligated for cloning and, the ligation products will be sent for sequencing.

5. Outcomes and benefits

The benefits of the proposed research project, which brings together the Unconventional Gas communities with the Contaminated Land communities (for which there is considerable mature science), will be to develop solutions for CBM and shale gas operations. The ultimate translation of a huge body of scientific knowledge on contaminated land bioremediation to the Unconventional Gas industry will provide tangible benefits and the potential for development of new partnerships across the world.

Data acquired from our biodegradation studies will provide an example of how to translate knowledge from Contaminated Land remediation to proposed mechanisms by which microbes activate biodegradation of coal-gas contaminants to methane. Furthermore, the proposed research project will help to achieve a continued protection of groundwater resources and supplies, the facilitation of responsible CBM and shale gas development and consistency with current regulations about water quality.

The proposed research project will provide also:

- Consistent protocols for sampling and analysing produced water from CBM and shale gas wells;
- Scientific information to support achievement of the outcomes;
- A regulatory basis for water well testing and baseline data collection for CBM and shale gas development.

Further putative outcomes of the research might include the development of more sensitive methods for the identification of prokaryotes in coal-bed methane. In addition, by characterising the microbial population in CBM wells, we aim to limit the presence of non-desirable bacteria in the formation, well and associated facilities (i.e. SRB which negatively affect methanogens and causes HS souring problems for the infrastructure).

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Zinder, Stephen H., and Markus Koch, 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. *Archives of Microbiology,* 138.3: 263-272.

Zinder, S., 1993. Physiological ecology of methanogens. *Methanogenesis*, pp.128–206.

B2. Coal-bed methane produced water: sampling procedures

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Sampling guidelines

In general, collection of samples should include steps to prevent contamination and to preserve the samples for later analysis; therefore, it is essential to collect samples as near the wellhead as possible and to isolate them from the atmosphere (minimal or no headspace in the glass containers).

When collecting a water sample for microbial and chemical analyses, it is important to sterilize each tool and glassware by (1) autoclaving or (2) combusting at 450°C to prevent chemical and/or biological contamination. If sterilization is not possible, rinsing the glass containers with solvents is a valid alternative.

The glass containers would be best if conditioned by rinsing with the formation water three times before the samples are collected. If the amount of produced water is too low to allow conditioning of the glass containers, the samples can be collected directly.

If biocides are injected into the well, it would be best to stop the addition few days before sampling when possible.

Apparatus:

1. Carboy, ~ 5L (or more), with bottom spigot;

2. Amber glass bottles, 1L with Teflon lined screw cap;
3. Tygon Tubing, and fittings for wellhead;

Water samples should be collected directly from active well-heads. Sample containers should be filled to the top so that minimal to no headspace is present. It is important to eliminate the headspace, since oxygen can be deleterious for both microbial and chemical analyses. If a minimal headspace is present, the sample containers are best stored and shipped upside-down, to minimize the exchange of gas between the headspace and the external environment.

Storing and shipping

Produced water samples should be kept at 4°C and packed prior to shipment using the following procedures (modified from US EPA guidelines, Art Masters, 2011):

1. Minimal or no headspace in all bottles. If present turn the bottles upside down;
2. Ensure that the lids on all bottles are tight (will not leak);
3. Label all bottles with water- and solvent-proof label. If not available cover the label with clear tape;
4. Place bottles in separate and appropriately sized polyethylene bags and seal the bags;
5. Select a cooler, then secure and tape the drain plug with fiber or duct tape inside and outside. Line the cooler with a large heavy duty plastic bag;
6. Place cushioning/absorbent material in the bottom of the cooler and then place the containers in the cooler with sufficient space to allow for the addition of cushioning between the containers;
7. Put "blue ice" (or ice that has been "double bagged" in heavy duty polyethylene bags properly sealed) on top of and/or between the containers. Fill all remaining space between the containers with absorbent material;

8. Securely fasten the top of the large bag with tape (preferably plastic electrical tape);
9. Close the cooler and securely tape (preferably with fiber tape) the top of the cooler shut. Chain-of-custody seals should be affixed to the top and sides of the cooler within the securing tape so that the cooler cannot be opened without breaking the seal.

References

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Appendix C

Appendices to Chapter 6

C1. Multidimensional GC-C-IRMS and GC-TC-IRMS analysis of coal tar samples

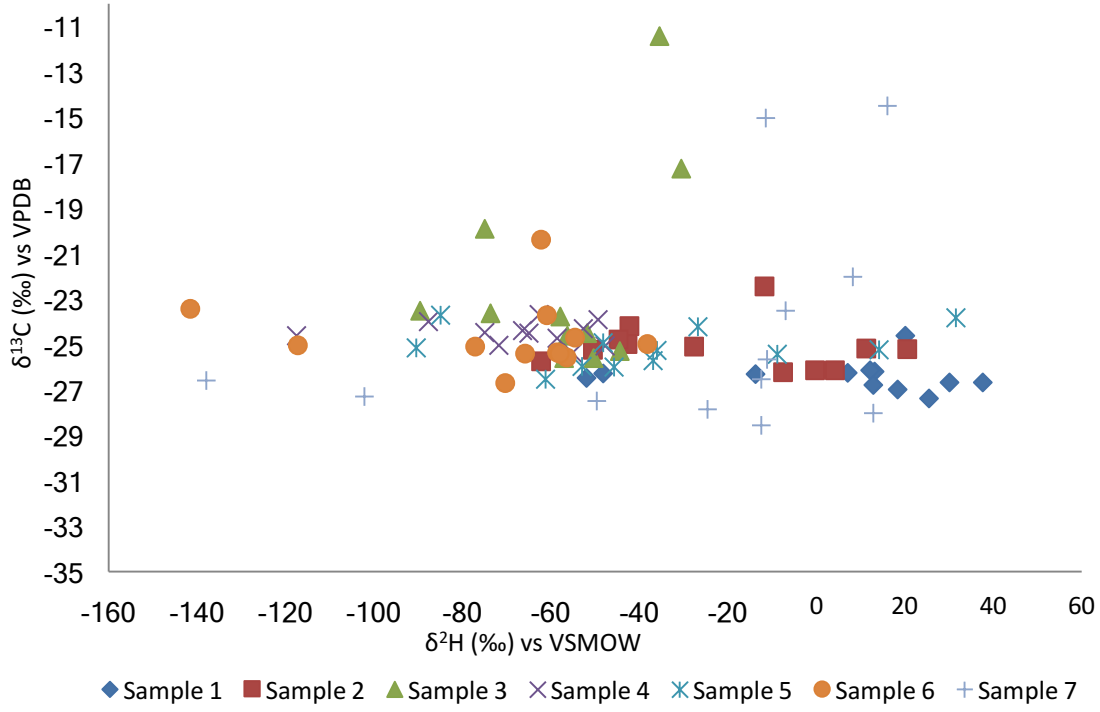

Figure C1. Dual isotope plot of $\delta^{13}C$ versus δ^{2} of selected PAHs for all samples tested in this study.

C2. Comparison of dual plot carbon and hydrogen isotope analysis with 454 pyrosequencing of coal tar S-I

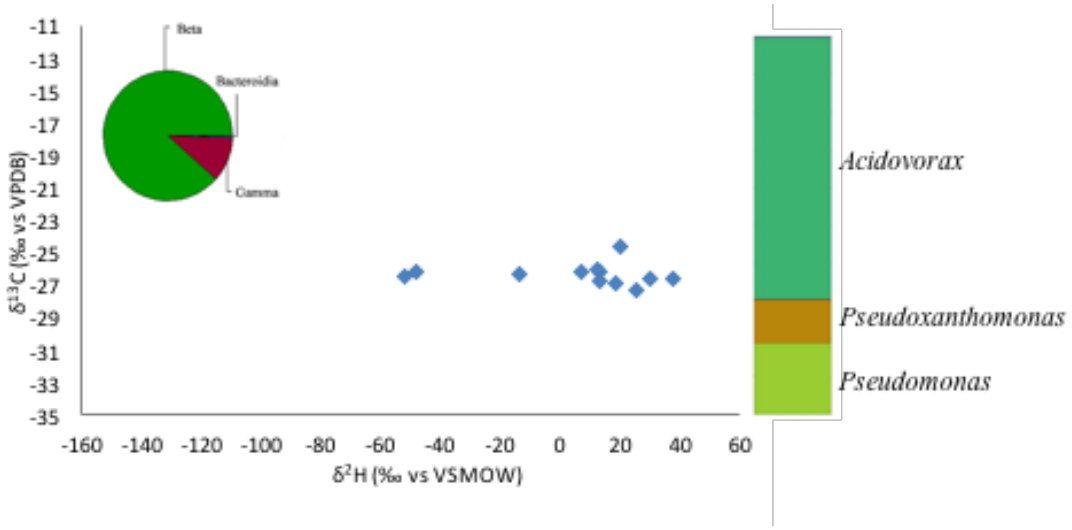

Figure C2. Dual plot showing carbon and hydrogen isotope ratio of S-I (within the 16 priority PAHs, only 12 were found above the analytical uncertainties). Pie chart showing taxonomy on a class level, bar chart on the right showing bacteria genera from pyrosequencing results

C3. Comparison of dual plot carbon and hydrogen isotope analysis with 454 pyrosequencing of coal tar S-II

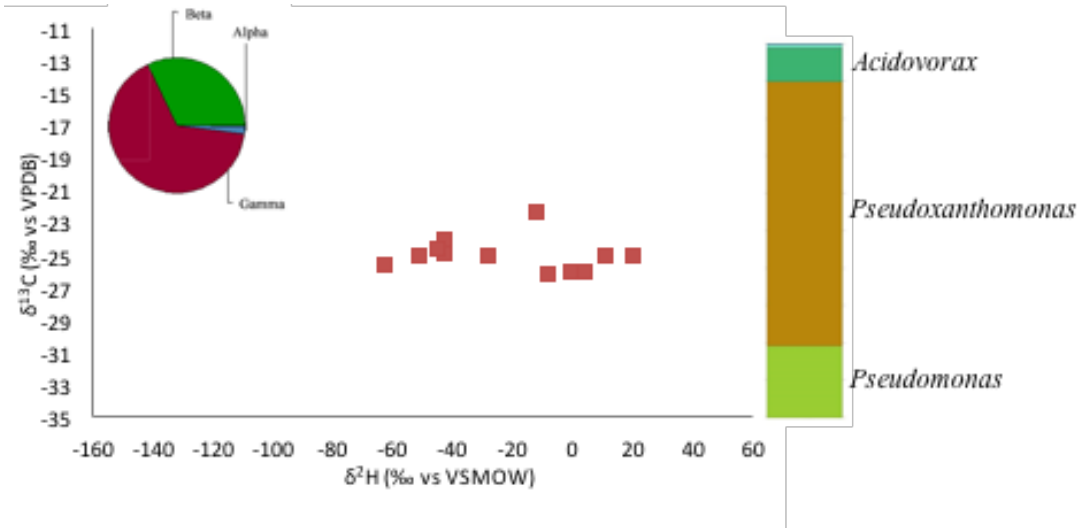

Figure C3. Dual plot showing carbon and hydrogen isotope ratio of S-II (within the 16 priority PAHs, only 12 were found above the analytical uncertainties). Pie chart showing taxonomy on a class level, bar chart on the right showing bacteria genera from pyrosequencing results.

C4. Comparison of dual plot carbon and hydrogen isotope analysis with 454 pyrosequencing of coal tar S-III

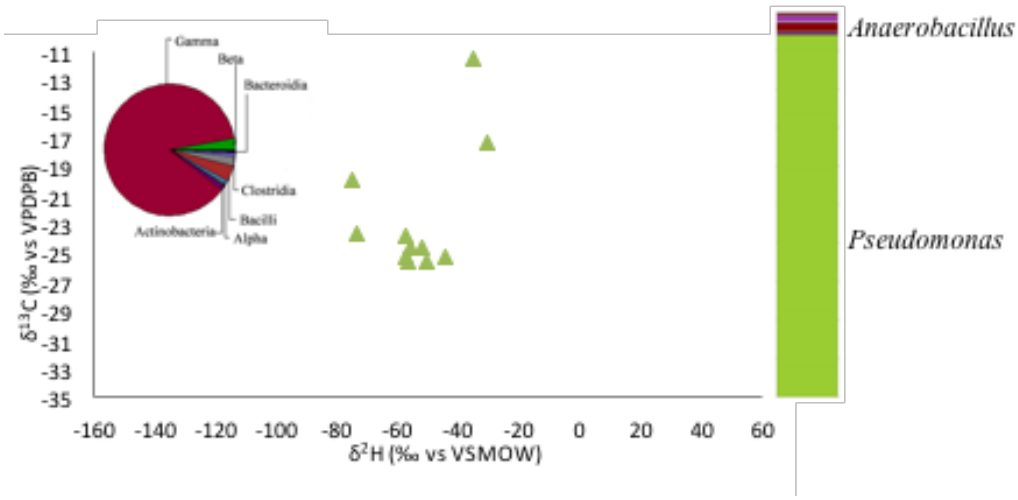

Figure C4. Dual plot showing carbon and hydrogen isotope ratio of S-III (within the 16 priority PAHs, only 12 were found above the analytical uncertainties). Pie chart showing taxonomy on a class level, bar chart on the right showing bacteria genera from pyrosequencing results.

C5. Comparison of dual plot carbon and hydrogen isotope analysis with 454 pyrosequencing of coal tar S-IV

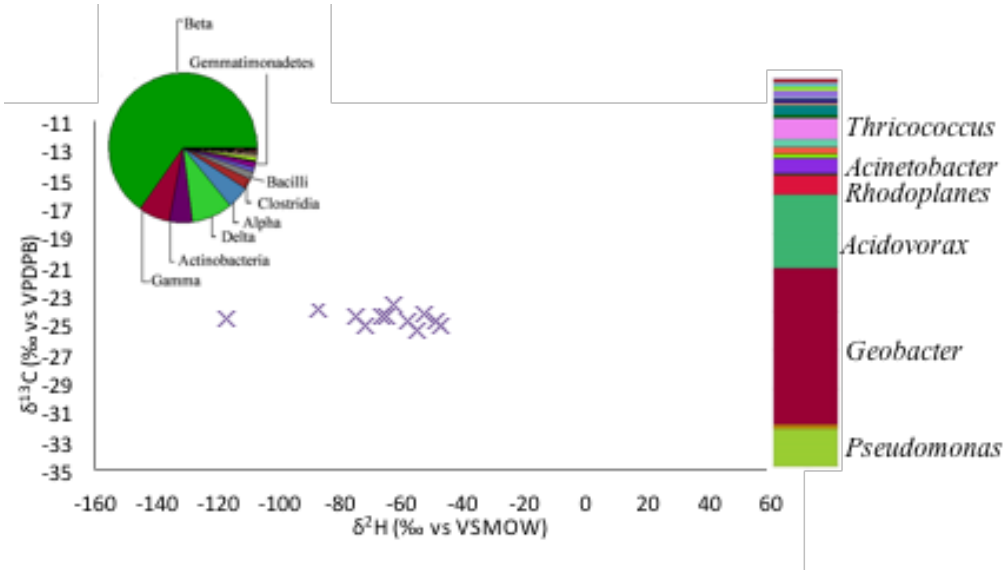

Figure C5. Dual plot showing carbon and hydrogen isotope ratio of S-IV (within the 16 priority PAHs, only 12 were found above the analytical uncertainties). Pie chart showing taxonomy on a class level, bar chart on the right showing bacteria genera from pyrosequencing results.

C6. Comparison of dual plot carbon and hydrogen isotope analysis with 454 pyrosequencing of coal tar S-V

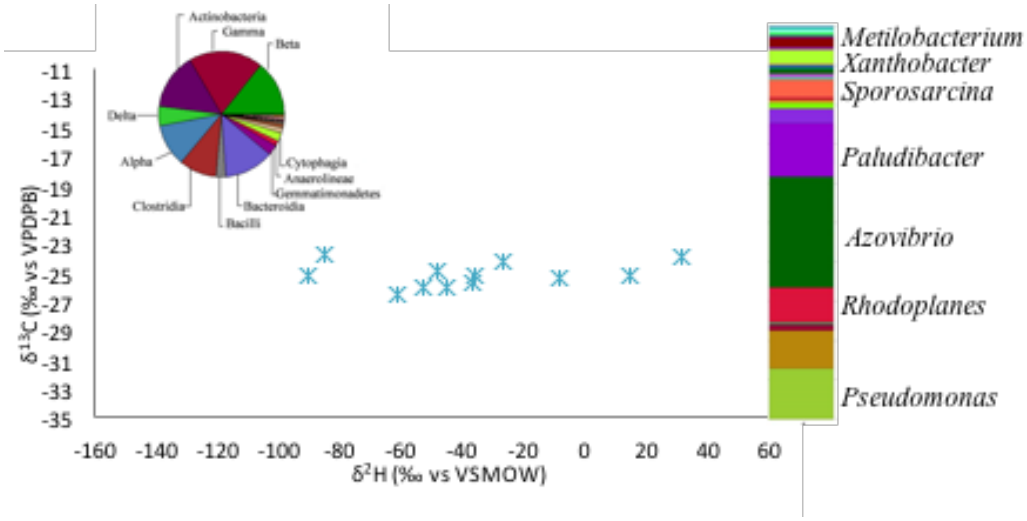

Figure C6. Dual plot showing carbon and hydrogen isotope ratio of S-V (within the 16 priority PAHs, only 12 were found above the analytical uncertainties). Pie chart showing taxonomy on a class level, bar chart on the right showing bacteria genera from pyrosequencing results.

C7. Comparison of dual plot carbon and hydrogen isotope analysis with 454 pyrosequencing of coal tar S-VI

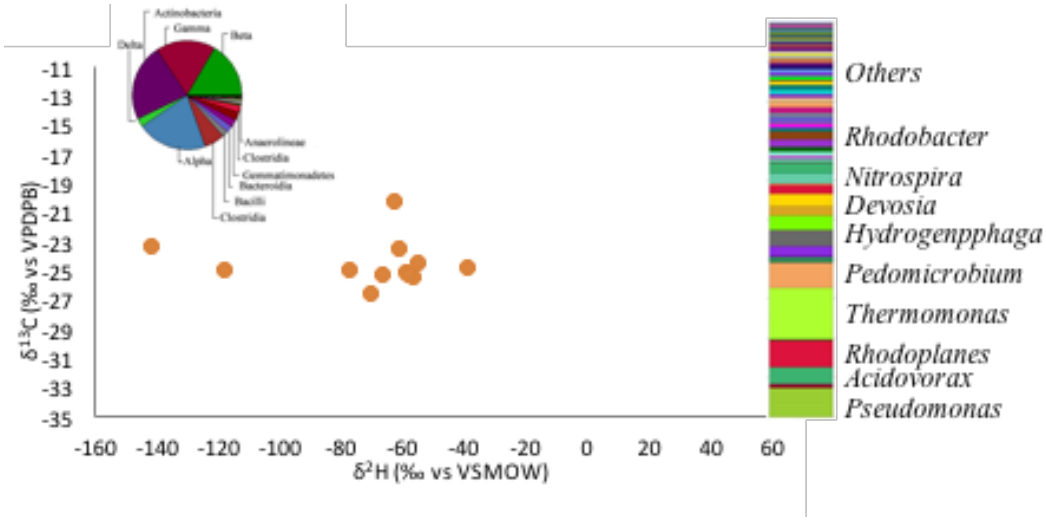

Figure C7. Dual plot showing carbon and hydrogen isotope ratio of S-VI (within the 16 priority PAHs, only 12 were found above the analytical uncertainties). Pie chart showing taxonomy on a class level, bar chart on the right showing bacteria genera from pyrosequencing results.

C8. Comparison of dual plot carbon and hydrogen isotope analysis with 454 pyrosequencing of coal tar S-VII

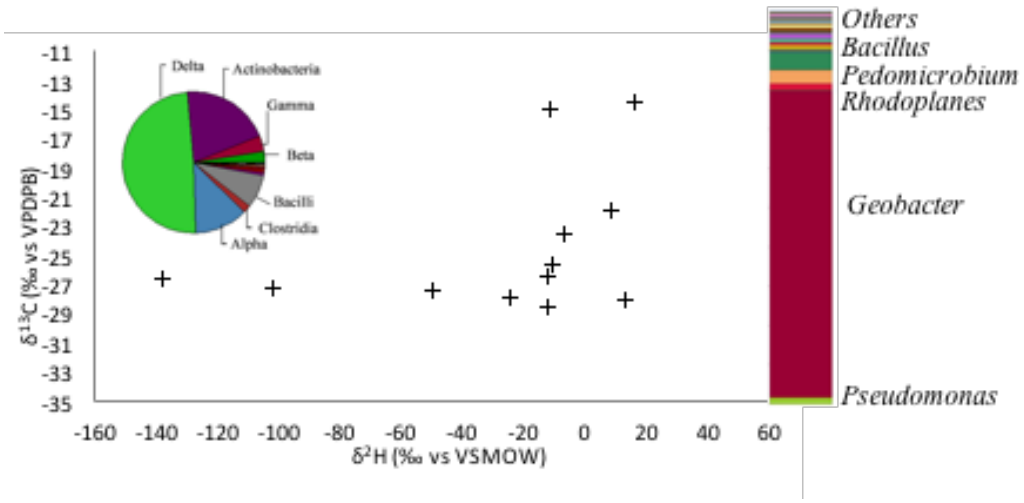

Figure C8. Dual plot showing carbon and hydrogen isotope ratio of S-VII (within the 16 priority PAHs, only 12 were found above the analytical uncertainties). Pie chart showing taxonomy on a class level, bar chart on the right showing bacteria genera from pyrosequencing results.

C9. Selected GC-MS, GC-C-IRMS and GC-TC-IRMS chromatograms of coal tar S-I

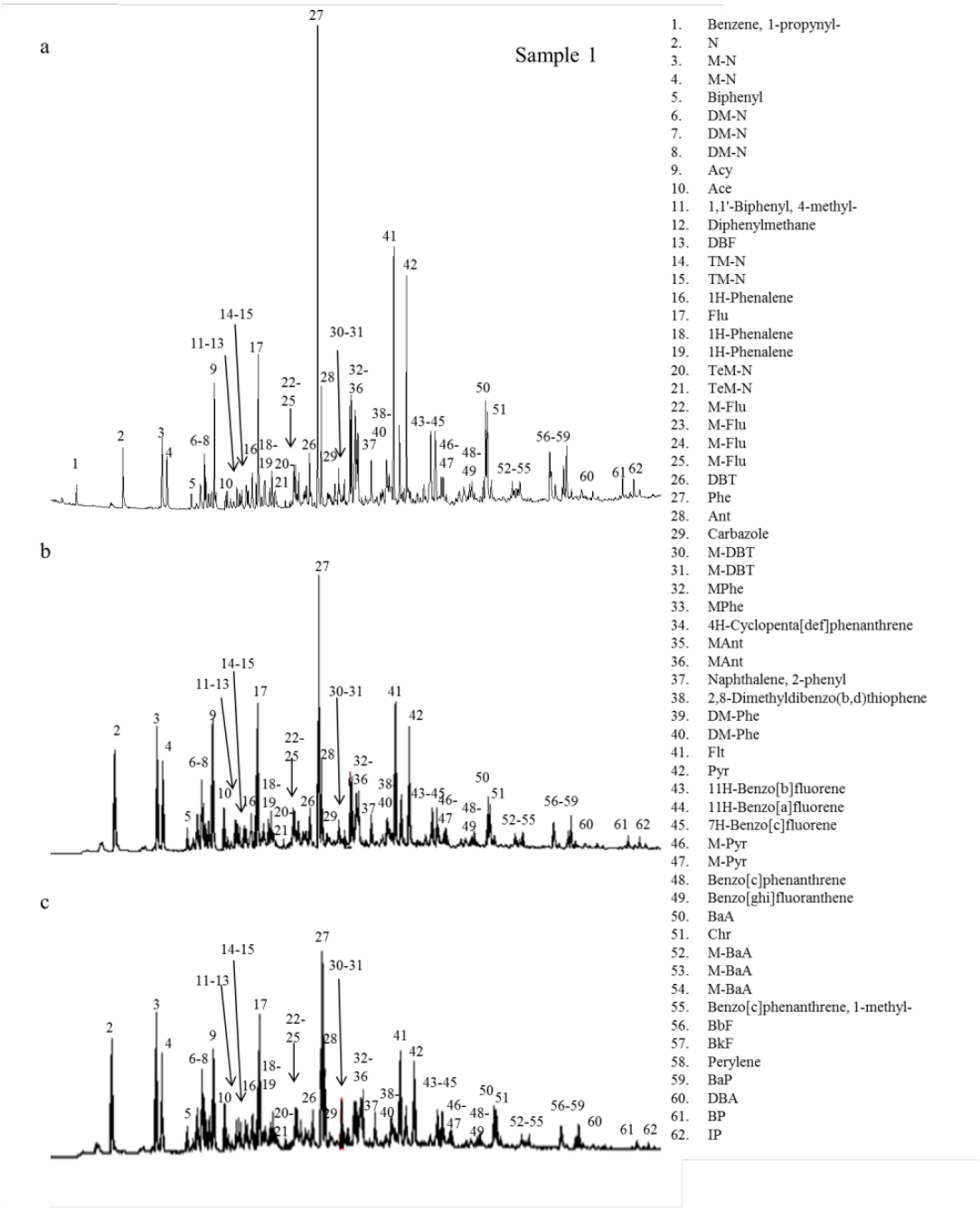

Figure C9. S-I: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

C10. Selected GC-MS, GC-C-IRMS and GC-TC-IRMS chromatograms of coal tar S-II

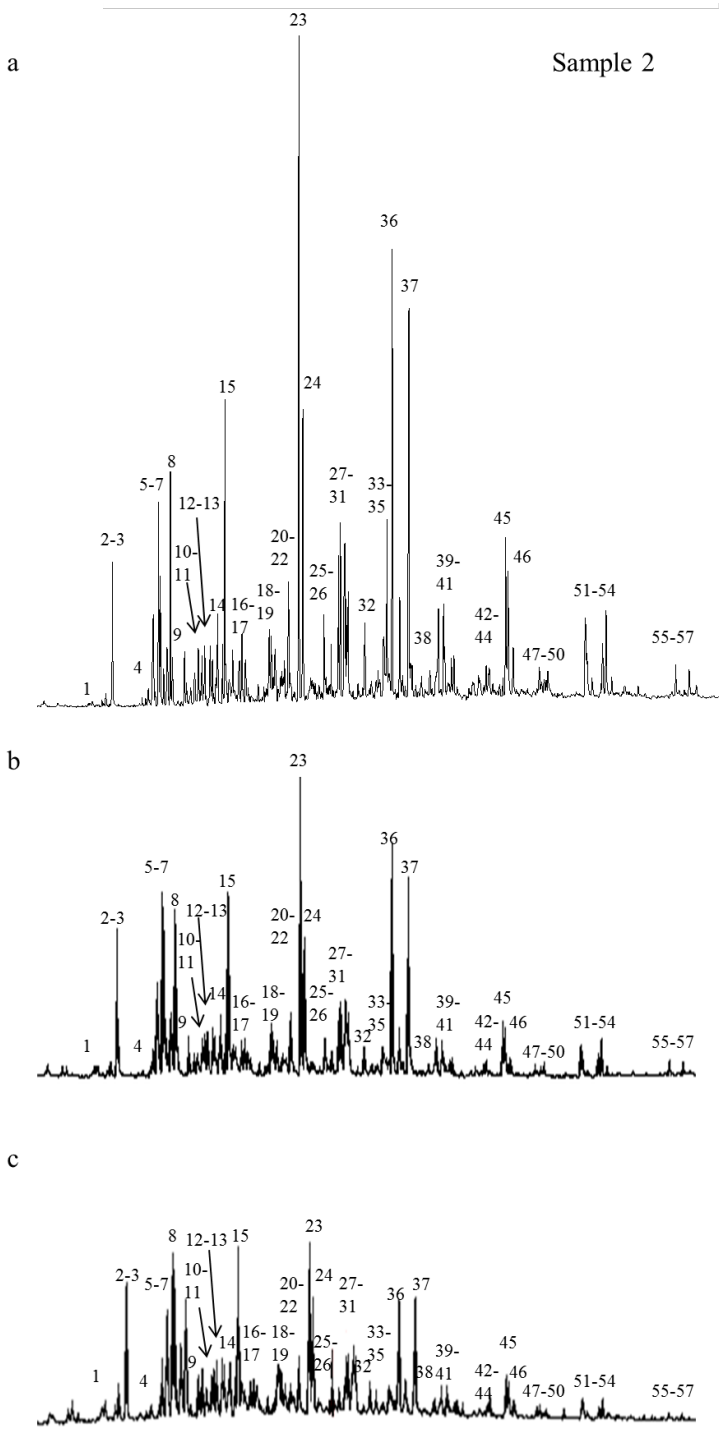

Figure C10. S-II: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

C11. Selected GC-MS, GC-C-IRMS and GC-TC-IRMS chromatograms of coal tar S-III

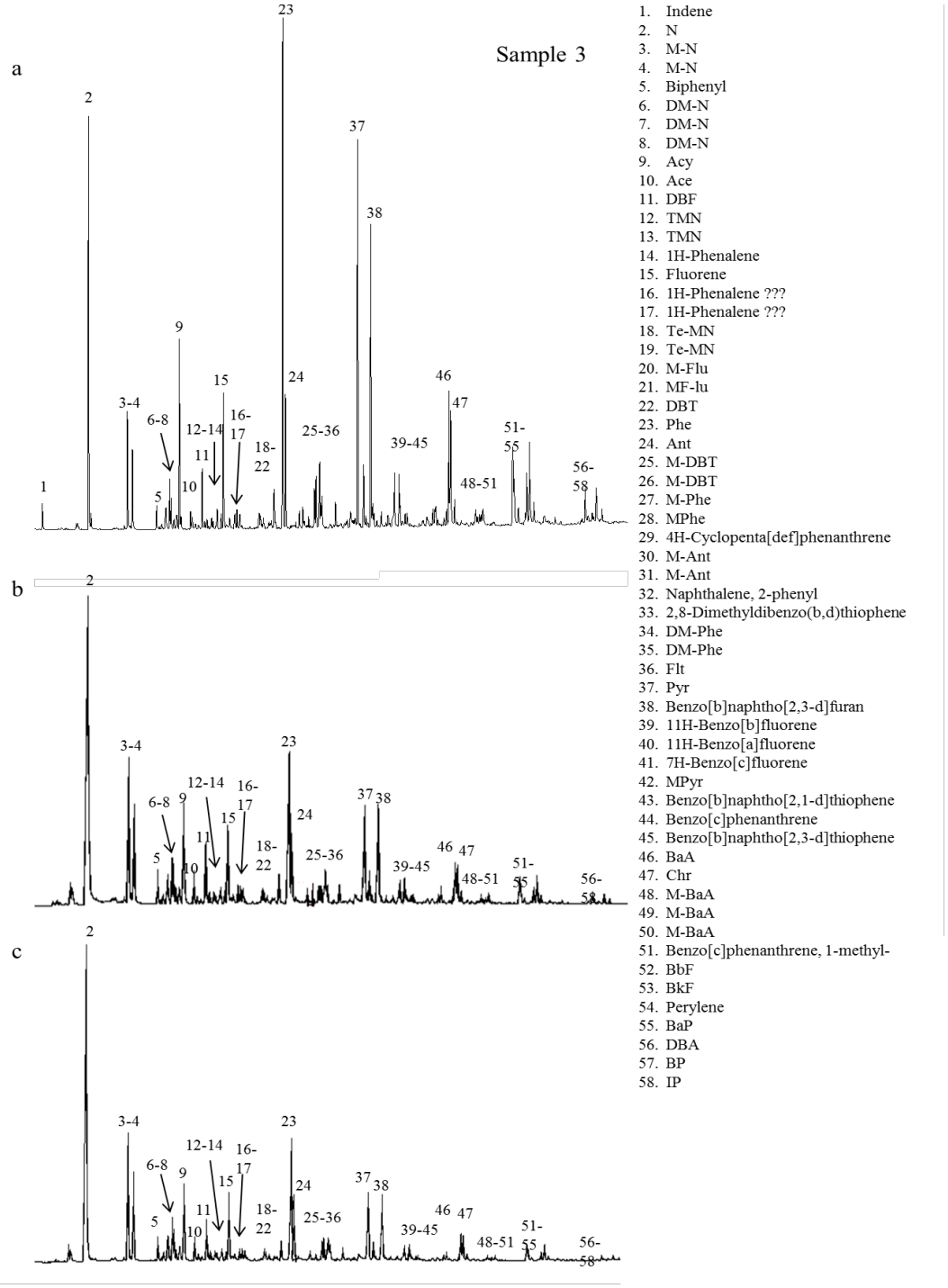

Figure C11. S-III: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

C12. Selected GC-MS, GC-C-IRMS and GC-TC-IRMS chromatograms of coal tar S-IV

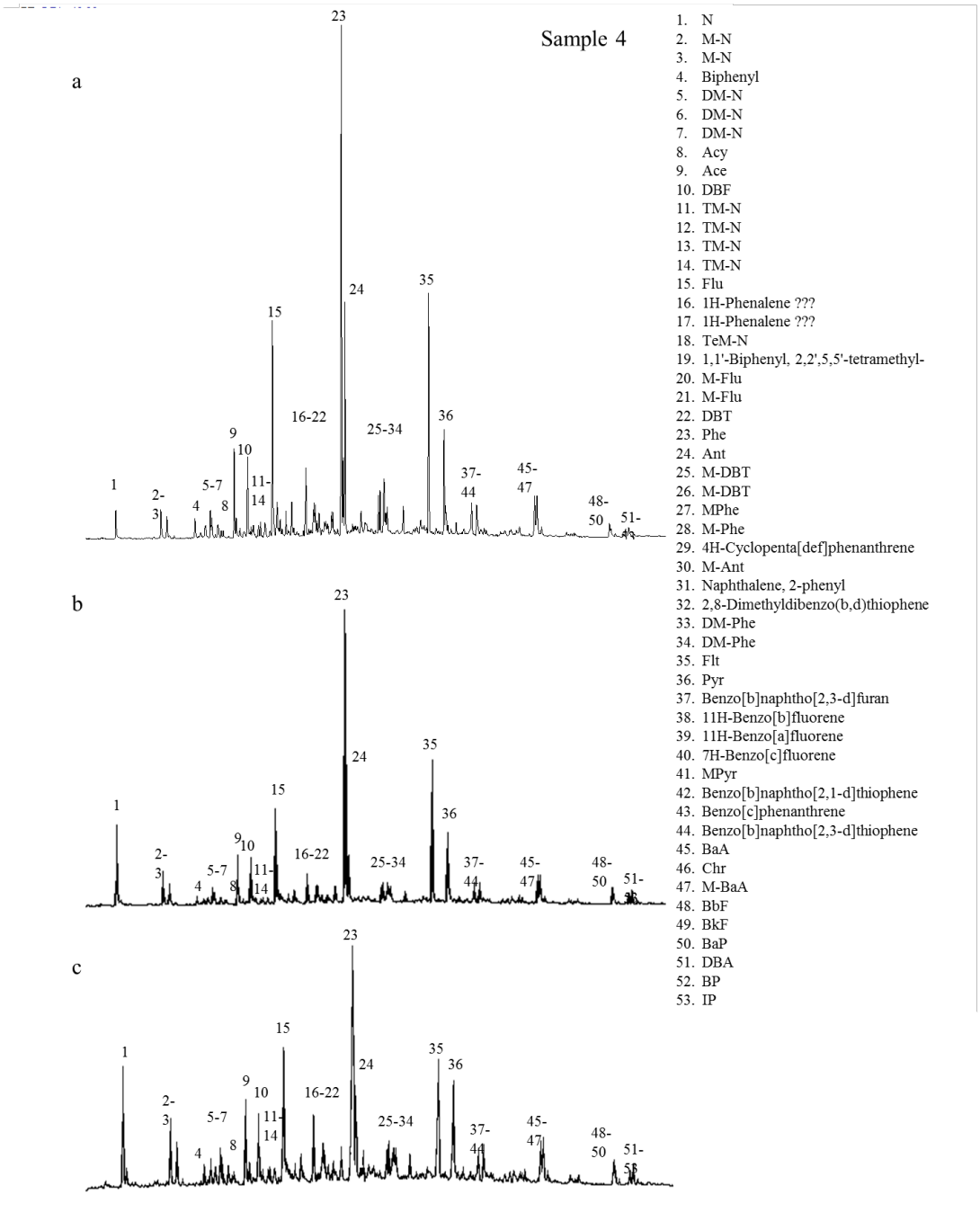

Figure C12. S-IV: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

C13. Selected GC-MS, GC-C-IRMS and GC-TC-IRMS chromatograms of coal tar S-V

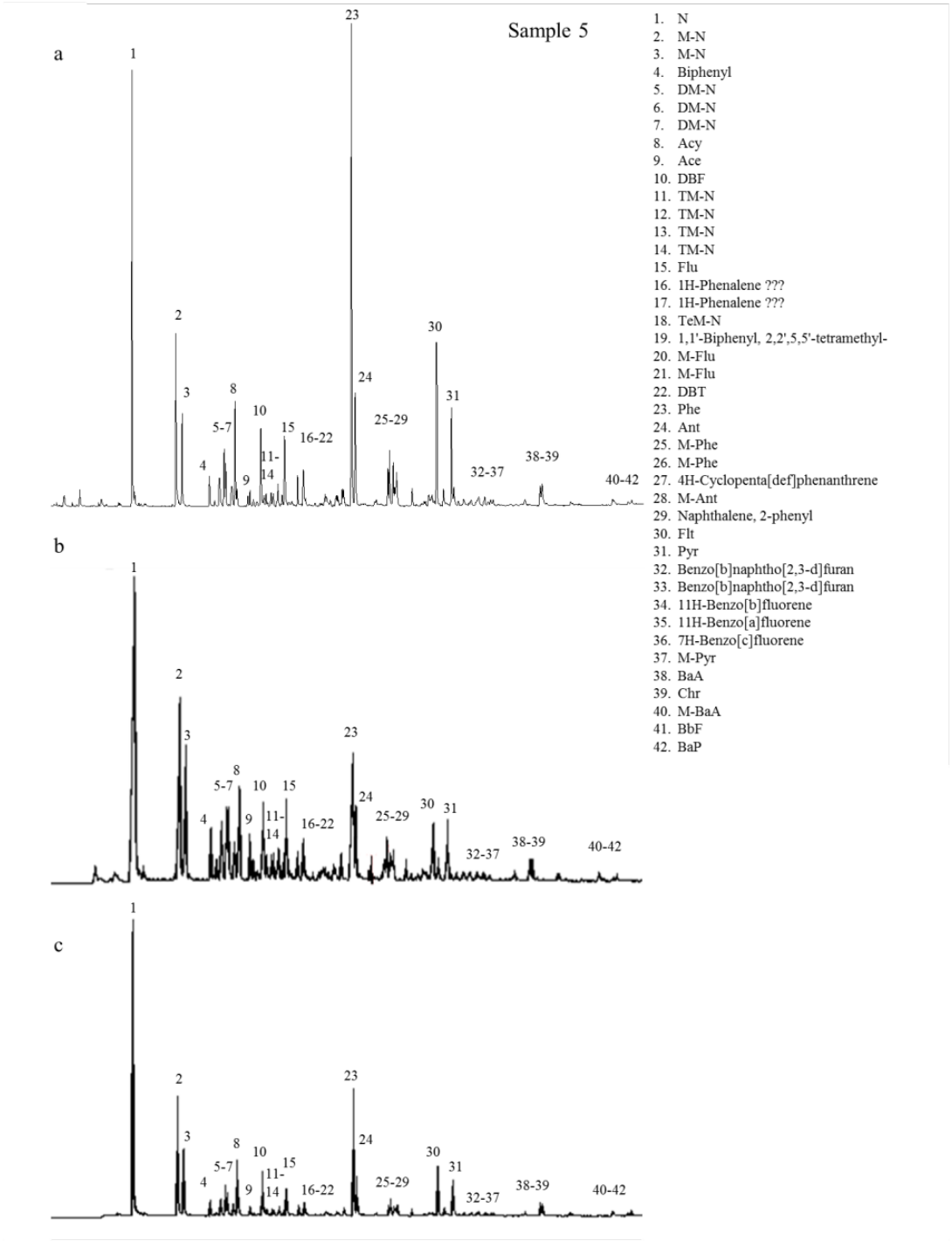

Figure C13. S-V: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

C14. Selected GC-MS, GC-C-IRMS and GC-TC-IRMS chromatograms of coal tar S-VI

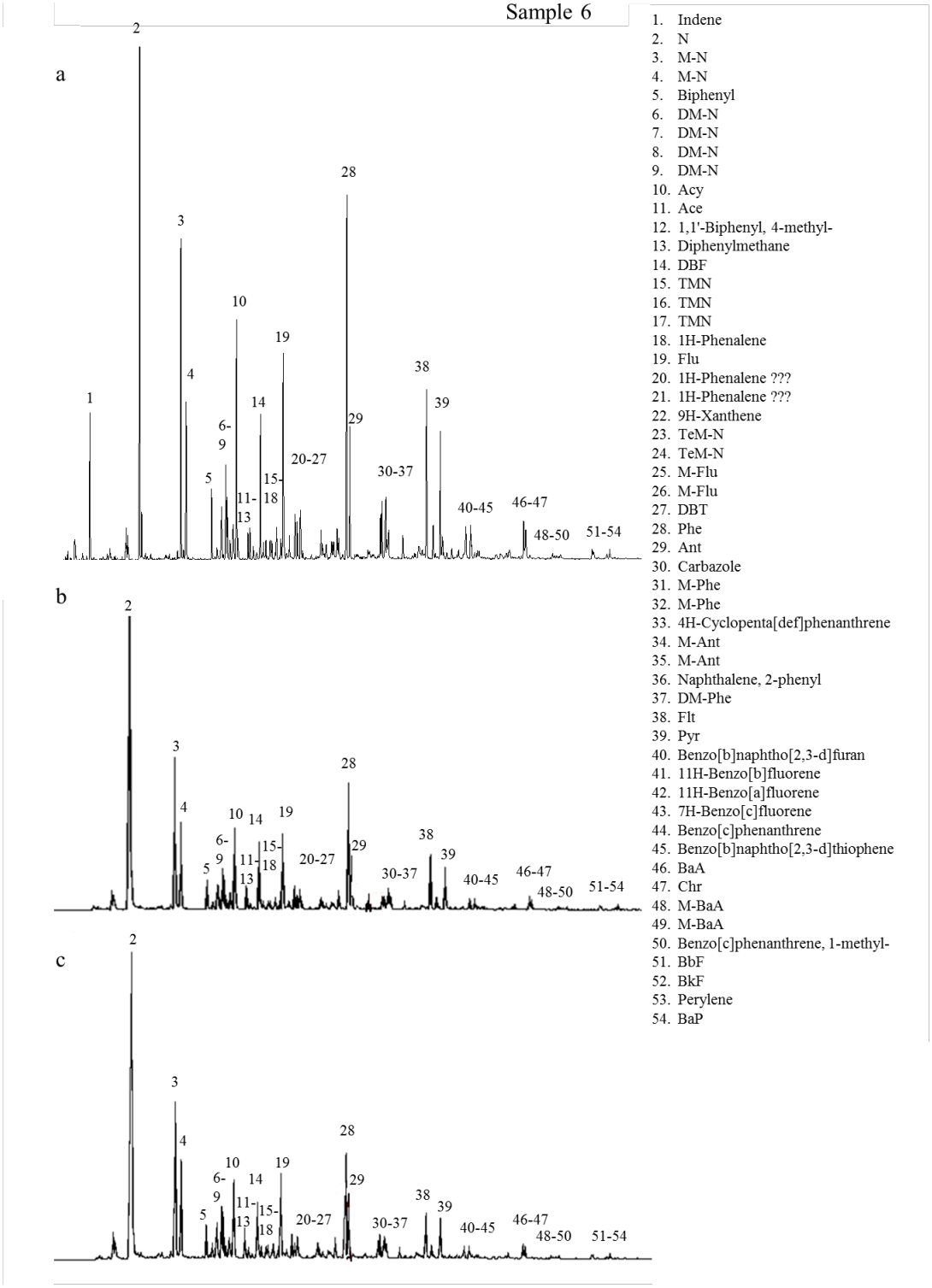

Figure C14. S-VI: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

C15. Selected GC-MS, GC-C-IRMS and GC-TC-IRMS chromatograms of coal tar S-VII

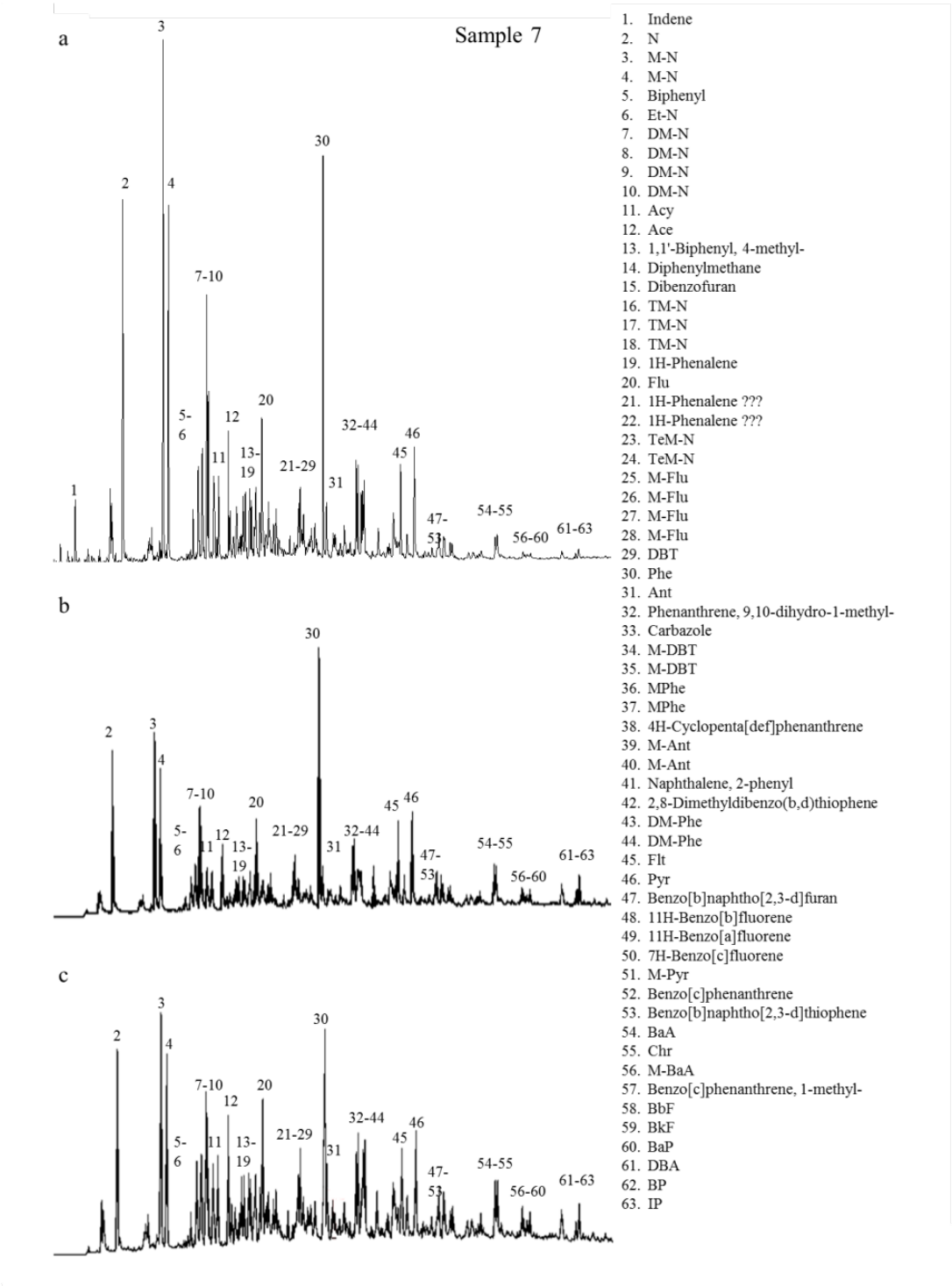

Figure C15. S-VII: Chromatograms comparison of (a) GC-MS analysis, (b) GC-C-IRMS analysis and (c) GC-TC-IRMS analysis.

Appendix D

Appendices to Chapter 7

D1. Microbial ecology of contaminated plumes

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1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic aromatic pollutants formed naturally during thermal geologic reactions and plant fossilisation. PAHs are also produced anthropogenically by coal liquefaction and gasification, creosote production, petroleum refining and the incomplete combustion of organic material (1). Although PAHs may undergo chemical oxidation, photolysis, adsorption and volatilisation, microbial degradation of PAHs is in most cases the major process

involved in the elimination of PAHs in the subsurface (2, 3). Several techniques have been developed, such as landfarming, biopile and bioslurry. These bioremediation processes are based on enhancing microbial activity through the optimisation of biodegradation conditions by aeration (addition of oxygen as electron acceptor), addition of nutrients or microbial consortia (biostimulation and bioaugmentation respectively) and control of pH, moisture and temperature (4- 6). Although biological treatment of PAHs in the subsurface has been extensively studied, the design of biological PAH-treatments remains largely empirical. Rate controlling parameters are often unknown. The bioavailability of hydrophobic compounds needs to be enhanced. Degradation rates are highly variable and cannot be predicted reliably. Treatment end points are uncertain. The ecological effects of residual, relatively insoluble PAHs that may remain after bio-treatment and the microbial communities involved in the biodegradation are partially unknown.

Typical approaches to establish the in-situ biodegradation potential include: (*i*) metabolite tracing and quantification of the concentration of key contaminants during biodegradation; (*ii*) the molecular characterisation of soil or groundwater samples, to verify the presence of indigenous biodegradative microorganisms; and (*iii*) compound specific isotope analysis (CSIA) for the direct assessment of biodegradation rates. The decrease over time of key contaminants is generally used to assess the effectiveness of the bio-treatment and to determine its end point, but additional data, such as the shift in the isotope ratio of carbon and hydrogen elements in PAHs and the change of the bacterial community structure (7) could give more valuable information for the monitoring of bio-treatments. In order to develop the most appropriate and cost-effective approaches to the bioremediation of a contaminated site, the microbial ecology of that site should be understood to the fullest extent possible. Cultivation-based (8-10) and cultivation-independent (11-16) techniques have been used to evaluate the microbial ecology of PAHs-degradation. However, culturing techniques greatly underestimates the diversity of the prokaryotic world (17) and fails to account for the complex interactions of the members of microbial communities with each other and with their native environment. Cultivation-independent techniques can help us better estimate the prokaryotic

diversity of complex systems (18, 19) where it can be difficult to establish which organisms are responsible for the degradation of particular contaminants.

2 Objectives

As part of a larger project investigating strategies for the bioremediation of PAH-contaminated soil from former manufactured gas plant (FMGP) sites in UK and USA, we developed and applied, for the first time, molecular tools for study of pure coal tar DNAPLs. We described the complex bacterial community structures adapted to extremely toxic substrates and demonstrated that the presence of bacteria in the pure organic-phase DNAPL does not represent a limit for bioremediation in-situ. Furthermore, we carried out multidimensional compound specific isotope analysis (CSIA) of carbon and hydrogen elements of PAHs in coal tar DNAPL, highlighting significant differences in the hydrogen isotope signature of unsubstituted- and alkylated-PAHs. This clear shift in the isotope signature is a strong indicator of biodegradation processes that can be used as biomarker for in-situ bioremediation monitoring and also for source apportionment of coal tar. To improve our understanding of the microbial ecology of hydrocarbon-contaminated plumes and to carry on our continuous work on bioremediation technologies, we intend to pursue the following overlapping objective:

1. Identify populations of PAH-degraders and heterotrophs in the bacterial community and follow changes in their diversity;
2. Analyse the biodegradation activities of the bacterial community upon selected PAHs;
3. Assess the biodegradation rates through changes of the carbon and hydrogen isotope signature of PAHs undergoing biodegradation;
4. Determine if some specific strains could be used as bio-indicators of the effectiveness and of the endpoint of the biological treatment.

To achieve these goals, a combination of powerful analytical tools such as high-throughput 16S rRNA gene sequencing, stable isotope probing (SIP) and metabolomic multidimensional CSIA will be used. Results of molecular and isotope analyses will be further analysed with multivariate statistical tools to confirm the validity and reproducibility of data.

3. Experimental methodologies

In this study, we will perform DNA-based SIP with ^{13}C-naphthalene, ^{13}C-phenanthrene and ^{13}C-pyrene, to investigate the degradation pathways of PAHs of increasing molecular weight (up to four aromatic rings). The methodologies can be divided in two main sessions, characterisation and SIP incubation. An outline of the experiments is given below.

1a. Microbial characterisation:

- Sampling;
- DNA extraction and amplification;
- High-throughput 16S rRNA gene sequencing;
- Pyrosequencing data analysis and multivariate statistics.

1b. Chemical and isotope signature characterisation:

- Accelerated solvent extraction, concentration and purification;
- Compound specific carbon and hydrogen analyses;
- GC-MS analyses;

- Multivariate statistics.

2. Microcosm experiments for stable isotope probing:

- Incubation with ^{13}C-Naphthalene, ^{13}C-Phenanthrene, ^{13}C-Pyrene;
- Carbon and hydrogen CSIA;
- GC-MS analyses;
- SIP;
    - DNA extraction and density gradient ultracentrifugation;
    - Clone library construction and/or DGGE;
    - High-throughput 16S rRNA gene sequencing;
    - Pyrosequencing data analysis;
- Multivariate statistics.

4 Anticipated Outcomes

Individual stable-isotope probing experiments will be performed with three different uniformly ^{13}C-labelled PAHs to investigate the bacterial guild responsible for PAHs degradation in contaminated plumes. Coupled with the high-throughput 16S rRNA gene analysis data, SIP of PAH-degrading bacteria in the contaminated plumes will represent a comprehensive investigation of bacteria capable of degrading 2-ring (naphthalene), 3-ring (phenanthrene) and 4-ring (pyrene) PAHs. Collectively, we expect detect a diverse range of bacteria spanning the *Alpha*-, *Beta*- and *Gamma*-*Proteobacteria*, as well as *Actinobacteria*, typically involved in the degradation of hydrocarbon. The construction of clone library will help to investigate a degree of differentiation between PAH degrading bacteria. Multidimentional CSIA will provide a direct assessment of the in-situ biodegradation potential, as well as reference data for the compound specific hydrogen analysis of polyaromatic

hydrocarbons. The combined approach of deep sequencing methods, SIP and multidimensional CSIA will also be used for source apportionment of hydrocarbon contaminant in-situ.

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Appendix E

Additional work on carbonization processes

E1. An Innovative agro-forestry supply chain for residual biomass: physicochemical characterisation of biochar from olive and hazelnut pellets

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An Innovative Agro-Forestry Supply Chain for Residual Biomass: Physicochemical Characterisation of Biochar from Olive and Hazelnut Pellets

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Abstract: Concerns about climate change and food productivity have spurred interest in biochar, a form of charred organic material typically used in agriculture to improve soil productivity and as a means of carbon sequestration. An innovative approach in agriculture is the use of agro-forestry waste for the production of soil fertilisers for agricultural purposes and as a source of energy. A common agricultural practice is to burn crop residues in the field to produce ashes that can be used as soil fertilisers. This approach is able to supply plants with certain nutrients, such as Ca, K, Mg, Na, B, S, and Mo. However, the low concentration of N and P in the ashes, together with the occasional presence of heavy metals (Ni, Pb, Cd, Se, Al, etc.), has a negative effect on soil and, therefore, crop productivity. This work describes the opportunity to create an innovative supply chain from agricultural waste biomass. Olive (*Olea europaea*) and hazelnut (*Corylus avellana*) pruning residues represent a major component of biomass waste in the area of Viterbo (Italy). In this study, we evaluated the production of biochar from these residues. Furthermore, a physicochemical characterisation of the produced biochar was performed to assess the quality of the two biochars according to the standards of the European Biochar Certificate (EBC). The results of this study indicate the cost-effective production of high-quality biochar from olive and hazelnut biomass residues.

Keywords: biochar; biomass; soil fertiliser; olive; hazelnut

1. Introduction

Biochar is a carbon-rich material produced by thermal decomposition of biomass under oxygen-limited conditions [1]. According to the International Biochar Initiative (IBI), biochar is primarily used for soil applications for both agricultural and environmental gains [2]. The IBI definition differentiates biochar from charcoal, whose use is as a fuel for heat, as an absorbent material, or as a reducing agent in metallurgical processes [1,3]. Thermo-chemical processes include (i) slow pyrolysis (conventional carbonization); (ii) fast pyrolysis; (iii) flash carbonization; and (iv) gasification [4–7]. During the last two decades, pyrolysis process received more attention from the scientific community, since it is an efficient method for converting biomass into bio-fuel [5,8]. The pyrolysis process and its

parameters, such as final temperature, pressure, heating rate, and residence time, greatly influence biochar quality [5]. The advantage of slow pyrolysis is to retain up to 50% of the carbon (C) feedstock in stable biochar [9], which makes it suitable as soil fertiliser. High-temperature pyrolysis (>550 °C) produces biochar with high aromatic content and, therefore, recalcitrant to decomposition [10]. Biochars produced through low-temperature processes (<550 °C) typically have a less-condensed C structure and are expected to give a better contribution to soil fertility [11]. The nature of the biomass feedstock also influences the properties of the produced biochar [3,12]. The relation between biochar properties and its potential to improve agricultural soils is a nascent focus area and the appropriate pyrolysis conditions are still unclear [13]. Numerous recent studies focused on methodologies for the chemical characterisation of biochars [13–15], other studies investigated the intrinsic potential of biochar as a soil amendment [16,17], although further efforts are required to obtain biochar with suitable properties [3]. One of the attractive characteristics of biochar as a soil amendment is its porous structure, which improves water retention and increases soil surface area [2]. Moreover, the concentration of biochar into soil has been related to an improved nutrient use efficiency, either through nutrients contained in biochar or through physicochemical processes that allow a better uptake of soil-inherent or fertiliser-derived nutrients [2]. The application of biochar increases physical and chemical qualities of soils, resulting in greater productivity of the agro-ecosystem [18]. Biochar, due to its biological and chemical stability, can also act as a C sink. The recalcitrance of biochar to microbial degradation enables the long-term sequestration of C in soil [2,19].

Biochar application in agriculture, positively affects the water holding capacity; this property derives from the distribution and the degree of cohesion of the pores in biochar, which depends on the particles size and aggregation, as well as the organic matter (OM) content. The effect of biochar on water holding capacity is dependent on both the high internal surface area of biochar and the capability to aggregate soil particles with OM, minerals, and microorganisms. The increase in soil porosity also allows a better percolation of excess water towards the deeper layers of the soil, therefore increasing ventilation.

This work aims to determine the opportunity to create an innovative supply chain from agricultural waste biomass, especially regarding olive (*Olea europaea*) and hazelnut (*Corylus avellana*) in order to evaluate the production of biochar from their pruning residues. Biomass residues in Mediterranean areas come mainly from agricultural and agro-industrial activities, as well as forest by-products. Only a few woody residues are used to produce fertilisers and as renewable energy resources [20]. In contrast, typical management strategies in the agricultural industry do not provide any valorisation of these biomasses, which are burnt in the field to prevent proliferation of plant diseases [20]. However, this landfill choice affects the soil structure since OM in woody biomass residues must be completely decomposed before used as fertiliser.

2. Materials and Methods

2.1. Biomass from Olive and Hazelnut Prunings

In the area of Viterbo, pruning residues from olive and hazelnut are rarely utilised as a source of energy in burning stoves or boilers; they are, instead, burnt in situ, therefore reducing the formation of soil organic carbon. During summer, besides pruning residues, suckers are removed before the harvest, representing another significant loss of biomass. Approximately 15 m^3 of biomass samples from both olive and hazelnut have been collected in farms of the Viterbo province. Recent studies [21,22] have investigated the possibility of enhancing olive and hazelnut residue waste management as a means to produce soil fertilisers and energy, therefore reducing the environmental impact of such residual organic wastes. Biomass from pruning crop operations (Figure 1a,b) represents an attractive resource that could be exploited for (i) fuel production (combustion and/or gasification) and (ii) biochar production (pyrolysis) that can be used as soil fertiliser.

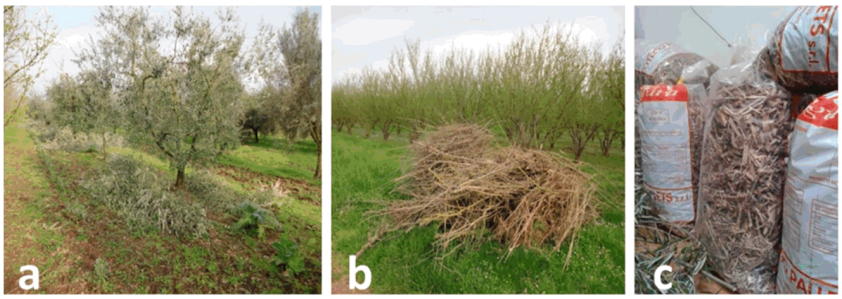

Figure 1. Pruning residues from (**a**) olive and (**b**) hazelnut after crop operations; and (**c**) bio-shredding.

A pelletization procedure was developed and applied on bio-shredding obtained from olive and hazelnut residues (Figure 1c). Pruning residues were collected on site and immediately transferred to the laboratory for sifting and exsiccation (Figure 2) until a water content of 15% was achieved. Final water content as low as 15% is necessary for further refining of the product and pellet production. The humidity concentration in the prunings is very notable, because we can improve the technical process for pellet production by biomass. In Italy there are not many companies and total supply chains that work the prunings for pellet production and for use of these residual agriculture sub-products (Figure 3).

Figure 2. Schematic of the pelletization process showing (**a**) pellet mill; (**b**) olive and hazelnut pellet; and (**c**) packaging.

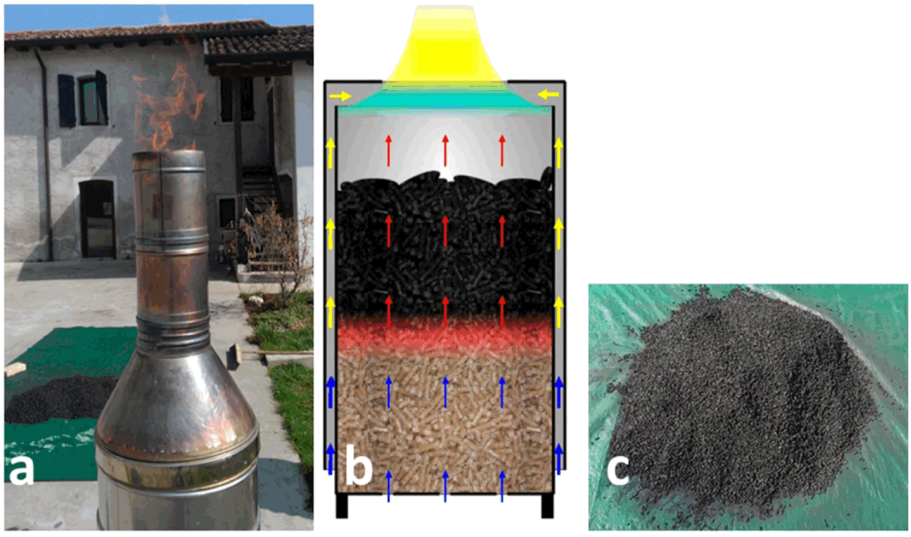

Figure 3. Biochar production from pellets showing (**a**) the Elsa Research carbonization system; (**b**) a schematic representation of the conversion process; and (**c**) the final product (biochar).

2.2. Pyrolysis Process

Pyrolysis of biomass is commonly considered as a thermo-chemical conversion process [7,23]. Pyrolysis is carried out under partial (or complete) absence of oxygen and relies on capturing the off-gases from thermal decomposition of the organic materials [19]. The physicochemical characteristics of biochar are determined by the type of feedstock and by the temperature of pyrolysis. For example, higher salt and ash contents are expected in wheat straw than in wood-derived biochar [24], and C content and N content are greater in pine chips than in poultry litter-derived biochar [25]. A higher pyrolysis temperature results in greater surface area, lower biochar recovery, higher ash content, elevated pH, minimal total surface charge [26], and lower cation exchange capacity [24]. Removal of volatile compounds at higher pyrolysis temperatures also cause biochars to have higher C content and lower hydrogen (H) and O content [26]. Pyrolysis of agro-forestry residues is typically carried out with temperatures between 400 and 800 °C. With these conditions, the feedstock is converted to liquid products (so-called tar or pyrolysis oil) and/or gas (syngas), which can be used as fuels or raw materials for subsequent chemical transformation. The residual solid carbonaceous material obtained (biochar) could be further refined to products, such as activated carbon.

2.3. Biochar Production form Olive and Hazelnut Pellets

The carbonisation system Elsa Research (Blucomb Ltd., based in Udine, Italy) was used to produce the biochar from olive and hazelnut pellets; biomass conversion was achieved by pyrolytic micro-gasification (Figure 3). The Elsa Research carbonisation system works with natural ventilation and does not require being powered by batteries or electricity. A chimney is typically used to increase the air draft for fuels that have difficulties igniting.

Physicochemical characterisation of the biochar obtained from the Elsa Research carbonisation system was performed at the European Biochar Institute, which released the EBC based on the quality of the biochar.

3. Results

3.1. From Biomass to Biochar: Conversion Rates Analyses

Auto-thermal conversion of biomass was carried out under natural ventilation. Quantitative analyses of pyrolysed biomass and produced biochar, as well as the conversion rates, are reported for 10 and four sessions of pyrolysis, respectively, for olive and hazelnut pellets (Table 1). A statistical comparison between olive and hazelnut performances during pyrolysis is reported in Table 2, showing the total conversion rates, mean, and standard deviation (SD) of the results obtained in the experiments.

Table 1. Conversion rates of biomass obtained from each pyrolysis session.

Olive				Hazelnut			
Session	Biomass (kg)	Biochar (kg)	Conversion Rate	Session	Biomass (kg)	Biochar (kg)	Conversion Rate
1	38.35	8.11	0.209	1	37.69	8.11	0.215
2	39.07	8.21	0.210	2	36.25	7.96	0.220
3	38.88	8.19	0.211	3	37.03	8.09	0.218
4	38.96	8.16	0.209	4	37.11	8.09	0.218
5	34.09	7.10	0.208				
6	39.02	8.23	0.211				
7	38.89	8.19	0.211				
8	38.93	8.19	0.210				
9	38.97	8.20	0.210				
10	38.81	8.13	0.209				

Table 2. Comparisons and statistical values of conversion rates of olive and hazelnut.

	Olive		Hazelnut	
	Biochar (kg)	**Conversion Rate**	**Biochar (kg)**	**Conversion Rate**
Total	384.47	80.71	148.08	32.25
Mean		0.210		0.218
SD		0.00088		0.00188

Further analyses were carried out to investigate the calorific power of the two biochars produced. Composition, structure, heat value of the gas, tar liquid, and semi-char solid products depend on the pyrolysis temperature [7]. Quantity and quality of resulting outputs from biomass pyrolysis are related to the chemical composition of the operating temperature and the feedstock [7,27]. The calorific values calculated were compared with those provided by the producers in order to make energy considerations on the process. The results obtained are consistent with other pyrolysis processes. The latter led to the volatilisation of a fraction of biomass with a calorific value ranging between 75% and 85% of the starting biomass. The calorific value is measured in terms of the high calorific value [28]. Table 3 distinguishes two types of calorific value (usually expressed in MJ/kg): (i) the higher calorific value that it is the amount of heat produced by a complete combustion of a mass unit of a sample, at constant volume, in an atmosphere rich of oxygen at standard conditions (25 °C, 101.3 kPa); and (ii) the lower calorific value (PCI) that does not include the heat of the condensation of water [28].

Table 3. Analysis of the calorific power of pyrolysis reaction for the two biochars produced in this study.

Olive Wood	**Units**	**Pellet**	**Biochar**
Higher calorific value	MJ/kg	19.47	31.71
Lower calorific value	MJ/kg	16.17	30.48
Calorific value from pyrolysis	MJ/kg	12.37	
Percentage of calorific value from pyrolysis	%	0.76	
Hazelnut Wood	Units	Pellet	Biochar
Higher calorific value	MJ/kg	19.02	26.62
Lower calorific value	MJ/kg	16.71	25.66
Calorific value from pyrolysis	MJ/kg	14.21	
Percentage of calorific value from pyrolysis	%	0.85	

Pyrolysis does not produce energy from heat; rather, it leads to the production of gas from biomass. In general, pyrolysis involves the heating of biomass to temperatures greater than 400 °C in the absence of oxygen [29]. At these temperatures, biomass thermally decomposes releasing a vapour phase and biochar (solid phase). On cooling the pyrolysis vapour, polar and high-molecular-weight compounds condense out as bio-oil (liquid phase) while low-molecular-weight volatile compounds remain in the gas phase (syngas) [6]. The physics and chemistry of pyrolysis process results are extremely complex, and are dependant depending on both the rector conditions and the nature of the biomass [29]. The combustion of gas in the Elsa Research system occurs in "close-coupled combustion" (micro-gasification). Biochars produced by Blucomb Ltd. (Udine, Italy) (spin-off) for the European project were analysed by Eurofins laboratories, accredited for the certification of the EBC. International biochar experts developed the EBC in order to consider it in the European context as a voluntary industrial standard [30]. The EBC guarantees a sustainable biochar production, with a low-risk use in agronomic systems. Biochar produced in accordance with the standards of the EBC fulfils all of the requirements of sustainable production and environmental impact by certifying (i) sustainable production and provision of biomass feedstock; (ii) energy efficient, low emission pyrolysis technique; (iii) low contaminant level in the biochar; and (iv) low hazard use and application of the biochar. These standards are in compliance with current environmental European regulations [31].

3.2. Elemental Analysis

The chemical composition of biochar is determined also by the source of biomass employed. Biochar produced from wood, for example, is denser and has higher C content (~80%) [32]. These properties reflect the chemical complexity of lignin, which makes it more resistant to thermal degradation. The elemental composition, plotted as H/C vs. O/C ratios (Figure 4), is often used to describe maturity, decomposition rate, and combustion behaviour of fossil chars and coal [33,34]. When applied to biochar, the H/C and O/C ratios can be suitable indicators of the degree of carbonisation. High ratios typically point to primary plant macromolecules, such as simple carbohydrates and cellulose [35]. An H/C ratio of ⩽0.2 indicates C of plant origin with elevated carbonisation [36].

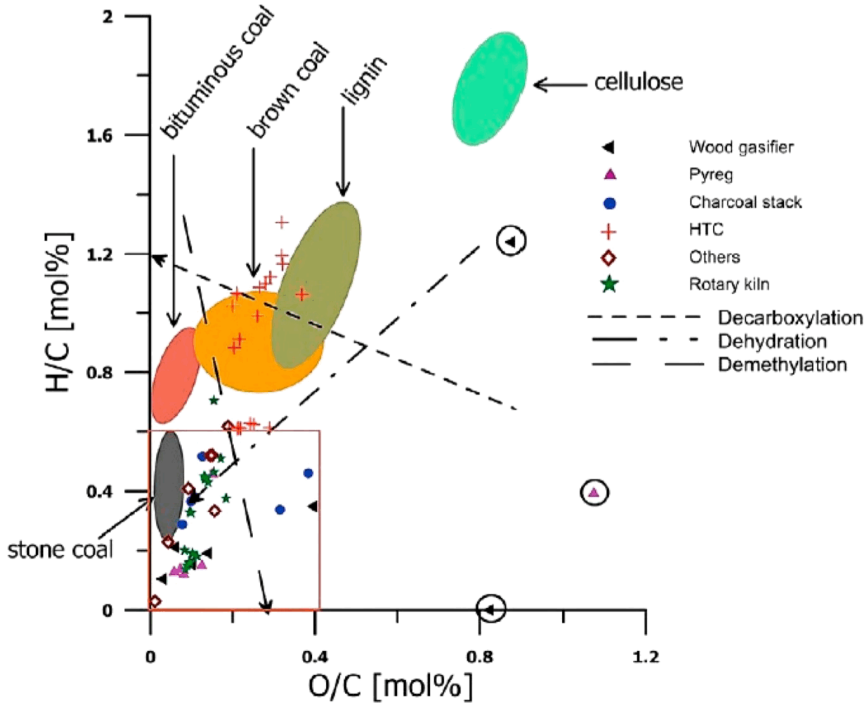

Figure 4. Example of Van Krevelen diagram of biochars obtained through different pyrolisis processes. The red square shows the optimum elemental ratio values of H/C and O/C for biochar production.

The O/C ratio is an indicator of the presence of polar functional groups, which influence the stability of biochar by preventing a dense, graphite-like structure of the material [37]. Therefore, the O/C ratio is useful to assess hydrophilicity and hydrophobicity of the charred material. The ratios of H, O, and C can also be used to differentiate between materials obtained by different processes. In the view of C sequestration and for material with complex aromatic structure and low presence of functional groups, optimum ratios of H/C and O/C are approximately ⩽0.6 and ⩽0.4, respectively [38]. Nitrogen in biochar is an important nutrient, its concentration is related to the concentration in the starting material, with values between 1.8 and 56 g·kg^{-1}, although N in biochar is in a form often not readily bioavailable [39]. The C/N ratio, an indicator of the bioavailability of an organic compound, is highly variable and ranges between 7 and 500 [38].

The results of elemental analyses of the two biochars investigated in this study are reported in Table 4. Both biochars are characterised by values well below the limits established by the EBC, in particular the olive and hazelnut biochars have high values of C and low H/C and O/C ratios. A low H/C ratio indicates that the produced biochars are also recalcitrant to microbial degradation. These results indicate that our production process yield high-quality biochars with a level of carbonisation that makes it suitable for C sequestration, as confirmed by the H/C ratios.

Table 4. Elemental analyses from EBC (Method DIN 51732).

Elements	Units	Hazelnut Biochar	Olive Biochar	EBC Biochar Base	EBC Biochar Premium
H (Hydrogen)	% w/w	1.21	1.58	-	-
C (Carbon, total)	% w/w	78.1	90.1	>50	>50
N (Nitrogen, total)	% w/w	0.64	0.42	-	-
O (Oxygen)	% w/w	1.2	1.7	-	-
Carbonate as CO_2	% w/w	2.62	1.17	-	-
Carbonate (organic)		75.5	89.8		
H/C ratio (molar)		0.18	0.21	<0.6	<0.6
O/C rate (molar)		0.012	0.014	<0.4	<0.4
Sulphur (total)	**% w/w**	**0.07**	**<0.03**		

3.3. Nutrients and Trace Elements

Biomass residues containing high concentrations of minerals, such as those obtained from herbaceous plants produce biochars with high ash content [32], maintain in the biochar matrix most of the nutrients present in the starting material (Table 5). These types of biochar have a lower total carbon (TC) content and cohesion than those obtained from wood-pruning biomass. The low C content, together with elevated concentrations of nutrients, makes biochars from herbaceous material more readily available for microorganisms [2]. The concentration of phosphorus (P) and potassium (K) in the biochar is related to the initial content in the feedstock. The content of P and K are typically between 2.7 and 480 g· kg^{-1} and 10 to 58 g· kg^{-1}, respectively [39].

Table 5. Determination from microwave digestion (method: DIN 22022-1).

Elements	Units	Methods	Hazelnut Biochar	Olive Biochar	EBC Biochar Base	EBC Biochar Premium
P (Phosphorus)	mg/kg	ISO 11885	590	330	-	-
Mg (Magnesium)	mg/kg	ISO 11885	2900	1400	-	-
Ca (Calcium)	mg/kg	ISO 11885	38,000	11,000	-	-
K (Potassium)	mg/kg	ISO 11885	5500	3500	-	-
Na (Sodium)	mg/kg	ISO 11885	2100	260	-	-
Fe (Iron)	mg/kg	ISO 11885	6500	1500	-	-
Si (Silicon)	mg/kg	ISO 11885	25,000	9700	-	-
S (Sulphur)	mg/kg	ISO 11885	910	200	-	-
Pb (Lead)	mg/kg	ISO 17294-2	66	20	<150	<120
Cd (Cadmium)	mg/kg	ISO 17294-2	<0.2	<0.2	<1.5	<1
Cu (Copper)	mg/kg	ISO 17294-2	100	6	<100	<100
Ni (Nickel)	mg/kg	ISO 17294-2	9	8	<50	<30
Hg (Mercury)	mg/kg	DIN EN 1483	<0.07	<0.07	<1	<1
Zn (Zinc)	mg/kg	ISO 17294-2	340	84	<400	<400
Cr (Chromium total)	mg/kg	ISO 17294-2	22	15	<90	<80
B (Boron)	mg/kg	ISO 17294-2	32	10	-	-
Mn (Manganese)	mg/kg	ISO 17294-2	350	380	-	-

EBC biochar base and premium report the limits required by the EBC protocol of certification. The total ash content ranged between 6.2% and 18.8% (w/w) for biochar from pellets of olive and hazelnut wood. The nutrient content is much greater in hazelnut biochar than olive, which was evident especially for Mg, Ca, Fe, S, Cu, and Zn. Biochar from hazelnut pellets could bring a greater contribution of nutrients to the soil and, therefore, be less resistant to microbial decomposition. Heavy metal content in both biochars was well below the EBC limits. Only Cu in the hazelnut biochar was close to the maximum value established by the EBC.

3.4. PAHs (Polycyclic Aromatic Hydrocarbons) Composition

PAHs are ubiquitous in the environment, being by-products of the incomplete combustion of organic material [40]. The chemical structure of PAHs makes them highly resistant to biodegradation and oxidation [41]. The presence of PAHs in pyrolytic reactions above 700 °C is well established [42], although they can be produced in pyrolysis reactions of less than 700 °C at low concentration [43]. It is, therefore, critical to ensure PAH concentrations remain below the limits established by the EBC. The 16 priority US EPA PAHs are typically used to assess the total PAH content; the limits established by the EBC are of <12 and <4 mg/kg for biochar standard and premium, respectively. The PAH composition of the two biochars analysed in this study (Table 6), shows that both biochars are well below the EBC limits, with values ranging from <0.1 to 1.1 mg/Kg.

Table 6. PAHs determination from toluene extract.

Elements	Units	Methods	Limits		Hazelnut Biochar	Olive Biochar
			GW 1 *	GW 2 *		
Naphthalene	mg/kg	DIN EN 15527	-	-	0.9	1.1
Acenaphthylene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Acenaphthene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Fluorene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Phenanthrene	mg/kg	DIN EN 15527	-	-	0.3	0.3
Anthracene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Fluoranthene	mg/kg	DIN EN 15527	-	-	0.1	0.1
Pyrene	mg/kg	DIN EN 15527	-	-	0.1	0.1
Benz(a)anthracene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Chrysene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Benzo(b)fluoranthene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Benzo(k)fluoranthene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Benzo(a)pyrene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Indeno(1,2,3-cd)pyrene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Dibenz(a,h)anthracene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
Benzo(g,h,i)perylene	mg/kg	DIN EN 15527	-	-	<0.1	<0.1
SUM PAHs (EPA)	mg/kg	calculated	<12	<4	1.20	1.60

* (GW 1 = quality level basic related dry bases; GW 2 = quality level premium related dry bases).

Total PAH content of the two biochars are 1.2 and 1.6 mg/kg for olive and hazelnut, respectively. Therefore, both biochars can be considered suitable for soil applications, since both are well below the EBC threshold limit of 4 mg/kg for biochar premium.

3.5. pH, Electrical Conductivity (EC), and Density

In general, the pH of biochar is relatively homogeneous and varies from neutral to basic pH. Feedstock of various origins produce biochar with an average pH between 6.2 and 9.6 [39]. Lower pH is typically found for biochars obtained from green pruning feedstock and organic waste, while the highest values are to be attributed to poultry litter biochar. The Table 7 reports the elements values, according to their pH, electrical conductivity, salt content and density.

The two biochar have a pH of 8.4 and 9.9 for olive and hazelnut, respectively. The EBC indicates a maximum limit of 10; therefore, biochar produced from these types of wood residues is slightly below the limit established by the certification. The EC is of particular importance when adding biochar to soils with high EC and salinity. The two biochars had an EC of 217 and 332 mS/cm, respectively, for olive and hazelnut (as shown in Table 7). Both values are very low and do not represent a real risk for the addition to soil even under conditions of high EC. In general, biochar has a lower density than soil, with an average of 0.4 g·cm^{-3} compared to a soil of medium texture, with average of 1.3 g·cm^{-3}. When adding biochar to soils with little ventilation, this property can help to reduce the density by

mitigating issues related to the compaction of soil. The olive and hazelnut biochars produced in this study have a density of 0.45 and 0.44 g·cm^{-3}, respectively.

Table 7. Elements value (pH, electrical conductivity, and density).

Elements	Units	Hazelnut Biochar	Olive Biochar
pH values ($CaCl_2$)	-	9.9	8.4
Electrical conductivity	µS/cm	332	217
Salt content	g/kg	0.655	1.18
Salt content cal. with bulk density	g/L	0.287	0.527

4. Conclusions

The two biochars analysed in this study show excellent physicochemical properties, which makes them suitable for agricultural applications. Both biochars can be certified as Biochar Premium according to the regulations of the EBC; this allows a potential commercialisation of the biochars, with higher prices than Biochar Base, typically less expensive, but with a higher content of PAHs. The benefits of using Biochar Premium as soil fertiliser includes improved productivity, increased water holding capacity of the soil (e.g., [44–46]), and a better retention of nutrients and agrochemicals in soils, all of which should offset initial investment and provide added profits per application. Biochar fuel commands a high-value application, offering numerous benefits, and an authentic alternative to develop the biomass utilization efficiency [4,47]. The added value of biochar is also linked to other issues, such as those involving agricultural and environmental sustainability. As claimed by many studies [1,16], biochar as a soil amendment can improve soil health and increase agricultural productivity with further environmental benefits related to global warming mitigation [16,48–52]. Based on our results, we intend to define an agro-forestry chain to use the residual waste biomass for the production of high quality biochar for agronomic and commercial purposes. We are proceeding to evaluate the properties of biochar for soil improvement.

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