

**Department of Applied Chemistry**

**Polar Constituents of Oils and Bitumens – New Applications  
to Petroleum Geochemistry and (Palaeo)Biogeochemistry**

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**This thesis is presented for the degree of  
Doctor of Philosophy  
of  
Curtin University of Technology**

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**Dedicated to Lisa**

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## Declaration

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To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

Christian Olivier Eduard Hallmann

Perth, July 1<sup>st</sup> 2008

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Polar organic molecules are defined by differences in electronegativity between their atomic constituents and the resulting asymmetrical structures. They represent the basic chemical building blocks of life. Having a strong affinity to water (H<sub>2</sub>O), which is essential for life on Earth, polar molecules are studied by the discipline of biochemistry and their origin, distribution, and function in living systems is relatively well understood. Polar constituents of sedimentary organic matter and petroleum have been previously studied but they are, in general, yet far from being understood. They can be present as primary biogenic molecules, rearranged biogenic molecules, or secondary functionalized hydrocarbons. The studies compiled in this thesis use selected polar organic compounds as molecular tools: phospholipids as indicators of biomass, high-molecular-weight polycyclic aromatic hydrocarbons as combustion markers, phenols as indicators of oil-water interaction processes, and carboxylic acids in general.

Chapter 2 studies the biological oxidation of petroleum accumulations; a process mediated by microbes that inhabit the deep subsurface and affect the long-term storage of living carbon as sedimentary biomass of the 'deep biosphere'. The results presented in chapter 2 suggest that intact bacterial cells are present in biodegraded petroleum, as indicated by the detection of membrane lipid fragments, termed phospholipids, in these oil samples. Carboxylic acids released from phospholipids (i.e. phospholipid fatty acids, PLFA) in oil samples vary in concentration (~2.0 - ~10.0 µg/g oil) and composition (*i*-C<sub>14:0</sub> dominated vs. *i*-C<sub>15:0</sub> and *i*-C<sub>17:0</sub> dominated) during progressive petroleum biodegradation, thereby showing that the microbial community increases in size during the removal of petroleum constituents, and that the community structure changes. Not one but at least two structurally different microbial consortia are shown to be responsible for petroleum degradation. Chapter 4 evaluates the rapid oxidation of biomass during the impact of an extraterrestrial bolide, which occurred during the late Neoproterozoic. The co-occurrence of a -3.5‰ negative sedimentary stable carbon

isotope excursion and a molecular combustion-marker anomaly (coronene; 0.48 ppb, relative to a 0.04 ppb background), which are followed by a diversification of Acritarch species, suggests that combustion of 'early' terrestrial and marginally-marine biomass might have caused extensive smoke and atmospheric dimming, as well as subsequent photosynthetic stress. Moreover, the sharp combustion marker anomaly can probably provide a long-sought chronostratigraphic marker for the late Neoproterozoic, when also detected in other locations around the globe. Chapter 5 evaluates the effects of petroleum interaction with water. For this purpose oils produced from one reservoir were monitored during a 335-day period following the rationale that oil-water interaction increases during petroleum production. Based on a selective depletion of volatile aromatics and invariant phenol concentrations the results exclude both evaporative and oil-water partitioning processes. Petroleum compositional changes, recorded mainly in the low-molecular-weight aromatic and phenol fractions, were tentatively attributed to abiotic oxidation processes. Furthermore, methodological advances in the analysis of carboxylic acids of low molecular weight, evaluated for the execution of the other studies, are presented in chapter 4. Overall, the presented results shed more light on carbon export fluxes from different sedimentary carbon reservoirs by shedding new light on deep biosphere metabolism, elucidating the significance of the Neoproterozoic Acraman impact event, and contributing to our knowledge of petroleum destruction through its interaction with water in sedimentary basins. Moreover, they show that, in contrast to traditional belief, NSO compounds in oils and bitumens can form useful molecular tools to study questions in petroleum geochemistry, biogeochemistry, and palaeobiogeochemistry. Understanding the size of carbon reservoirs and fluxes on Earth, as well as the mechanisms that cause these carbon fluxes, can increase our appreciation of global biogeochemical cycling and, in turn, explain ecosystem dynamics, past evolutionary events, and predict future change of current climatic conditions.

## Preface & Acknowledgements

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Driving North over a lonely Pilbara highway I get to write down the final lines of this thesis on an 'outback-stained' paper notebook. The last weeks before a doctoral thesis submission deadline are not the ideal period for a fieldtrip, but the opportunity to visit the 'Trendall locality', home to some of Earth's oldest microfossils, was just too tempting. Although my doctoral candidature did not start with a research inclination towards palaeontology, during the last three years I had the unique opportunity to shape the direction of my research focus and initiate studies that cover different areas within the field of organic geochemistry. While rather unorthodox, this approach has helped me to find my own scientific path, which I will continue after the submission of this thesis. Many achievements would not have been possible without the help and support of colleagues and friends. The attempt to acknowledge them all follows here but I apologize in advance for anyone I have forgotten – you can be sure that your help was appreciated!

First of all I thank Kliti Grice for taking me in to her group and, most of all, for giving me all the scientific freedom I could have asked for. Many discussions helped me to '*keep focused!*' and even outside Uni I could always count on her help when I needed it. Thanks! In this incipency, I also thank Ben van Aarssen and Trevor Bastow, who actually 'lured' me into Curtin University after a number of beers during the 2003 IMOG in Krakow. Ben became my thesis supervisor until he left Curtin for Chevron, but continued to be an adjunct supervisor of my project. Andrew Murray, who became my co-supervisor, is responsible for introducing me to Ben and Trevor and I thank him for advice and discussions on the petroleum side of this thesis.

Only two months after the start of my PhD I left Perth to visit the University of Newcastle upon Tyne, UK, where I learned the basics of molecular microbial ecology, funded by a Marie Curie fellowship scheme. I am grateful to Ian Head for hosting me in his lab and for valuable discussions on the topic, to Geoff Abbott and Yvonne Hall for organizing everything, and most of all to Arlene Rowan, who taught me the practical aspects of laboratory work in molecular microbial ecology. Many thanks go also to Angie Brown, who I could always bother with (what must have been the most stupid) questions on microbiology, and all the other members of the team for their helpfulness: Joanna Baptista, Neil Gray, Darryl Nelson, Fiona Read, Ivana Brito, Russell Davenport, Emma Bowen, and Helen Talbot. Maik Inthorn, Henning Peters, Johan Kool, and Viktoria Feigl made my stay a true pleasure with the many evenings we spent together, improving our cooking skills or watching the latest episodes of 'Tiki Bar TV'. Thanks also to Ameera Al-Hassan for being a great housemate, and teaching me the basics of Arabian language and Kuwaiti cuisine.

My second outbound research project was a 'home match', conducted at the University of Cologne. I thank the EAOG for their financial support and Lorenz Schwark for hosting me and for laying the fate of his lab in my hands - fortunately everything survived unscathed. I do miss the strict routine of 11.30 am lunch breaks and the subsequent coffee discussions with Nicole Juraschek, Eva Lehndorff, Anne Zacke, and Hanna Cieszynski. Thanks to Oliver Paech and Sarah Wurth for making sure that life in the lab didn't get boring. Hanna and Piotr Cieszynski are thanked for their enormous help with getting the 'Engine' (GC-MS) to work properly.

The last three years in Perth are an unforgettable experience, and mainly shaped by the many fantastic people I have met. I thank all of you, in particular (in tentative chronological order): Ryan Chester, my housemates from the 'Pinedale Palace', in particular Coleen Lee and Ben Halde, Christiane and Sebastian Vitzthum von Eckstaedt, the Kaur sisters Gurpreet and Jaspreet, Clare Gibson, Birgit Nabbefeld, Clara Loi, Paul Pringle, Jono Morton, Ercin Maslen, Christoph Hellmann, Christiane Eiserbeck and Ramon Wenzel, and the great German poet Thomas Schumacher.

I am extremely grateful for the help I received from colleagues. Geoff Chidlow, Kieran Pierce, and Sue Wang are thanked for flawless analytical support. All members of our group, in particular Daniel Dawson and Amy Bowater, are thanked for methodological advice and help during daily lab work. Khaled Arouri, Chris Boreham, James Burgess, Bob Davis, Volker Diekmann, Simon George, Paul Greenwood, Kath Grey, Erdem Idiz, Amy Kelly, David McKirdy, Dariusz Strapoc, Roger Summons, Paul Taylor, Herbert Volk, and Lynn Webster (in alphabetical order) are thanked for sharing samples, discussing problems, or helping with additional analyses.

My family, in particular my parents and my brother Matthias, are thanked for their ineffable love, continuous interest, and support.

At last I wish to thank all the fantastic people who joined the Eendracht expedition, in particular my 'buddy' Ottover and my 'surrogate buddy' Zoja, who made this trip one of the very best excursion I have ever participated in and a perfect finale to my years in Perth. What better concluding words can I find than *Chelsea, Chelsea...*

Christian Hallmann  
Perth, June 2008



This thesis is assembled by publications, either published, in press, in review, or to be submitted, which form the individual chapters and are listed below.

### CHAPTER 2

Hallmann, C., Schwark, L., and Grice, K. Community dynamics of anaerobic bacteria in deep petroleum reservoirs. *Nature Geoscience* **1** (9), 588-591 (2008).

### CHAPTER 3

Hallmann, C., van Aarssen, B.G.K., and Grice, K. Relative efficiency of free fatty acid butyl esterification. Choice of catalyst and derivatisation procedure. *Journal of Chromatography A* **1998-1999**, 14-20 (2008).

### CHAPTER 4

Hallmann, C., Grey, K., Webster, L., McKirdy, D., and Grice, K. Molecular signature of the Neoproterozoic Acraman impact event. *Earth and Planetary Science Letters*. Accepted for publication.

### CHAPTER 5

Hallmann, C., Davis, R., Grice, K., van Aarssen, B., and Murray, A. Comprehensive evaluation of 4D-reservoir geochemistry. Molecular and isotopic response of low-molecular-weight petroleum constituents.

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The work presented in this thesis was primarily designed, experimentally executed, interpreted, and written by the first author of the individual manuscripts. Contribution by colleagues is described here.

### CHAPTER 2

Christian Hallmann, Kliti Grice, and Lorenz Schwark designed experiments. Christian Hallmann performed experiments and interpreted results. Lorenz Schwark provided samples and analytical facilities. Christian Hallmann, Lorenz Schwark, and Kliti Grice wrote the paper. Paul Greenwood is acknowledged for discussions on experimental methodology. The project was executed at the University of Cologne and funded by a travel grant from the European Association of Organic Geochemists to Christian Hallmann.

### CHAPTER 3

Christian Hallmann designed experiments, performed experiments, interpreted results, and wrote the paper. Ben van Aarssen provided intellectual input through discussions on analytical methodology. Ben van Aarssen and Kliti Grice contributed to scientific discussions and writing the paper, and provided analytical facilities.

### CHAPTER 4

Christian Hallmann designed experiments, performed experiments, interpreted results, and wrote the paper. David McKirdy, Lynn Webster, and Kathleen Grey provided samples. David McKirdy, Kathleen Grey, and Kliti Grice provided intellectual input through discussions on results and contributed to writing the paper. Kliti Grice provided analytical facilities.

## CHAPTER 5

Christian Hallmann, Ben van Aarssen, and Robert Davis designed experiments. Christian Hallmann performed experiments, interpreted results, and wrote the paper. Kliti Grice contributed to writing the paper and provided scientific feedback.

Manuscripts and abstracts based on research that was conducted during the preparation of this thesis, but not included in the thesis.

### Peer-reviewed

Grice, K., Lu, H., Atahan, P., **Hallmann, C.**, James, E., Greenwood, P., Maslen, E. and Dodson, J. Perylene: a Molecular Marker for Lignin Degradation and the Evolution of Vascular Plants. *Geochimica et Cosmochimica Acta*. In Review.

Maslen, E., Grice, K., Gale, J., **Hallmann, C.**, and Horsfield, B. Crocetane: A potential marker of photic zone euxinia in thermally mature sediments and crude oils of Devonian age. *Organic Geochemistry* **40**, 1-11 (2009)

Schwark, L. and **Hallmann, C.** Secular variation of alkyl-carbazoles in oils and sediments. In Preparation.

Brown, A., Gray, N., Rowan, A., Aitken, C., Jones, D., Roeling, W., **Hallmann, C.**, Larter, S., Bowler, B., and Head, I. Anaerobic degradation of crude oil via sulphate-reduction in a laboratory microcosm study. In Preparation.

## Abstracts

**Hallmann, C.**, Grice, K., and Schwark, L. (2008) Microbial population dynamics in deep oil reservoirs – Insights from microbial membrane lipids. *GRC Organic Geochemistry, Holderness, NH (invited). Oral presentation.*

Grice, K., Lu, H., Atahan, P., **Hallmann, C.**, James, E., Greenwood, P., Dodson, J. (2008) Perylene: a molecular marker for lignin degradation and a signal for the rise of vascular plants. *Australian Earth Sciences Conference, Perth. Keynote presentation.*

**Hallmann, C.**, Grey, K., Webster, L., McKirdy, D., and Grice, K. (2008) Signature and significance of the Neoproterozoic Acraman impact event. *Australian Organic Geochemistry Conference, Adelaide. Poster presentation.*

**Hallmann, C.**, van Aarssen, B., Davis, R., and Murray, A. (2007) 4D reservoir geochemistry – The behaviour of phenols and carboxylic acids during petroleum production. *23<sup>rd</sup> International Meeting on Organic Geochemistry, Torquay, England. Poster presentation.*

**Hallmann, C.**, van Aarssen, B., and Grice, K. (2007) Rapid and efficient protocol for the semi-quantitative analysis of C<sub>2+</sub> carboxylic acids in crude oils. *23<sup>rd</sup> International Meeting on Organic Geochemistry, Torquay, England. Poster presentation.*

Byrne, K., McKirdy, D., **Hallmann, C.**, Arouri, K., Schwark, L., Tingate, P., and Boulton, P. (2007) Molecular signatures of sequentially extracted residual oils reveal Cambrian petroleum in the Cooper and Eromanga Basins, South Australia. *23<sup>rd</sup> International Meeting on Organic Geochemistry, Torquay, England. Poster presentation.*

**Hallmann, C.**, Grice, K., and van Aarssen, B. (2007) Carbon isotopic determination of the origin of crude oil carboxylic acids. *23<sup>rd</sup> International Meeting on Organic Geochemistry, Torquay, England. Poster presentation.*

Schwark, L. and **Hallmann, C.** (2007) Secular variation in the relative composition of dimethylated carbazoles. *23<sup>rd</sup> International Meeting on Organic Geochemistry, Torquay, England. Poster presentation.*

**Hallmann, C.**, Grice, K., and Schwark, L. (2007) Molecular and isotopic study of acidic metabolites in the deep biosphere employing petroleum reservoirs as naturally occurring bioreactors. *Goldschmidt Conference, Cologne, Germany. Oral presentation.*

- Hallmann, C.**, Arouri, K., McKirdy, D., and Schwark, L. (2006) A new perspective on exploring the Cooper/Eromanga petroleum province – Evidence of oil charging from the Warburton Basin. *APPEA Conference and Exhibition, Gold Coast, Australia. Oral presentation.*
- Hallmann, C.**, McKirdy, D. and Schwark, L. (2006) Variations in gross geochemical and carbazole signature of sequentially extracted bitumens from an intact pore system reflect adsorption processes during initial reservoir wetting. *Australian Organic Geochemistry Conference, Perth, Australia, Book of abstracts, p.91-92. Oral presentation.*
- Rowan, A.K., Aitken, C.M., **Hallmann, C.**, Gray, N.D., Kolukirik, M., Bennett, B., Larter, S.R., Jones, D.M., Head, I.M. (2006) Differential patterns of crude oil degradation under sulfate-reducing and methanogenic conditions. *GRC on Organic Geochemistry, Holderness, NH (unpublished). Poster presentation.*
- Hallmann, C.** and van Aarssen, B. (2006) Rearranged and secondarily functionalised hydrocarbons as thermally stable biological markers. *GRC on the Origin of Life (unpublished). Poster presentation.*
- Hallmann, C.** (2005) Polar hetero compounds in oil-source rock correlation studies. *AAPG-GIA*. Available from: [http://www.searchanddiscovery.com/documents/abstracts/2005grants\\_in\\_aid/hallmann.htm](http://www.searchanddiscovery.com/documents/abstracts/2005grants_in_aid/hallmann.htm)



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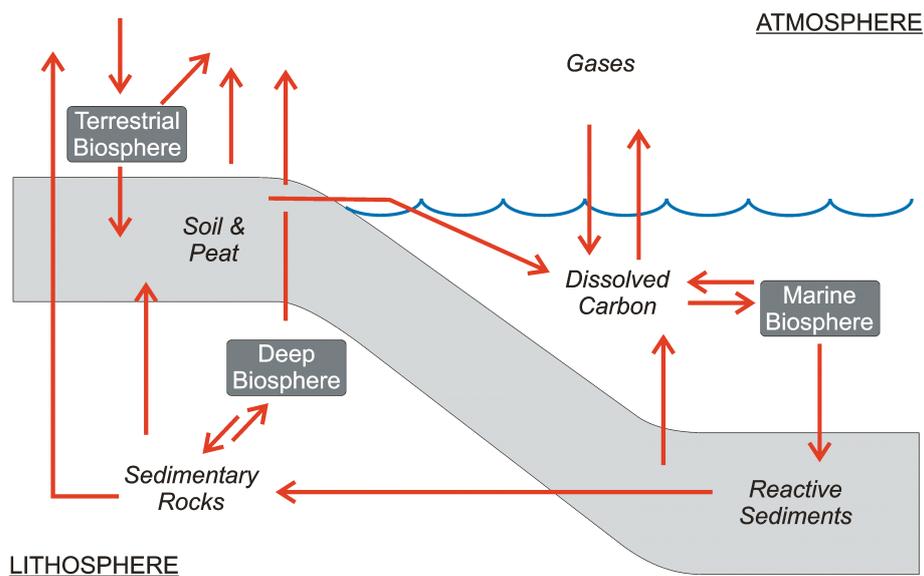
## Introduction and overview.

### **Organic geochemistry and the carbon cycle**

Carbon is the chemical basis for the evolution of life on Earth. It is the major chemical constituent of most organic matter, from petroleum to the complex molecules (DNA, RNA, and proteins) that control genetic reproduction in organisms. Carbon is stored in several major sinks: (i) biochemicals in living and dead organisms in the biosphere (ii) gaseous carbon dioxide in the atmosphere (iii) in the oceans it occurs as dissolved (inorganic) atmospheric carbon dioxide ( $\text{CO}_2\text{-H}_2\text{CO}_3\text{-HCO}_3^-\text{CO}_3^{2-}$  equilibrium) and as settling calcite shells of marine organisms, and (iv) sedimentary organic matter, carbonates, and fossil fuel. The largest store (66,000,000 to 100,000,000 billions of metric tones) of carbon is trapped in sedimentary rocks. Carbon is transferred between the biosphere, geosphere, and atmosphere by various fluxes (Killops and Killops, 2005; Berner, 2004; [Figure 1.1](#)). Understanding the magnitude of carbon reservoirs and fluxes, as well as flux mechanisms, is important when reconstructing the dynamics of biogeochemical cycling on Earth. Since  $\text{CO}_2$  is one of the most important greenhouse gases, the size and dynamics of the atmospheric carbon reservoir have received significant attention in recent years. A greater understanding of  $\text{CO}_2$  cycling on Earth can help to understand ecosystem dynamics and can be used to reconstruct past evolutionary events, and predict future climatic trends.

The discipline of organic (bio)geochemistry, which forms the core of the studies presented in this thesis, studies the carbon of living and dead biomass; its fate, sequestration, and release – broadly, the cycling of carbon on Earth.

The studies presented in this thesis focus on carbon export fluxes in different sedimentary sub-reservoirs of the carbon cycle: the biogenic (Chapter 2) and abiotic (Chapter 5) conversion of fossil fuels to atmospheric methane and CO<sub>2</sub>, and the pyrolytic conversion of sedimentary organic matter to atmospheric CO<sub>2</sub> (Chapter 4). Combined, these studies try to shed more light on present and past carbon dynamics. The remainder of this chapter will briefly present the concept and results of the individual studies and illustrate them within the context of the global carbon cycle. The individual studies are closely related by a methodological approach of using specific polar organic compounds as molecular tools to answer the questions posed (see below).



**FIGURE 1.1** Simplified depiction of the global biogeochemical carbon cycle. Only selected carbon fluxes are shown. Modified after Kempe (1979) and Killops and Killops (2005).

## **Sedimentary carbon fluxes**

Sedimentary rocks represent Earth's largest carbon reservoir (Kempe, 1979; Killops and Killops, 2005), but can be further subdivided into carbonate rocks, organic-rich source rocks, disperse sedimentary organic matter, (shallow) reactive sediments, sedimentary petroleum accumulations, and biomass of the deep biosphere (e.g. Killops and Killops, 2005). The deep biosphere is of particular interest since it forms part of both the lithosphere and the biosphere.

### Petroleum reservoirs

Petroleum source rocks are sedimentary deposits that contain organic matter of sufficient quality and quantity to allow the expulsion of petroleum under certain pressure and temperature conditions (Peters and Moldowan, 1993; Hunt, 1996). This is in contrast to disperse sedimentary organic matter, which does not suffice to expel petroleum of commercial significance. Source rocks form a sedimentary carbon reservoir but can be subject to a carbon export flux when they release carbon in the form of bitumen that migrates away from the source rock in the form of petroleum (Peters and Moldowan, 1993; Hunt, 1996; Killops and Killops, 2005). When trapped in the subsurface, petroleum forms a fossil carbon reservoir again. Seal failure or tectonic uplift can however re-release the stored petroleum to the surface (Magoon and Dow, 1994) where it will be subject to biological oxidation (Atlas, 1995), photo-oxidation (Lee, 2003), and evaporation (McDonald et al., 1983); processes that eventually convert the carbon into CO<sub>2</sub> thereby allowing it to be added back to the atmospheric carbon reservoir. Carbon export from petroleum reservoirs can also occur by direct oxidation processes in the subsurface, however with notably slower reaction rates.

### Biotic oxidation of petroleum

One of the processes capable of inducing a carbon export flux from petroleum reservoirs is the subsurface biological degradation of petroleum (i.e. *biodegradation*; e.g. Peters and Moldowan, 1993 and references therein; Head et al., 2003). Complex consortia of anaerobic microbes conquer the physicochemical boundaries of life deep beneath the Earth's surface (e.g. D'Hondt et al., 2002;

Kashefi and Lovley, 2003; Schippers et al., 2005) and use sedimentary carbon, sedimentary bitumen and, if present, petroleum, as a growth substrate (Head et al., 2003; Jørgensen and D'Hondt, 2006). The catabolism of certain petroleum constituents can result in the generation of large quantities of biogenic methane (Larter et al., 2005), smaller amounts of larger hydrocarbon gases (Hinrichs et al., 2006), and low-molecular-weight metabolites (such as organic acids) as by-products (e.g. Wilkes et al., 2003; Aitken et al., 2004). Under ideal circumstances the methane can accumulate as a gas cap above the oil leg (Peters and Moldowan, 1993; Hunt, 1996), but often the increased reservoir pressure induces seal failure and allows the methane to escape and migrate up-dip, where it can be biogenically oxidized (Orphan et al., 2001) to CO<sub>2</sub> and be added back to the atmospheric carbon reservoir. If no seal failure occurs, gas cap expansion can cause the oil leg to pass the reservoir spill point, thereby inducing petroleum remigration (Magoon and Dow, 1994; Hunt, 1996) and again leading to eventual oxidation and atmospheric escape. Moreover, the low-molecular-weight metabolites produced during biodegradation are capable of fuelling the deep microbial biosphere (Parkes et al., 2007) or being transported away after the partitioning into formation waters due to their increased polarity after biogenic oxidation (Jones et al., 2001).

Little is yet known on the factors that control the extent and activity of this deep biosphere. An increased understanding of the topic would be beneficial for the petroleum industry if biodegradation could be efficiently predicted pre-drill (Head et al., 2003), but it would also allow us to construct a more precise model of carbon reservoirs and fluxes on Earth, thereby improving our ability to predict climate dynamics. **Chapter 2** focuses on the dynamics of petroleum degrading bacteria in deep petroleum reservoirs. It presents the first geochemical evidence for the existence of phospholipids in petroleum, indicative of intact bacterial cells, and shows how the bacterial community changes in size and structure during the progressive biological degradation of petroleum. The composition of phospholipid fatty acids reported in **chapter 2** allows speculations on the bacterial members of microbial consortia that are active during, respectively beyond, the degradation of petroleum *n*-alkanes and contributes to the identification of the yet largely unknown deep microbiosphere.

### Abiotic oxidation of petroleum

Petroleum constituents can also be removed from reservoir petroleum accumulations through abiotic processes. Hydrophilic constituents of petroleum are typically found in oil field formation waters (Dale et al., 1995; Love et al., 2003), but their origin is contested. A partitioning equilibrium was suggested (Lafargue and Barker, 1988), causing the transfer of hydrophilic components from the petroleum phase to the adjacent water phase; a process commonly referred to as water washing. After partitioning components can be transported away by subsurface water flow. A metastable equilibrium between petroleum, water, and sediments, was proposed (e.g. Helgeson et al., 1993), which would involve the chemical conversion of petroleum constituents, followed or not by partitioning equilibrium processes. **Chapter 5** is based on the assumption that the interaction of oil and water can be most efficiently studied in the subsurface (*in situ*), during periods of enhanced oil-water contact, since laboratory studies of water washing proved to be inefficient models of subsurface processes (de Hemptinne et al., 2001). During the production of petroleum, a rise of the oil-water contact (OWC) is often observed (e.g. Staples et al., 2005) and interpreted to result in an increased surface contact area between the oil phase and the water phase. The samples studied in **chapter 5** represent a time series of oils produced over a period of 335 days from the same reservoir. Compounds thought to be sensitive to oil-water interaction were monitored and the results presented in **chapter 5** exclude water washing and evaporation processes as mechanisms acting in subsurface reservoirs during petroleum production. A change in petroleum composition was tentatively attributed to abiotic oxidation processes, which would support the theory of a metastable equilibrium, and adds valuable knowledge on the mode of carbon export flux from petroleum reservoirs.

### Rapid oxidation of biomass and reactive sediments

The majority of dead organic matter (OM), i.e. necromass, in aquatic environments is remineralized back to CO<sub>2</sub>, and only a small fraction of less than 0.1% on average (Holser et al., 1988) eventually accumulates in sediments. Remineralization of OM causes a slow but steady addition of CO<sub>2</sub> to the atmosphere, more or less in pace with biological CO<sub>2</sub> uptake. Certain events can however oxidize large amounts biomass, necromass, and sedimentary OM and

cause a massive input of CO<sub>2</sub> to the atmosphere, with consequences for climatic conditions and the biosphere. On a small scale, forest fires are such events. Operating on a much larger scale, and therefore more significant, are magmatic intrusions into organic-rich strata and extraterrestrial bolide impacts (van Breugel, 2006, and references therein). Tectonic events and changes in sea level can have profound effects on the weathering-induced release of carbon stored in carbonate rocks.

Negative shifts in sedimentary stable carbon isotope ratios ( $\delta^{13}\text{C}$ ) are often attributed to an increase in atmospheric CO<sub>2</sub> and subsequent climate warming (e.g. van Breugel, 2006). **Chapter 4** describes Ediacaran samples from the interior of Australia that record an extraterrestrial bolide impact, a negative stable carbon isotope excursion, and a radiation of extinct Acritarch species. Moreover, the studied sediments briefly predate the explosive radiation of animal species that occurred during the latest Neoproterozoic (Marshall, 2006). The results presented in **chapter 4** correlate the impact event to the negative  $\delta^{13}\text{C}$  shift by suggesting a massive combustion of early terrestrial and marginally marine OM (i.e. biomass, necromass, and reactive sedimentary OM) caused by the impact of an extraterrestrial bolide. Molecular combustion markers (e.g. coronene) suggest long-term soot fallout, which would have caused environmental pressure on phototrophs and might be related to the observed Acritarch radiation. The results presented in **chapter 4** provide an important analogue for the study of younger impact events and their consequences for climate and biosphere dynamics.

### The Deep Biosphere

Until the 1970s, life on Earth was believed to be restricted to the surface, upper oceans, and the shallow subsurface (i.e. soil). This view changed dramatically when Corliss and colleagues in 1979 described a complex ecosystem near hydrothermal vents of the Galápagos rift area (Corliss et al., 1979). The animal communities appeared to be based on sulfur-oxidizing bacteria that performed chemosynthesis rather than photosynthesis, and sparked interest in life in hostile and extreme environments. One of these environments is the deep subsurface, where microbes live independent of sunlight under conditions of increased temperature and pressure (Gold, 1992). Almost 30 years later, the deep biosphere forms a prime research object and is recognized to represent a major carbon reservoir, possibly

accounting for > 90 % of global prokaryotic biomass (Head et al., 2003). Nevertheless, questions regarding the identity, function, and physiology of microbes in the deep subsurface still remain largely unanswered. The deep biosphere is thought to heavily influence biogeochemical cycles (Whitman et al., 1998), in particular those related to biological nitrogen and phosphorus (Head et al., 2003), and possibly exerts control on climate dynamics (IODP, 2001). A greater comprehension of subsurface microbial dynamics is therefore of high importance to apprehend the future extent of Earth's current 'climate crisis'. The answers sought are partially dependent on improving current analytical techniques (e.g. Kuypers and Jørgensen, 2007). The discovery of microbial membrane lipids in oils as reported in **chapter 2** represents one such innovation. Studies focusing on the deep biosphere used to analyze sediments or oil field waters. While the former are typically characterized by low cell counts, the latter are prone to contamination from drilling fluids. Biodegraded petroleum was until recently never considered a focus of microbiological studies because molecular biological techniques are severely restricted in the presence of petroleum. By showing that petroleum reservoirs can be utilized as 'naturally-occurring bioreactors' to study the metabolism and ecology of microbes inhabiting the deep subsurface in sufficient detail using organic geochemical methods, the results presented in **chapter 2** present a methodological advance that is expected to stimulate future research on the microbiology of deep petroleum reservoirs.

## **Polar Constituents of Oils and Bitumens**

The work presented in this thesis focuses on specific polar constituents of oils and bitumens and uses these as molecular tools to solve geochemical problems.

### Concept of polarity

The term 'polar' is relatively vaguely defined but generally refers to dipole-dipole intermolecular forces between the atomic constituents of a molecule. Polarity is therefore strongly dependent on the difference in electronegativity between atoms in a molecule and the resulting asymmetry of the compounds structure (McMurry, 2003). One of the most polar compounds in nature is water (H<sub>2</sub>O). The fact that life on Earth depends on water has strong implications for the importance

of molecular polarity, making it roughly synonymous with a terminology of hydrophilic and hydrophobic.

### Defunctionalization of polar biomolecules

Recently deposited and reactive sediments contain the organic remainders of primary producers and sediment dwelling microbes (e.g. Ourisson et al., 1984; Summons, 1993). Under certain conditions of oxygen deficiency, a small part of the organic matter may escape remineralization and become preserved and buried with the sediments. The bulk chemical composition of this bio- and necromass is dominated by polar non-hydrocarbons. Most biomolecules are characterized by a highly polar or amphiphilic character, governed by the basic structure of all living cells on Earth: an aqueous cytoplasm that is shielded from the also aqueous extracellular environment by a hydrophobic cell wall, which mostly consists of an amphiphilic lipid bilayer (Madigan and Martinko, 2006).

The majority of sinking and settling organic matter is subject to rapid biochemical and physicochemical decomposition. Following a 'degradation–recondensation' pathway, some biodegradation products of primary biopolymers can recondense progressively to form a geopolymer, thereby escaping mineralization (Vandenbroucke and Largeau, 2007). These random abiotic recondensation reactions include Maillard reactions (Maillard, 1916), condensation and polymerization of phenol oxidation products (Kononova, 1966), the cross-linking of polyunsaturated fatty acids (Harvey et al., 1983), and sulfur vulcanization reactions (e.g. Sinninghe Damsté et al., 1998; Sandison et al., 2002), amongst others (Vandenbroucke and Largeau, 2007). Recalcitrant structural biopolymers that are present in algae (algaenan), higher plant cuticles (cutan) or periderm (suberan) are not affected by microbial enzymatic attack and accumulate following a 'selective preservation' pathway (Tegelaar et al., 1989).

A portion of functionalized biomolecules undergoes defunctionalization and rearrangement, which results in an array of partly or entirely defunctionalized breakdown products that do not become incorporated into the kerogen (e.g. Brocks and Summons, 2003) but are present as solvent-extractable bitumen throughout and beyond diagenesis.

## Primary and Secondary NSO Compounds

Primary, i.e. biogenic, polar molecules usually do not survive diagenesis without significant alteration, and can thus not be found within the sedimentary carbon reservoir. Exceptions are biolipids that are produced by microbes inhabiting the deep subsurface. Due to extremely slow metabolism (Jørgensen and D'Hondt, 2006), the deep biosphere does not produce a notable addition of organic necromass to subsurface environments as suggested by Gold (1998). However, forming an enormous reservoir of living biomass in the form of microbes – dormant, sporulated, or alive – intact membrane lipids (bacterial phospholipids and archaeal ether lipids) can be extracted from deep sediments and oil reservoirs as described in **chapter 2**.

Initial mineralization and defunctionalization leads to a reduction of the heteroatom content of sedimentary organic matter. A small percentage of polar organic molecules is however involved in recondensation reactions and becomes preserved, either incorporated in the kerogen, or as free compounds within the sedimentary bitumen. Nitrogenous compounds, for example amino acids from protein biodegradation and amino sugars from bacterial cell walls, can easily be incorporated during oxidative polymerisation of phenols (Stevenson and Butler, 1969). Furthermore, inorganic hetero-atoms can become incorporated in organic compounds during rearrangement and recondensation. A good example are sulfides (mainly HS<sup>-</sup> and H<sub>2</sub>S), produced by bacterial reduction of sulfate, which are capable of reacting with functional groups leading to an incorporation into the kerogen matrix through vulcanization reactions (e.g. Sinninghe-Damsté et al., 1998; Sandison et al., 2002). Initial defunctionalization is often paralleled by fragmentation and ring opening, which destroys any recognizable link between a sedimentary component and its biological precursor. Post-diagenetic polar compounds are therefore often rejected as carriers of a modified primary biological signal. In recent years, however, it has been found that polar constituents of bitumens can yield valuable information on biogenic input and depositional conditions. Remnants of the nitrogen-bearing chlorophyll and bacteriochlorophyll molecules (i.e. porphyrins; e.g. Barwise and Roberts, 1984) and their oxidative degradation products (Grice et al., 1996) are widely accepted to carry information on the primary producers, depending on the mode of alkylation. Certain lignin-derived phenols, again recognized by a specific alkylation pattern and stable carbon isotopic value, can be used to reconstruct past vegetation (e.g. Huang et al., 1999). The carbazole

molecular backbone bears resemblance to certain phyto-alkaloids. Certain dimethylated carbazoles can be used as source indicators in oil-source rock correlation studies (Hallmann et al., 2007) and show tentative parallels to the evolution of higher plants (Schwark and Hallmann, unpublished results), which was elaborated as a part of the research performed during the preparation of this thesis.

Post-diagenetic oxidation of hydrocarbons to form polar compounds is debatable (Love et al., 2003), but has been proposed. It generally involves a state of thermodynamical metastable equilibrium (Helgeson et al., 1993) and has been addressed in **chapter 5** of this thesis.

The results presented in the following chapters largely focus on selected polar constituents of oils and bitumens and show that these compounds can be used as valuable molecular tools to answer a variety of geochemical questions. Although in many cases little is known on their mode of formation or rearrangement during diagenesis, the results presented here commend future research using a similar approach.

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## Community dynamics of anaerobic bacteria in deep petroleum reservoirs.

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**Abstract** - The nature, activity, and metabolism of microbes inhabiting the deep subsurface are a matter of intense on-going debate<sup>1-7</sup>. Primarily limited by temperature<sup>8</sup>, little is known about secondary factors that restrict or enhance microbial activity<sup>9,10</sup> or about the extent of a habitable environment in the deep subsurface. In particular, the degraders of chemically inert organic substrates remain elusive<sup>9</sup>. Petroleum reservoirs can be regarded as natural bioreactors and are ideally suited for the study of microbial metabolism in the deep subsurface. Here we report fatty acids from purified crude oil fractions, indicating the presence of intact phospholipids and suggesting that indigenous bacteria inhabit petroleum reservoirs in sediment depths of up to 2000 m. Results show that both size and structure of the bacterial community vary during progressive petroleum biodegradation. A major change occurs only after the degradation of *n*-alkanes, the main petroleum constituents<sup>11</sup>, indicating a succession of microbial consortia. The here reported results release petroleum fluids for further microbiological studies, which can lead to novel insights about bacterial metabolism and diversity in this habitat of the deep subsurface.

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Abstract reference key: <sup>1</sup> Schippers et al., 2005; <sup>2</sup> Jørgensen & d'Hondt, 2006; <sup>3</sup> Parkes et al., 2007; <sup>4</sup> Aitken et al., 2004; <sup>5</sup> Parkes et al., 2005; <sup>6</sup> De Long, 2004; <sup>7</sup> Rueter et al., 1994; <sup>8</sup> Wilhelms et al., 2001; <sup>9</sup> Head et al., 2003; <sup>10</sup> Roeling et al., 2003; <sup>11</sup> Peters & Moldowan, 1993.

## **Introduction**

Study of the deep-subsurface microbiosphere has become a focus of intense research (Aitken et al., 2004; De Long, 2004; Parkes et al., 2005; Schippers et al., 2005; Jørgensen and D'Hondt, 2006; Parkes et al., 2007; Jones et al., 2008). Microbial habitats were shown to extend to great depths beneath the Earth's surface based on the presence of live bacterial cells (Schippers et al., 2005). At the same time, new extremes in the physicochemical boundaries for life are continually being reported (Kashefi and Lovley, 2003). Although cell counts rapidly decrease in sub-seafloor sediments, small numbers of prokaryotes can persist for long periods by sporulation (Lappin-Scott and Costerton, 1990) until continuous sedimentary subsidence transports them beyond the temperature limit for life (Wilhelms et al., 2001). Shallow sediments are rapidly depleted in nutrients and labile organic compounds, forcing microbes to slow down metabolism (Jørgensen and D'Hondt, 2006). At greater depths, microbes capable of catabolizing macromolecular organic compounds produce volatile fatty acids (VFA) and molecular hydrogen, which provides energy for themselves and other bacteria (Parkes et al., 2007). Such low molecular weight substrates are capable of sustaining the sulfate-reducing bacteria (SRB) and acetoclastic/hydrogenotrophic methanogenic Archaea commonly found in deep sediments (Head et al., 2003). When petroleum is released from organic rich rocks at greater depth and migrates into the habitable range of a sedimentary column, it represents a more abundant growth substrate for microorganisms than macromolecular organic matter and VFA. The occurrence of biodegraded shallow petroleum accumulations (Head et al., 2003; Roeling et al., 2003) provides evidence that under certain circumstances this oil is used as a microbial growth substrate. While microbial life in sub-seafloor sediments has recently received much attention, little is known about deep-subsurface prokaryotic behavior in the presence of oil. Studies of petroleum degradation in deep reservoirs can yield useful information on the catabolic behavior of microbes in other subsurface environments, where low amounts of substrate and low cell numbers preclude detailed analyses. We investigated microbial dynamics in petroleum reservoirs through the analysis of bacterial fatty acids indicative of intact phospholipids (PLFA) and thus microbial cells, in oil samples. The relative amount and distribution of selected PLFA sheds light on microbial populations inhabiting deep-subsurface petroleum reservoirs and their dynamics during the progressive biodegradation of oil.

## Results and discussion

Series of oil samples from three basins were analyzed. Each series contains biodegraded oils from shallow reservoirs and pristine oils from deeper reservoirs, where elevated temperatures inhibit microbial life (Wilhelms et al., 2001). The genetic relationship of biodegraded to pristine oil couples was verified using the distribution of triterpenoids and biodegradation-resistant aromatic steroids (Wardroper et al., 1984), thereby attesting changes in oils to be caused by microbial action and not by initial variations in chemical composition. The extent of biodegradation was determined by assessing the quasi-stepwise disappearance of compound classes. Based hereon, oil samples were categorized on a scale from 0 to 10 as proposed by Peters and Moldowan (PM; Peters and Moldowan, 1993).

Investigating microbes in oils by culturing methods carries the risk of a bias since subsurface conditions cannot be effectively reproduced in the laboratory (Head et al., 2003). Molecular methods that involve complimentary base pairing of oligonucleotides to DNA or RNA (e.g. polymerase chain reaction [PCR], or fluorescence in-situ hybridization [FISH]) have been conducted on oil-associated waters but are generally inhibited by abundant polar organic matter (Harry et al., 1999). While these methods consequently have severe limitations when applied to oils, analysis of oil-associated waters carries a high risk of contamination from drilling fluids (Roeling et al., 2003).

We approached the question of microbial activity by searching for membrane lipid fragments from intact cells and here present results that indicate the presence of indigenous PLFA in crude oils from deep reservoirs. Phospholipids are the principal building blocks in the cell membrane of all prokaryotes and eukaryotes (Madigan and Martinko, 2006). They are not preserved but turned over rapidly during metabolism (White et al., 1979) and decompose outside the cell. Phospholipids are therefore molecular indicators of live cells (Vestal and White, 1989) and proxies for their biomass. In the laboratory, fatty acid tails can be chemically released from the phospholipid parent molecule (see Supplementary Material) and utilized to characterize the parent organism or, in case of environmental samples, microbial community structure (Yao et al., 2006). The ubiquitous presence of microbes requires a strict analytical protocol to exclude sample contamination during retrieval, storage, and handling. The microbes studied

here were shown to be indigenous to the reservoirs based on several lines of evidence, as described in detail in the Supplementary Online Material.

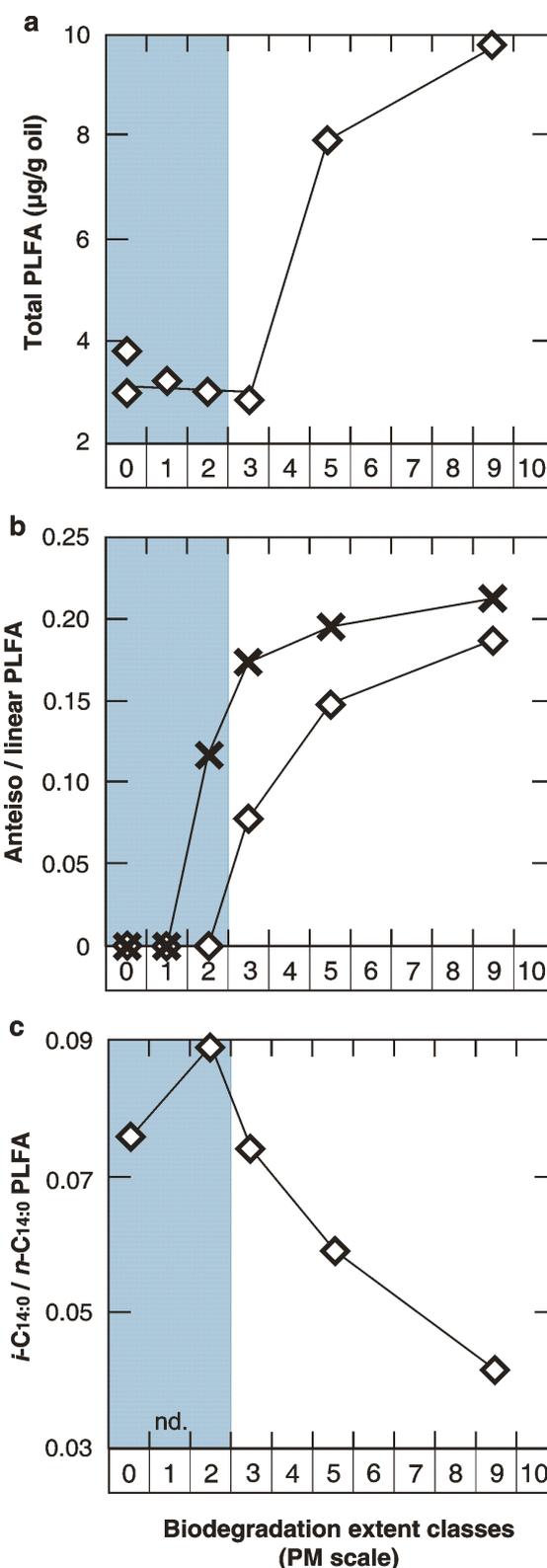
**FIGURE 2.1** Microbial community changes during progressive petroleum degradation. Increase of total and diagnostic phospholipid fatty acids (PLFA) during progressive biological degradation of oils as classified on the PM scale (Peters and Moldowan, 1993).

(a) Total PLFA are a taxonomically non-specific proxy for biomass and increase after PM stage 3.

(b) Relative increase of *ai*-C<sub>15:0</sub> over linear *n*-C<sub>15:0</sub> (×) and *ai*-C<sub>17:0</sub> over linear *n*-C<sub>17:0</sub> (◇).

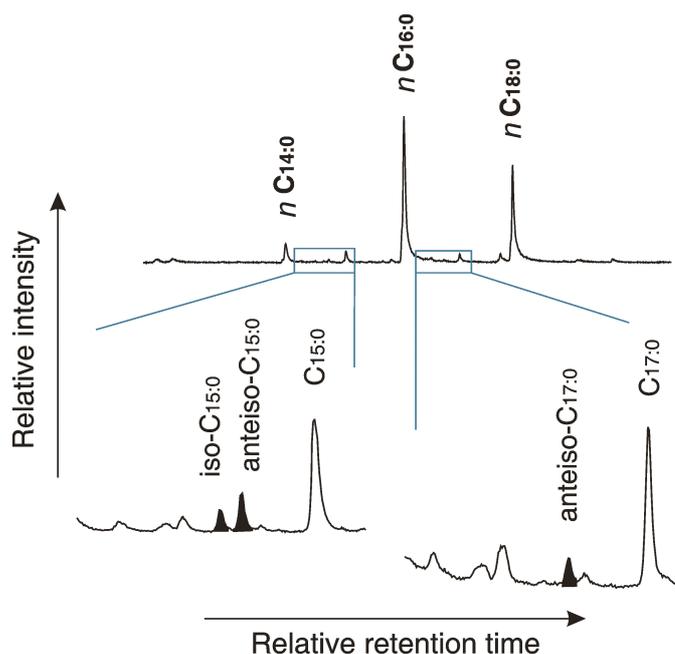
(c) The relative amount of *i*-C<sub>14:0</sub> follows an opposite trend.

Shaded areas indicate the stages of *n*-alkane removal. Nd., not



The concentration of total PLFA was found to be higher in biodegraded oils than in syngeneic pristine oils (Figure 2.1 a), indicating a larger amount of viable biomass associated with petroleum biodegradation. Calculation of bacterial cell numbers (Green and Scow, 2000) using terminally methyl-branched PLFA as bacterial markers (see Supplementary Methods for conversion factors) yields values of 7.2–7.6 log<sub>10</sub> cells (g oil<sup>-1</sup>) for the most heavily degraded oil (PM 9), which correlate well with cell numbers reported for deep seafloor sediments (Parkes et al., 2005; Schippers et al., 2005), whereas cell numbers in non-biodegraded oils are 0 or below detection limit (5.3–5.8 log<sub>10</sub> cells [g oil<sup>-1</sup>]). A number of PLFA, such as hexadecanoic (*n*-C<sub>16:0</sub>) and octadecanoic (*n*-C<sub>18:0</sub>) acid are ubiquitous in all living organisms (Madigan and Martinko, 2006) but others carry more taxonomic value and can reveal details on environmental conditions under which microbial communities thrive. Their main advantage lies in the non culture-biased fingerprinting of entire microbial communities and the monitoring of changes therein, be they taxonomical or physiological (Bossio and Scow, 1998). Terminally methyl-branched *iso*- (*i*-) and *anteiso*- (*ai*-) PLFA frequently predominate in Gram-positive bacteria (Vestal and White, 1989; Bossio and Scow, 1998), but are absent in Gram-negative bacteria, except in SRB (Chang et al., 2001; Li et al., 2007). SRB are a phenotypic group largely situated within the delta subgroup of Gram-negative proteobacteria and exclusively comprise obligate anaerobes. A range of terminally methyl-branched PLFA (C<sub>14</sub>-C<sub>17</sub>) was found in the studied oils (Figure 2.2).

The distribution of PLFA indicates structural changes in the bacterial community during progressive biodegradation of oil, with a major step occurring after the removal of *n*-alkanes from the oils (Figure 2.1). Bacterial biomass, approximated by total PLFA, is constant during initial biodegradation stages, but strongly increases after the disappearance of *n*-alkanes (Figure 2.1 a), parallel to a relative increase of the terminally methyl-branched PLFA *ai*-C<sub>15:0</sub> and *ai*-C<sub>17:0</sub> (Figure 2.1 b). The relative amount of *i*-C<sub>14:0</sub> follows a reversed trend and increases during the early stages of biodegradation, but drops quickly after *n*-alkanes have been removed (Figure 2.1 c). This suggests that it mainly derives from a different precursor organism than *ai*-C<sub>15:0</sub> and *ai*-C<sub>17:0</sub> and indicates the presence of two end member consortia.



**FIGURE 2.2** Bacterial PLFA in crude oils. Chromatogram ( $m/z$  74.1) showing the distribution of phospholipid-derived fatty acids (PLFA; analyzed as methyl esters by GC-MS) from live microbes in biodegraded oil. Selected terminally branched PLFA (shaded) are diagnostic markers for Gram-positive bacteria.

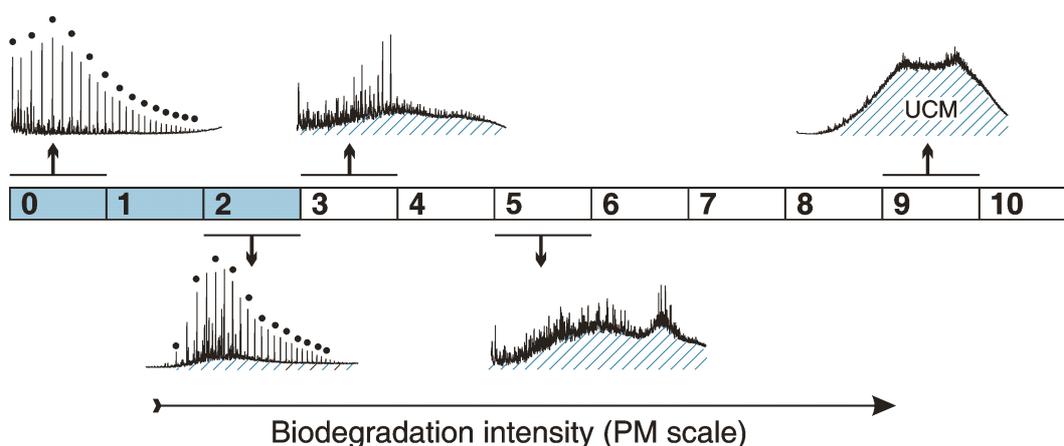
The microbes most often reported from petroleum reservoirs are SRB (Head et al., 2003). This finding might however be biased since many of these studies focused on gas souring in reservoirs that had undergone seawater injection. Although some SRB are capable of degrading *n*-alkanes in vitro (Rueter et al., 1994), SRB typically rely on small organic substrates, such as volatile fatty acids (VFA), or molecular hydrogen as an electron donor (Rueter et al., 1994). The breakdown of larger and/or inert compounds is thought to be achieved by hydrolytic and fermenting bacteria (Roeling et al., 2003), which carry a wider range of metabolic features (Ollivier and Cayol, 2005). The majority of fermenters are Gram-positive bacteria (Madigan and Martinko, 2006), whose most prominent representatives in the deep subsurface are *Clostridiaea* that predominantly engage in fermenting processes (Voordouw et al., 1996). Based on cumulative evidence, it was recently suggested that the majority of microbes in the deep subsurface could be involved in fermentative processes (Teske, 2006). Methanogenesis is the most likely process occurring during subsurface *n*-alkane biodegradation (Bekins et al.,

1999; Jones et al., 2008) and involves the interaction of CO<sub>2</sub>-reducing Archaea and alkane-oxidising *Syntrophus* sp. (Jones et al., 2008). The high relative amounts of *i*-C<sub>14:0</sub> present during the stage of *n*-alkane removal and its subsequent decrease (Figure 2.1) could reflect the bacterial component of this consortium responsible for initial biodegradation and destruction of *n*-alkanes.

Parallel to the removal of *n*-alkanes during petroleum biodegradation, a large array of increasingly recalcitrant compounds becomes concentrated (Peters and Moldowan, 1993; Head et al., 2003). These components are cumulatively classified as an 'unresolved complex mixture' (UCM). During progressive biodegradation of oil, the UCM increases at the expense of degraded components (Peters and Moldowan, 1993; Head et al., 2003) (Figure 2.3). The disappearance of *n*-alkanes is marked by a change in community structure. Biodegradation beyond *n*-alkane removal is paralleled by a strong increase in bacterial biomass and reflects the growth of organisms that may be involved in degrading more recalcitrant compounds not catabolized by the alkane-degrading community (Figure 2.1). Degradation of recalcitrant compounds has been suggested to involve fermenting bacteria (Roeling et al., 2003), which are often Gram-positives (Madigan and Martinko, 2006). Active degradation and carbon assimilation by Gram-positives is expected to cause an increase in terminally methyl-branched PLFA (Vestal and White, 1989; Bossio and Scow, 1998), which was observed in the samples presented here (Figure 2.1), thereby strongly supporting this hypothesis.

The presence of bacterial fatty acids indicative of phospholipids from strict anaerobes confirms the oxygen-deficient nature of the deep biosphere. In the presence of minute amounts of oxygen, anaerobic respiration would be outcompeted by energetically more favorable aerobic respiration. Although shallow sediments are rapidly depleted in oxygen (De Long, 2004), no convincing data exist on anaerobic hydrocarbon degraders from petroleum reservoirs (Roeling et al., 2003). Anaerobic bacteria have been reported from petroleum reservoirs (Head et al., 2003) but only one thermophilic hydrocarbon degrader was identified that could be capable of living in deep reservoirs (Rueter et al., 1994). Metabolites of anaerobic alkane degradation were found in petroleum-contaminated anaerobic aquifers (Gieg and Suflita, 2002) but never in petroleum reservoirs, possibly due to the transitory nature of metabolites and low metabolic activity of subsurface

microbes (Jørgensen and D'Hondt, 2006). A first line of evidence for in-situ anaerobic biodegradation of petroleum was provided by the presence of metabolites of anaerobic naphthalene degradation in biodegraded oils (Aitken et al., 2004). The evidence presented here for fatty acids from anaerobic bacteria indigenous to oil reservoirs provides further support for anaerobic metabolism in subsurface oil reservoirs.



**FIGURE 2.3** Compositional alteration of oil during progressive biodegradation. Gas chromatograms showing the studied oil samples and their ranks on the biodegradation scale of Peters and Moldowan (1993). Removal of *n*-alkanes is paralleled by the relative enrichment of an ‘unresolved complex mixture’ (UCM). Black dots on the chromatograms of the non-biodegraded oils indicate odd-numbered *n*-alkanes. The grey shaded area (PM 0–2) on the scale indicates the timing of *n*-alkane removal.

## Conclusions

Studies of bacterial metabolism in the deep biosphere used to be limited to rock and water samples. The presence of a large array of organic compounds makes petroleum reservoirs ideal habitats to study the anaerobic metabolism of bacteria in the deep subsurface. This paper reports the first detection of bacterial fatty acids in phospholipid fractions of crude oils and commends these fluids for further studies.

Results show that a succession of microorganisms is responsible for progressive subsurface petroleum biodegradation and reveal a concomitant increase in biomass. Further studies using this approach are expected to reveal novel details of bacterial metabolism and diversity in this habitat of the deep subsurface.

### **Methods summary**

We present data on bacterial fatty acids derived from phospholipid fractions, and interpret these as phospholipid-derived fatty acids (PLFA), as will be explained below. PLFA are markers for living bacteria and can be used to profile community structure. The ubiquitous presence of microbes requires extreme caution that must be taken during collection, storage and processing of samples. More details of this method can be found in the Supplementary Information Section.

#### Sample collection, storage, and processing

Oils were topped with nitrogen after collection, frozen, stored at -20 °C in the dark, and analyzed immediately after thawing. Aliquots of 0.6–0.8 g oil were fractionated using liquid-liquid extractions and silica chromatography to produce a fraction consisting predominantly of intact phospholipids. Phospholipids were saponified to yield fatty acid methyl esters (FAME), and analyzed by gas chromatography (GC) coupled to mass spectrometry (MS).

#### Calculation of cell numbers

Bacterial cell numbers were calculated assuming conversion factors between 2 and  $6 \times 10^4$  cells per pmol PLFA (Green and Scow, 2000).

#### Originality of PLFA

We analyzed phospholipid-released fatty acid tails (PLFA) instead of intact phospholipids (IPL). The latter analysis would require the use of liquid chromatography (LC)-MS instead of GC-MS. LC has disadvantages: i) lower chromatographic resolution than GC (generally worsening the detection limit) and ii)

need to detect lower sample concentrations (phospholipids contain a variety of both, fatty acid tails and head groups, leading to more complexity than in PLFA). Using an extensive workup procedure, samples can be purified until a fraction is obtained that contains only phospholipids as a source of fatty acids. Saponification of this fraction thus yields fatty acids that only originate from phospholipids.

Oils can contain other sources of fatty acids than phospholipids, including i) free fatty acids (FFA), and ii) asphaltenes. Asphaltenes are macromolecular components of petroleum, which can occlude fatty acids and other petroleum hydrocarbons within their structure. Saponification of asphaltenes could thus also yield fatty acids. Experiments conducted to test the presence of either of these fatty acid sources within the phospholipid fraction led to negative results. The possibility that the analyzed PLFA originate from sources other than intact phospholipids can thus be excluded.

#### *Free fatty acids (FFA).*

Oil spiked with  $d_{39}$ -icosanoic acid, an FFA, was separated using the method described above to check for the presence of non-PLFA (free) fatty acids within the phospholipid fraction. No trace of the spiked FFA was found within the phospholipid fraction ([Supplementary Figure 2.2](#)), showing that fatty acids released from the phospholipid fraction contain no contribution from FFA.

#### *Asphaltenes.*

Asphaltenes were precipitated from a biodegraded oil, purified, and subjected to the silica chromatography part of the workup procedure. Whilst the amounts of asphaltenes separated during the test trial were ca. 3–10 times less than those present in the oils analysed for PLFA, the coloration on the silica was notably stronger ([Supplementary Figure 2.3](#)) than that produced by the phospholipid fractions. This suggests that during the first liquid-liquid extraction steps, asphaltenes and phospholipids do not partition into the same fraction. Liquid chromatography of the asphaltenes followed by gravimetric analysis yielded quantitative evidence that no asphaltenes occur within the phospholipid fraction after silica chromatography ([Supplementary Table 2.1](#)). The PLFA analyzed are

consequently not released from asphaltenes but represent microbial cell components.

### Exclusion of contamination

Microbes studied were shown to be indigenous to the reservoir based on several lines of evidence.

- 1) Microbial contamination during drilling and after sampling must be expected to be i) aerobic and ii) contain both, prokaryotic and eukaryotic components (Madigan and Martinko, 2006). PLFA reported in this study are anaerobic and samples did not contain marker lipids for eukaryotes or aerobic prokaryotes.
- 2) Increase of PLFA parallel to the intensity of biological degradation suggests that PLFA are indigenous to the oils. Post-sampling contamination would add similar amounts of contaminants to all samples, which is not the case.
- 3) During biodegradation, a large amount of the oil is converted to gas. Biodegradation resistant compounds are concentrated during this process. The discrepancy between main hydrocarbon removal stages (PM 2–3) and PLFA increase (PM 3–5) indicates that PLFA do not increase simply due to a concentration effect.

### Laboratory artefacts

Procedural blanks using squalene as a substitute for oil showed low amounts of ubiquitous linear saturated fatty acids in the range  $C_{14}$ – $C_{18}$ , with  $n$ - $C_{16:0}$  and  $n$ - $C_{18:0}$  dominating, as well as a phthalate peak ([Supplementary Figure 2.2](#)). The blank was subtracted from the analyzed samples before quantification. Terminally methyl-branched acids, interpreted in this study to describe subsurface microbial dynamics, were not present in any procedural blanks.

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## **Supplementary Information**

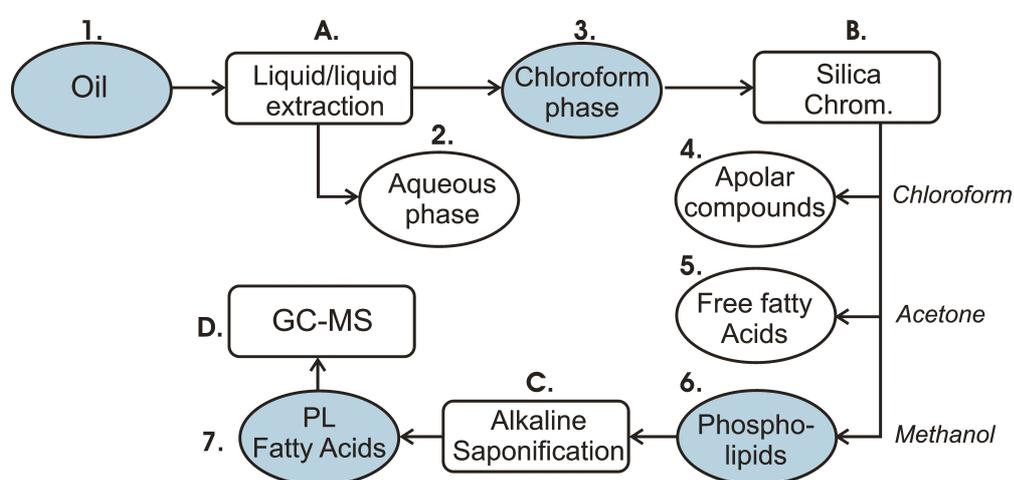
### Samples

For propriety reasons the exact depth and location of reservoirs cannot be disclosed. Middle Eastern oil samples were generated from Tithonian (latest Jurassic) carbonate source rocks and recovered from Tertiary carbonate reservoir rocks. Heavily biodegraded oils (PM 9; Peters and Moldowan, 1993) were found at reservoir depths around 150 m below surface, while deeper reservoirs at approximately 700 m below surface hosted oils that were unaffected (PM 0; Peters and Moldowan, 1993). The reservoirs are part of an uplifted fault belt and the deeper reservoirs are believed to having been sterilized during deepest burial (>3400 m). Central European oil samples were generated from Toarcian (Lower Jurassic) marine clastic source rocks and recovered from Dogger (Middle Jurassic) deltaic sandstone reservoir rocks. Reservoir depths varied between 1286 m and 1448 m. Oils from the deepest reservoirs were unaffected by biodegradation (PM 0; Peters and Moldowan, 1993), while shallower reservoir oils were slightly degraded (PM 2 and 3; Peters and Moldowan, 1993). South American oil samples

were generated from limnic Aptian source rocks within a rift sequence and recovered from either clastic Oligocene or Cretaceous carbonate reservoir rocks. Reservoir depths were approximately 3000 m for non-degraded and 2000 m for degraded oils. Reservoir temperatures were approximately 85°C in the reservoir containing the least biodegraded oils (PM 1; Peters and Moldowan, 1993) and approximately 50°C in the reservoirs containing heavily biodegraded oils (PM 5; Peters and Moldowan, 1993).

### Sample cleanup and analysis

Oil samples were collected in 1 L Schott bottles, topped with a nitrogen atmosphere, frozen, and stored at -20°C in the dark. Analyses were performed immediately after the samples reached room temperature. The sample workup procedure is depicted in [Supplementary Figure 2.1](#) showing a flow chart of processes and resulting fractions. Numbers in [Supplementary Figure 2.1](#) reflect sample fractions and letters reflect cleanup processes.



**SUPPLEMENTARY FIGURE 2.1** Simplified flow chart of sample clean-up procedure used to purify phospholipid (PL) fatty acids (PLFA). Letters represent processes and numbers represent sample fractions. Phospholipids and PLFA, are present in blue shaded fractions only.

Aliquots of 0.62–0.87 g oil (Supplementary Figure 2.1; Fraction 1) and a procedural blank using pure squalene as an oil surrogate were dissolved in chloroform (CHCl<sub>3</sub>, 12.5 mL), added to methanol (MeOH, 25 mL) and phosphate buffer (10 mL; 8.7 g K<sub>2</sub>HPO<sub>4</sub> L<sup>-1</sup>), and ultrasonicated. Mixtures were cooled and phases were separated by centrifugation (3500 rpm, 5 minutes). The supernatant was removed and added to CHCl<sub>3</sub> (3 mL) and water (3 mL). Water was deionized, heat-sterilized and triple solvent extracted before use. After 30 minutes of resting, phases were separated as described above and the lower CHCl<sub>3</sub> phase was removed. The procedure was repeated once and CHCl<sub>3</sub> phases were combined, dried over anhydrous MgSO<sub>4</sub> and concentrated using a rotary evaporator. The concentrated extract (Supplementary Figure 2.1; Fraction 3) was fractionated by column chromatography on 120 mg activated silica (60 Å, 0.045–0.063 mm) that was conditioned with MeOH (2 mL) and CHCl<sub>3</sub> (3 mL). A neutral fraction was eluted with CHCl<sub>3</sub> (2 mL; Supplementary Figure 2.1; Fraction 4), a free fatty acid containing fraction with acetone (2 mL; Supplementary Figure 2.1; Fraction 5), and intact phospholipids with MeOH (2 mL; Supplementary Figure 2.1; Fraction 6). Each fraction was observed to elute a distinctive, light coloured band from the column. The phospholipid fraction (Supplementary Figure 2.1; Fraction 6) was dried, resuspended in MeOH/Toluene (200 mL; 1:1 vol./vol.), and saponified at 75°C with a 0.2 M KOH/MeOH solution (0.5 mL) to trans-esterify phospholipid fatty acids (PLFA) to fatty acid methyl esters (FAME). After cooling to room temperature the mixture was neutralized with a 0.2 M aqueous acetic acid solution (0.5 mL), and water (1 mL) was added. Phospholipid-released, methylated fatty acids (FAME) were extracted with CHCl<sub>3</sub> (3x 1 mL). The FAME fraction (Supplementary Figure 2.1; Fraction 7) was dried over anhydrous MgSO<sub>4</sub>, and concentrated before further analysis.

Gas chromatography (GC) and mass spectrometry (MS) were performed using a Hewlett Packard (HP) 5890 Series II GC coupled to a HP 5989A MS. The GC was fitted with a DB-35ms fused silica capillary column (25 m; 0.2 mm inner diameter; 0.33 mm film thickness) and operated with helium carrier gas in constant flow mode (31 cm sec.<sup>-1</sup>). Samples were injected in splitless mode (290°C, 1 min. purge) using an HP 7673 GC/SFE autosampler system. The GC temperature was held at 80°C for 2 minutes, raised to 180°C at 10° min.<sup>-1</sup>, followed by 6°/min.<sup>-1</sup> to 330°C, and held isothermal for 15 minutes. The MS was operated with a source temperature of

200°C and a quadrupole temperature of 100°C. Compounds were detected in full scan ( $m/z$  40-550) and selective ion monitoring (SIM) modes. Analytes were identified by comparison of retention times and mass spectra with authentic standards and quantified using deuterated carboxylic acid standards, assuming a uniform response.

#### Calculation of cell numbers

Bacterial cell numbers were calculated assuming conversion factors between 2 and  $6 \times 10^4$  cells per pmol PLFA (Green and Scow, 2000). The detection limit for PLFA using the analytical methods employed in this study was calculated to cell number values of 5.3 and  $5.8 \log_{10}$  cells ( $\text{g oil}^{-1}$ ). Cell counts of zero for non-biodegraded oils therefore do not attest the absence of microbial cells but merely indicate that cell numbers are below the detection limit, i.e. lower than 5.3, respectively  $5.8 \log_{10}$  cells ( $\text{g oil}^{-1}$ ).

#### Analytical approach

This study analyzed phospholipid-released fatty acid tails (PLFA) instead of intact phospholipids (IPL). Analysis of intact phospholipids would require the use of liquid chromatography (LC)-MS, instead of GC-MS, and has a number of significant disadvantages, as will be discussed.

LC-MS has the disadvantages of i) lower chromatographic resolution than GC and ii) the need to detect lower sample concentrations. The consequence of i) is a degraded detection limit (requires a signal three times as strong as background noise). Point ii) is caused by the fact that phospholipids contain various fatty acid tail and head groups, leading to a greater diversity in phospholipids than in PLFA. . Each phospholipid yields two fatty acids upon saponification. In phospholipids, the fatty acid tails can be linked to various polar head groups, resulting in a much greater variety of intact phospholipids than phospholipid fatty acids (PLFA) in the same sample (Fang et al., 2000). In studying the deep biosphere, quantification of intact phospholipids by LC-MS can thus be critical when dealing with concentrations near the detection limit (Zink et al., 2008). Furthermore, Zink et al. (2008) showed

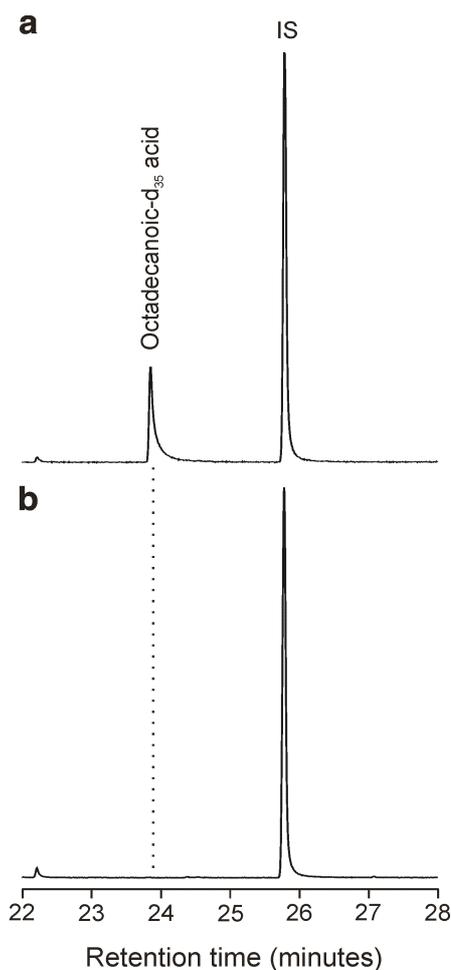
that the quantification of intact phospholipids after analysis by LC-MS yielded non-optimal relative standard deviations ranging from 4.7 to 30%.

The indirect study of living bacteria by analyzing PLFA released from their membrane lipids has wide application in the fields of environmental microbiology and soil science. PLFA-estimated biomass correlates well with total cell counts (Thomas et al., 1997). When discarding three outliers (characterised by significantly elevated standard deviation), the  $R^2$  value of cell counts (log cells g<sup>-1</sup>) obtained by direct counting and PLFA analysis is 0.76 (calculated using data from Thomas et al., 1997). Balkwill et al. (1988) compared the estimated biomass obtained by direct microscopic counts of stained cells with that obtained by chemical measures of microbial biomass. The study focussed on an uncontaminated subsurface aquifer dominated by coccobacillary bacteria of relatively uniform size. Biomass estimates were based on PLFA, lipid phosphate, cell wall muramic acid, adenosine triphosphate (ATP), and direct cell counts, amongst others; all gave essentially identical estimates of cell numbers and dry weight (Balkwill et al., 1988).

#### Originality of PLFA

The workup procedure used in this study was designed to purify the samples until a fraction is obtained that can only contain phospholipids as a source of fatty acids. Saponification of these fractions yields fatty acids that can only originate from phospholipids. This methodology has found routine application in microbial ecology and soil science since the 1970s and is well documented in the literature.

Oil samples can contain fatty acids from sources other than phospholipids: i) free fatty acids, and ii) asphaltenes. Free fatty acids in petroleum mostly originate from the secondary oxidation of petroleum hydrocarbons in reservoirs under elevated temperature and pressure conditions. During the silica chromatography step ([Supplementary Figure 2.1](#); procedure B) phospholipids and free fatty acids are quantitatively separated from each other. This has been successfully tested and is described below (see: '*Free fatty acids*').



**SUPPLEMENTARY FIGURE 2.2** Total ion chromatogram ( $m/z$  40-550) showing (a) the free fatty acid fraction (Fraction 5, [Supplementary Figure 2.1](#)), and (b) the PLFA fraction (Fraction 6, [Supplementary Figure 2.1](#)) of an oil sample spiked with  $d_{35}$ - $n$ -octadecanoic acid. The absence of the free fatty acid in the PLFA fraction supports the methodology. Acids were analyzed as methyl esters by GC-MS. IS, internal standard (added before GC-MS).

Asphaltenes are the macromolecular component of crude oils that is insoluble in paraffins, methanol, or water. Fatty acids and other petroleum compounds can be occluded within the asphaltene structure and released upon saponification. If asphaltenes were present in the phospholipid fraction, they could represent a non-phospholipid source of fatty acids. Experiments were performed to test the presence

of asphaltenes in the phospholipid fraction. Results described below show the analyzed PLFA originated solely from intact phospholipids.

#### *Free fatty acids*

An oil sample spiked with  $d_{39}$ -icosanoic acid, a free fatty acid, was separated using the method described above to check for breakthrough of non-PLFA fatty acids during sample work-up. No trace of the spiked free fatty acid was found in the phospholipid fraction ([Supplementary Figure 2.2](#)) indicating that fatty acids released from the phospholipid fraction contain no contribution of free fatty acids.

Solvent fraction	Asphaltene recovery	
	mg	%
Chloroform	0.1	5
Acetone	0.1	5
Methanol *	0	0

**SUPPLEMENTARY TABLE 2.1** Recovery of asphaltenes after silica chromatography (Procedure B, [Supplementary Figure 2.1](#)). An aliquot of 2 mg purified asphaltenes was fractionated. Most asphaltenes (90%) remain immobilized on the silica column and no asphaltenes co-elute with the phospholipid (methanol) fraction indicated by an asterisk.

#### *Asphaltenes*

To test the presence of asphaltenes in the phospholipid fraction, we purified an asphaltene fraction from a biodegraded crude oil and subjected it to the silica chromatography part of the workup procedure.

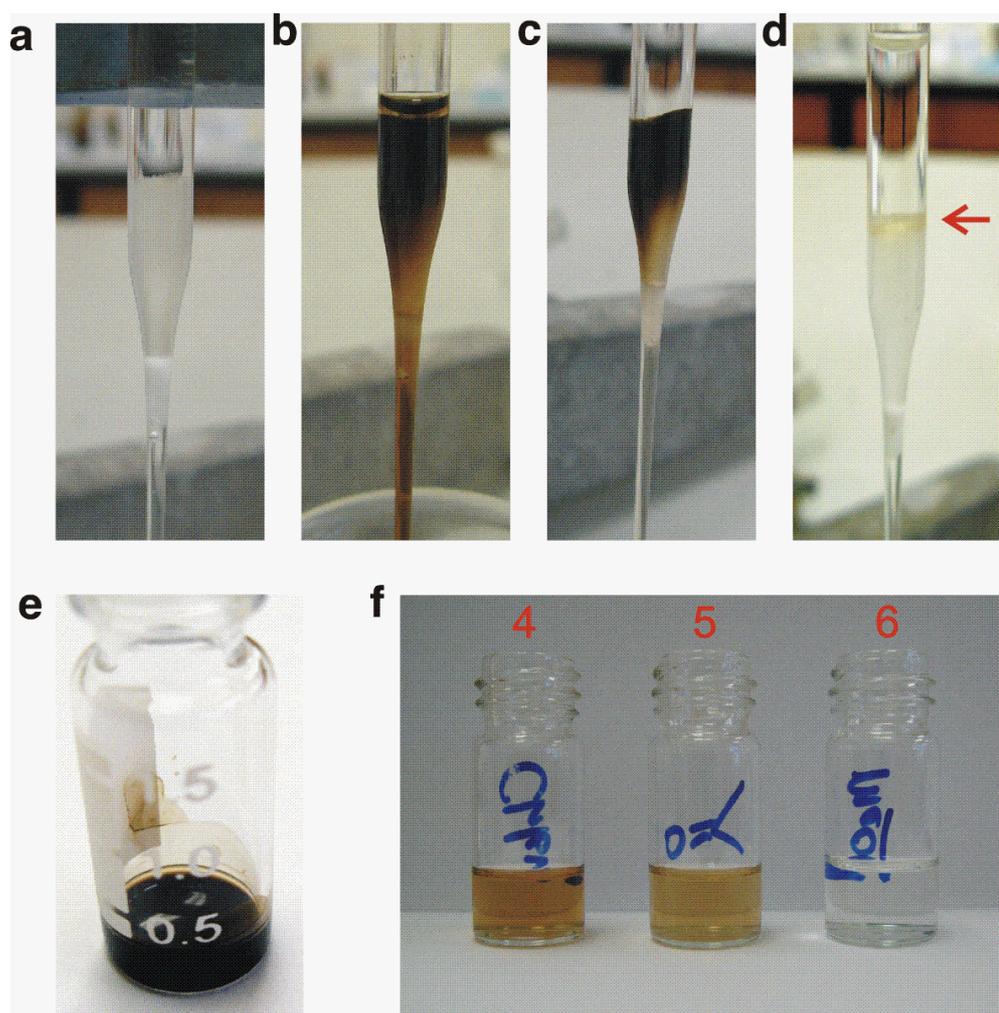
Asphaltenes in a viscous biodegraded oil (PM7; Peters and Moldowan, 1993) were precipitated by dissolving the oil in a minimum amount of dichloromethane and adding a 60-fold excess of cold (ca. 4°C) *n*-pentane. The mixture was agitated by ultrasonication for five minutes and suspended asphaltenes were precipitated by centrifugation of the suspension at 3800 revolutions per minute (rpm) for 15

minutes. The supernatant solvent was decanted, leaving only the asphaltene precipitate in the centrifugation tube. Asphaltenes were re-dissolved in a minimum amount of chloroform and the whole process, starting with the addition of a 60-fold excess of *n*-pentane, was repeated two times. Asphaltenes were dried, dissolved in dichloromethane and transferred to a sample vial. Dry asphaltenes had a black brittle appearance with a slightly lustrous shine.

The asphaltene content of the analyzed oil samples ranged between ca. 1% wt. and ca. 7% wt. The phospholipids analyzed in this study were isolated from oil samples weighing 0.62–0.87 g. The minimum amount of asphaltenes in any sample was thus 6.2 mg, while most samples contained around 30 mg of asphaltenes. We subjected 2 mg of pure asphaltenes, dissolved in a minimum amount of chloroform, to the silica chromatography separation ([Supplementary Figure 2.1](#); procedure B). This amount is at least three times smaller than that in any of the analyzed oils.

The dissolved asphaltenes had an intense dark color ([Supplementary Figure 2.3 e](#)). Adsorption of the asphaltene sample to the silica ([Supplementary Figure 2.3 a](#), silica before adsorption of asphaltenes) produced an intensely dark-colored band ([Supplementary Figure 2.3 b](#), silica after adsorption of asphaltenes, during the elution of the chloroform fraction). The coloration produced by this small amount of asphaltenes (significantly lower than the amount of asphaltenes in the analyzed oils) was considerably more intense than that produced by the phospholipid fractions of the samples analyzed in this study ([Supplementary Figure 2.3 d](#), typical coloration of phospholipid fractions adsorbed to the silica), which only produced a slight yellow-brownish band. This suggests that during the first step of the workup procedure ([Supplementary Figure 2.1](#), procedure A), asphaltenes are not recovered in the same fraction as phospholipids.

Asphaltenes were eluted from the silica column with chloroform, acetone, and methanol as described in '*Sample cleanup and analysis*'. Fractions were transferred to pre-weighed vials, evaporated to dryness and weighed to assess the elution behavior of asphaltenes. Results are presented in [Supplementary Table 2.1](#). Of the 2 mg of pure asphaltenes applied to the column, only 0.1 mg were recovered in the chloroform fraction and 0.1 mg in the acetone fraction. The methanol fraction contained no asphaltenes. The precision of the scale used was determined to be  $\pm 0.089$  mg ( $n = 5$  measurements). This shows that the majority of asphaltenes (90%) remains adsorbed to the silica and does not elute with the organic solvents.



**SUPPLEMENTARY FIGURE 2.3** Asphaltene elution behavior during phospholipid purification by silica gel chromatography (Procedure B, [Supplementary Figure 2.1](#)). (a) Silica column before the addition of asphaltenes. (b) Asphaltenes (2 mg) adsorbed on the silica column, shown during the elution of fraction 4 with chloroform ([Supplementary Figure 2.1](#)). Note the intense coloration. (c) Asphaltenes remaining immobilized on the silica after the elution of all solvents. (d) Typical coloration of phospholipid fractions (arrow) analyzed when adsorbed on silica. (e) Purified asphaltenes (2 mg) dissolved in chloroform (300  $\mu$ L). (f) Recovered solvent fractions (see [Supplementary Figure 2.1](#) for numbering): 4. chloroform (2 mL), 5. acetone (2 mL), 6. methanol (2 mL). Phospholipids are recovered from the methanol fraction. Asphaltenes mainly remain immobilized on the silica (90%). No asphaltenes elute in the methanol fraction ([Supplementary Table 2.1](#)).

Supplementary Figure 2.3 c shows the dark coloration of the silica remaining after all solvent elutions caused by asphaltenes that are immobilized on the silica. Supplementary figure 2.3 f shows the 3 fractions next to each other (numbering corresponds to Supplementary Figure 2.1): 4. chloroform (2 mL); 5. acetone (2 mL); 6. methanol (2 mL). Phospholipids are recovered in the methanol fraction (Nr. 6). Visual analysis of the fractions yields supporting evidence that no asphaltenes occur in the methanol fraction, thus excluding an asphaltene origin of the PLFA analyzed.

### Exclusion of contamination

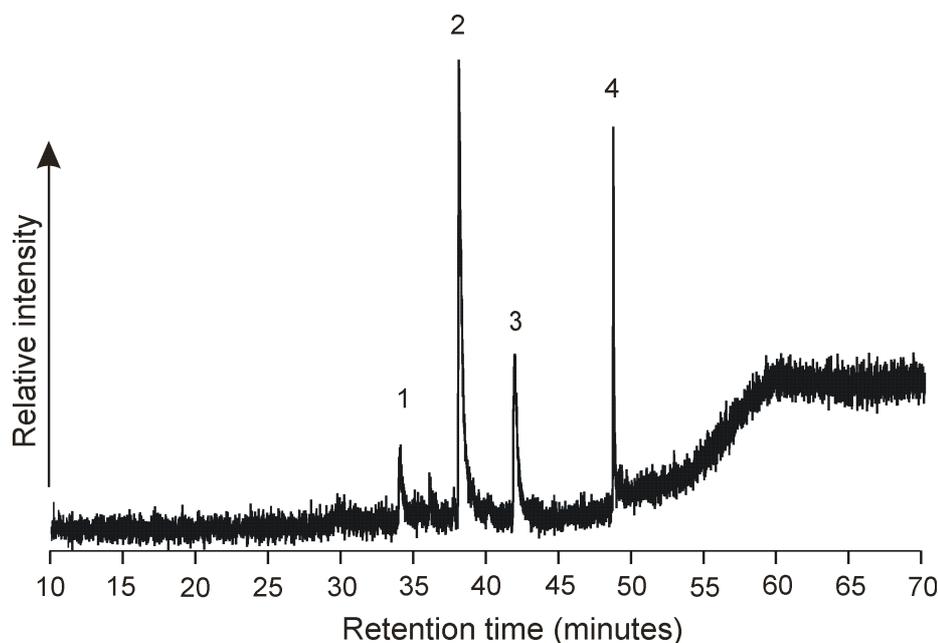
#### *Contamination during drilling*

Microbes studied were shown to be indigenous to the reservoir based on of several lines of evidence explained below. In contrast to petroleum formation waters, oils are far less susceptible to contamination from drilling fluids. The analysis of subsurface microbial communities using genetic methods must target the waters co-produced with petroleum, since the binding of molecular probes to DNA is highly problematic in the presence of polar organic matter (such as the petroleum substrate). The advantage of PLFA profiling is that it can target the oil, which is thought to be unaffected by microbes from the surface.

1. Any microbial contamination after sampling can be expected to be i) aerobic and to ii) contain both, prokaryotic and eukaryotic components (Madigan and Martinko, 2006). Contamination by eukaryotes and aerobic prokaryotes can be monitored by determining the presence of certain marker lipids, such as C<sub>16:1</sub> ω7-*cis* for aerobic prokaryotes, and the tri-unsaturated PLFA C<sub>18:3</sub> ω6-*cis* for fungi. Additionally, all phospholipid-released polyunsaturated fatty acids (PUFA) were considered to represent eukaryotic input. None of the mentioned PLFA or PUFA was found in any of the samples studied, thereby excluding contamination from aerobic microbes or eukaryotes, and suggesting that the PLFA in the samples must originate from anaerobic prokaryotes in the samples. We conclude that the PLFA derive from anaerobic microorganisms indigenous to deep petroleum reservoirs.

2. Results of this study show that the total amount PLFA per gram varies between samples. Samples from the same petroleum system having different biodegradation intensity show variations in their PLFA content. This suggests that

PLFA are indigenous to the oils and do not represent surface microbial contamination, which would add similar amounts of contaminants to all samples. Additionally, the increase of total PLFA with the level of biodegradation suggests that the PLFA originate from biomass of the microbes responsible for the biodegradation.



**SUPPLEMENTARY FIGURE 2.4** Procedural blank. Mass fragmentogram ( $m/z$  74.1) showing the PLFA fraction (Fraction 7, [Supplementary Figure 2.1](#)) of a procedural blank, analysed as methyl esters by GC-MS. Compound identification: 1. *n*-tetradecanoic acid, 2. *n*-hexadecanoic acid, 3. *n*-octadecanoic acid, 4. phthalate (contaminant). Note the low concentration of compounds as indicated by high background noise, and the absence of bacterial methyl-branched acids.

#### Laboratory artefacts

To assess contamination introduced during the laboratory workup procedure, procedural blanks using squalene as a substitute for oil were analyzed parallel to the samples. Procedural blanks showed low amounts of ubiquitous linear saturated fatty acids in the range  $C_{14}$ – $C_{18}$ , with *n*- $C_{16:0}$  and *n*- $C_{18:0}$  dominating, as well as a phthalate peak ([Supplementary Figure 2.4](#)). The blank was subtracted from the

analyzed samples before quantification. Terminally methyl-branched acids, interpreted in this study to indicate subsurface microbial activity, were not present in any procedural blanks.

#### Exclusion of a 'concentration effect'

During biodegradation of petroleum, a large amount of the original oil is catabolized to smaller, mostly as yet uncharacterized molecules, and eventually to carbon dioxide and methane (Larter et al., 2005). Compounds resistant to biodegradation are concentrated during this process. The main removal of compounds occurs before stage 2 to 3 on the PM (Peters and Moldowan, 1993) biodegradation scale (Figure 2.3). However, an increase in both total and terminally branched PLFA in the samples studied is observed between PM stages 3 and 5, while their concentration remains constant and low during PM stages 1 to 3 (Figure 2.3). Phospholipids are not recalcitrant but easily degraded. This discrepancy between main hydrocarbon destruction and PLFA increase excludes a concentration effect, but instead reflects the growth of degradative microbes in the petroleum reservoir.

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## Relative efficiency of free fatty acid butyl esterification. Choice of catalyst and derivatization procedure.

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**Abstract** - The conversion efficiency of alkanolic, alkenolic, branched, alicyclic, aromatic, keto-substituted, and dioic carboxylic acids to their corresponding butyl esters was compared under different reaction conditions (time, temperature, catalyst). We show that boron trifluoride is generally a more efficient catalyst than sulphuric acid. However, optimum derivatization conditions vary strongly for different acids and no single derivatization protocol can be employed without certain losses. Therefore, care must be taken when the simultaneous quantitative analysis of different types of carboxylic acids in one sample is envisaged. Addition of water-scavenging reagents to the reaction mixture caused the formation of artefacts and selectively decreased reaction yields.

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### Introduction

The analysis of long-chain fatty acids (>C<sub>12</sub>) by gas chromatography coupled to mass spectrometry (GC-MS) is a routine procedure in many branches of biological and Earth sciences (e.g. Szmigielska et al., 1996; Jøstensen and Landfald, 1997; Naraoka and Ishiwatari, 2000). Separating carboxylated

compounds by GC is complicated by their relatively high polarity and therefore derivatization prior to analysis is commonly needed. A popular method to derivatize carboxylic acids is esterification to fatty acid methyl esters (FAMES), a technique that has been thoroughly reviewed over the years (e.g. Christie, 1993; Craske, 1993; Liu, 1994; Eder, 1995). This procedure is however not very suitable for analyzing carboxylic acids of low molecular weight (<C<sub>12</sub>) since their increased volatility after derivatization can lead to unquantifiable losses related to evaporation. GC analysis of free fatty acids yields substandard results (Vičanová et al., 1994) and is restricted to mono-carboxylic acids of low molecular weight. Interaction with the capillary GC column phase leads to peak tailing and less than optimal separation (Cochran, 1975). Due to sample losses in the GC system, the analysis of free fatty acids cannot be performed reliably when dealing with minor amounts of sample (Vičanová et al., 1994). Although polar stationary phases such as those based on polyethylene glycol (PEG) or acid phases can be used in capillary GC columns to enhance separation, the maximum temperatures at which they can be operated precludes the analysis of compounds with higher boiling points. Derivatization methods forming propyl or butyl esters have been known for a long time (Craig et al., 1963; Salanitro and Muirhead, 1975) but gained popularity recently. Due to their decreased volatility, butyl esters allow the simultaneous analysis of both low- and high molecular weight fatty acids (e.g. Yoshioka and Ishiwatari, 2005; Taber et al., 2006).

Derivatization protocols reported in the literature differ widely in terms of catalyst reagent, derivatization time, and temperature. Still, very little comparative data is available. The most commonly used catalyst is boron trifluoride (BF<sub>3</sub>), which has become popular as a derivatization reagent since the early 1960s (Metcalf and Schmitz, 1961; Morrison and Smith, 1964). However, it decomposes rapidly when not adequately stored (Liu, 1994), poses a health concern (Rusch et al., 1986), and issues exist about its quality in derivatization reactions (Orgambide et al., 1993; Yurawecz et al., 1993). In particular, it can unpredictably form artefacts (Rusch et al., 1986; Yurawecz et al., 1993), especially when oxidized upon atmospheric exposure. These facts are even annotated in the product specification of commercially available BF<sub>3</sub>-butanol mixtures (Sigma-Aldrich Co., 1998). In theory, any Brønsted or Lewis acid can be used as a catalyst during the alkylation of an organic acid in the presence of an alcohol and sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) can be an attractive alternative to BF<sub>3</sub> (Christie, 1993).

In the present study we examined the relative efficiency of different butylation procedures. The amount of alcohol, catalyst ( $\text{BF}_3$  or  $\text{H}_2\text{SO}_4$ ), reaction time, and reaction temperature were investigated. In addition, we studied the importance of water-scavenging additives in butylation reactions.

## Experimental

### Materials

Dichloromethane (DCM, analytical-reagent grade) and *n*-pentane were purchased from Mallinckrodt (Sydney, Australia). Diethyl ether (photospectroscopic grade, inhibitor free), 1-butanol, and *n*-butanol/ $\text{BF}_3$  (10 % w/w) were purchased from Sigma-Aldrich (Sydney, Australia), concentrated sulphuric acid from Mallinckrodt (Sydney, Australia), and carboxylic acids (Table 3.1) from Sigma Aldrich (Sydney, Australia) and Fluka (Sydney, Australia). All solvents were used without further purification, except *n*-pentane, which was distilled before use. Magnesium sulfate ( $\text{MgSO}_4$ ) was pre-extracted with DCM and dried for >24 h. at 240 °C. Water was purified using an *Elga* (Clayton, Australia) 'Purelab ultra' apparatus. Glassware was annealed at 600 °C and cleaned with DCM before use.

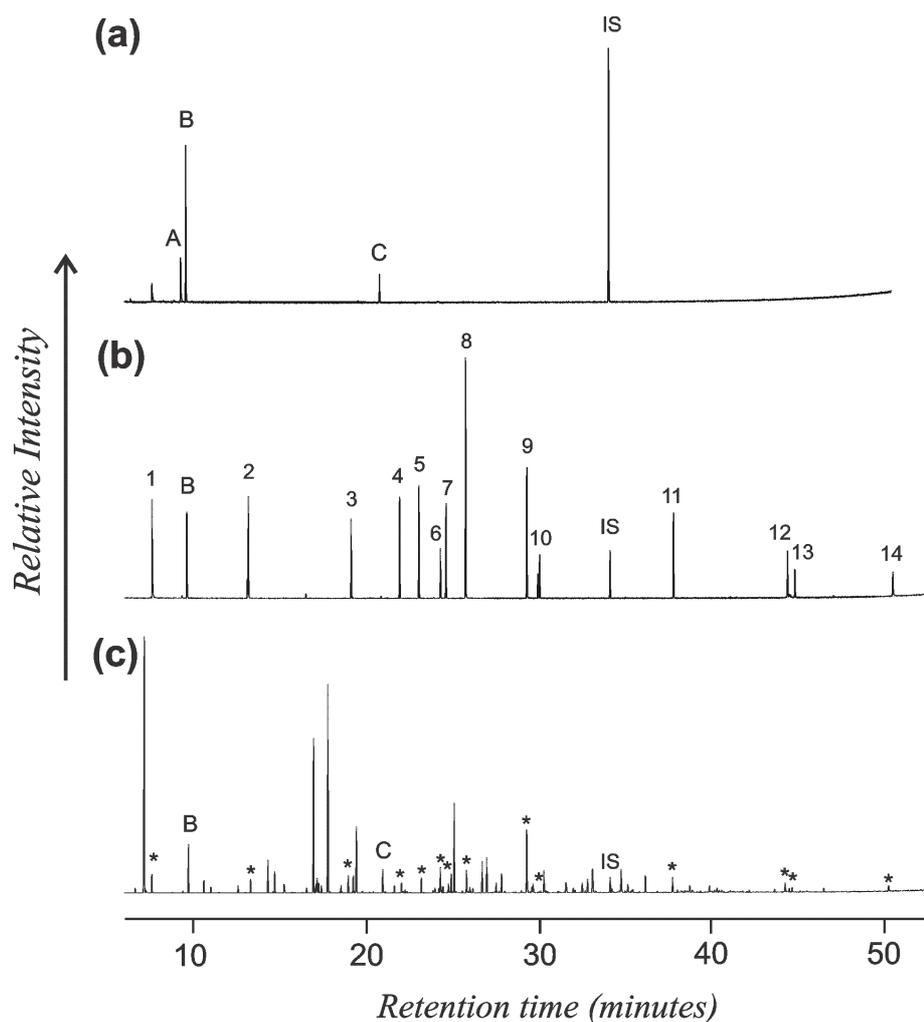
### Analytical protocol

An aliquot of a mixture of 14 organic acid standards dissolved in DCM (Table 3.1) was processed under varying conditions of esterification to compare fatty acid butyl ester yields. Reactions varied in terms of temperature (65 °C or 100 °C) and time (15, 60, or 120 min.). Additionally, one reaction was carried out at 100 °C for 15 h. This resulted in 7 different reaction schemes, labelled alphabetically from A to G (Tables 3.2 and 3.3). Each reaction scheme was conducted with three different amounts of *n*-butanol (100, 200, or 500  $\mu\text{L}$ ) and two different catalysts ( $\text{BF}_3$  or  $\text{H}_2\text{SO}_4$ ), resulting in a total of 42 measurements of butyl ester yield for each carboxylic acid. In addition, reaction schemes C and B were repeated with 100  $\mu\text{L}$  *n*-butanol, and 200  $\mu\text{L}$  *n*-butanol, respectively, and with 200 mg of anhydrous  $\text{MgSO}_4$  added to the reaction vessel. Reaction scheme B was repeated with the addition of 50  $\mu\text{L}$  2,2-dimethoxypropane.

	<i>Carboxylic acid</i>	<i>Concentration</i> [mg/mL]	<i>Amount used</i> [mg/50 $\mu$ L]
1	Acetic	2.320	0.116
2	Butanoic	1.500	0.075
3	Hexanoic	1.165	0.058
4	2-ethyl hexanoic	1.400	0.070
5	Cyclopentyl acetic	1.685	0.084
6	Benzoic	0.660	0.033
7	2-methyl-1-cyclohexane	1.480	0.074
8	Malonic (1,3-propanedioic)	2.910	0.146
9	Pyruvic ( $\alpha$ -keto propionic)	3.125	0.156
10	2,3-dimethylsuccinic	0.700	0.035
11	Tetradecanoic	1.145	0.057
12	Octadecanoic	0.500	0.025
13	Octadecenoic	1.650	0.083
14	Docosenoic	0.810	0.041
	SUM	21.050	1.053

**TABLE 3.1** Compounds used in this study to monitor the butylation efficiency of free carboxylic acids.

A volume of 50  $\mu$ L acid standard (1.05 mg total acids; Table 1) was added to an aliquot (100, 200, or 500  $\mu$ L) of *n*-butanol or *n*-butanol/BF<sub>3</sub> (10 % w/w) in a 5 mL screw-top (PTFE-lined) vial and *n*-pentane (1 mL) was added. In the case of the *n*-butanol samples, an aliquot of 10, 20, or 50  $\mu$ L H<sub>2</sub>SO<sub>4</sub> was added. The vial was sealed and heated (65 °C or 100 °C) for 15, 60, 120 min. or 15 h. in a temperature-controlled sand bath. After cooling to room temperature, the solution was rinsed 5 times with 1 mL purified water. To achieve separation of phases, the vials were centrifuged for 2 minutes at a speed of 2500 rpm. The final organic phase, still containing a small volume of water, was filtered through pre-extracted anhydrous MgSO<sub>4</sub> in a cotton-wool plugged Pasteur-pipette, and concentrated to a volume of approximately 1.5 mL under normal-atmospheric conditions to avoid sample loss. After addition of 17.36  $\mu$ g *n*-octadecane per sample as an internal standard, 1  $\mu$ L of the sample was analysed by GC-MS.

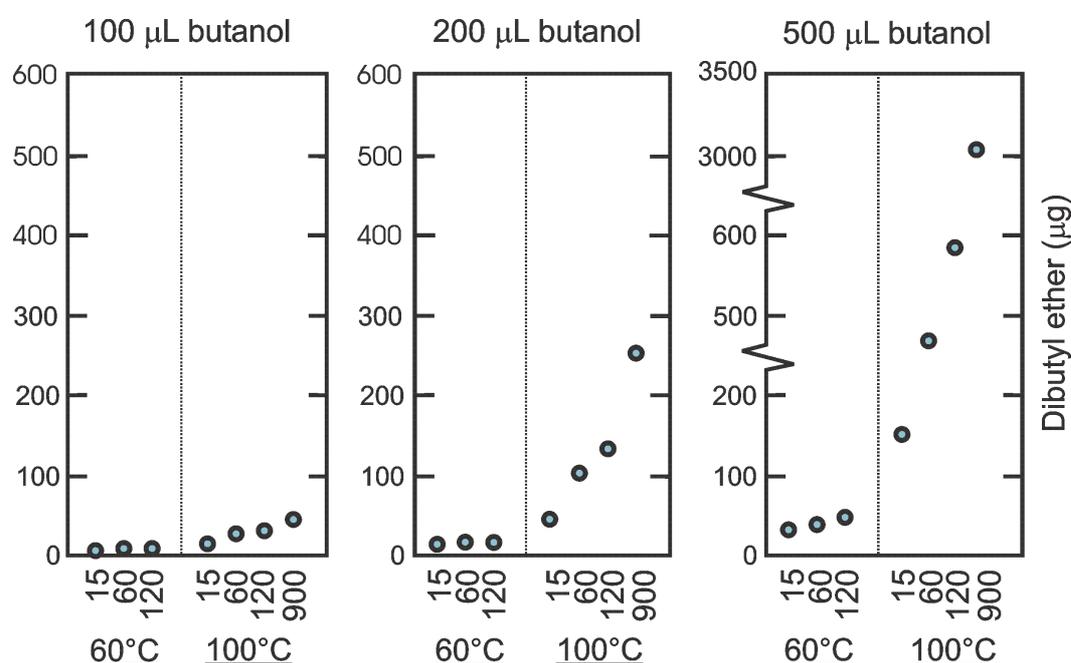


**FIGURE 3.1** Total ion chromatograms of (a) a procedural blank exhibiting the catalytic rearrangement products of *n*-butanol, (b) the butylated acid standard mixture, and (c) the butylated acid standard mixture, derivatized with addition of 2,2-dimethoxypropane. For the identification of carboxylic acids see [Table 3.1](#). Asterisks in (c) correspond to butyl esters in (b). A, 4-heptanone; B, di-*n*-butyl ether; C, 1,1'-dibutoxy butane; IS, internal standard (*n*-octadecane).

#### Gas chromatography and mass spectrometry

GC-MS was performed on a HP 6890 GC system coupled to a HP 5973 mass selective detector. Samples were injected using a 15:1 split ratio with a split flow of 19.5 mL/min. A Phenomenex ZB-5 (crosslinked 5% methylphenylsiloxane) capillary column of 60 m length, 0.25 mm inner diameter and 0.25  $\mu\text{m}$  film thickness was

used with helium as a carrier gas, flowing constantly at 1.3 mL/min. The oven temperature was held at 35 °C for 1 min., then raised with 5°C/min. to a final temperature of 310°C and held isothermal for 15 min. In electron impact (EI) mode, the MS was operated at an electron voltage of 70 eV and a source temperature of 230°C. Data was acquired in full scan ( $m/z$  50-550). Carboxylic acid butyl esters were quantified from the total ion chromatogram (TIC) relative to *n*-octadecane as an internal standard. No corrections were made for specific response factors of individual compounds.



**FIGURE 3.2** The amount of di-*n*-butyl ether, a rearrangement product of *n*-butanol, increases parallel to reaction time, temperature, and the amount of *n*-butanol used. Values are averages of reaction schemes using  $\text{BF}_3$  and  $\text{H}_2\text{SO}_4$  as catalysts.

## Results and Discussion

### Side reactions

All procedural blanks (Figure 3.1 a) and reaction mixtures (Figure 3.1 b) contained a number of products other than the expected butyl esters. These were

identified on the basis of their mass spectra as 4-heptanone, di-*n*-butyl ether, 3-methyl-2-pentanone and 1,1'-dibutoxy butane. Their presence in the procedural blanks shows that they are formed through the catalytic rearrangement of *n*-butanol in an acidic environment. The abundance of 1,1'-dibutoxy butane is closely related to the duration of the reaction, with a correlation of  $R^2 > 0.9$  for the linear relationship between reaction time and the concentration of dibutoxy butane (data not shown). The concentration of dibutyl ether, in contrast, is affected not only by reaction time but also by the amount of reagent (alcohol) and reaction temperature (Figure 3.2).

### Water scavengers

To force the Fischer esterification reaction to completion, water-scavenging reagents can be added (Molnar-Perl and Pinter-Szakacs, 1986; Sigma-Aldrich Co., 1998). We tested two of these. Anhydrous  $MgSO_4$  is a commonly used drying agent and 2,2-dimethoxy propane is a water scavenger, suggested to be used in derivatization reactions by commercial suppliers of  $BF_3$ -alcohol mixtures (Sigma-Aldrich Co., 1998).

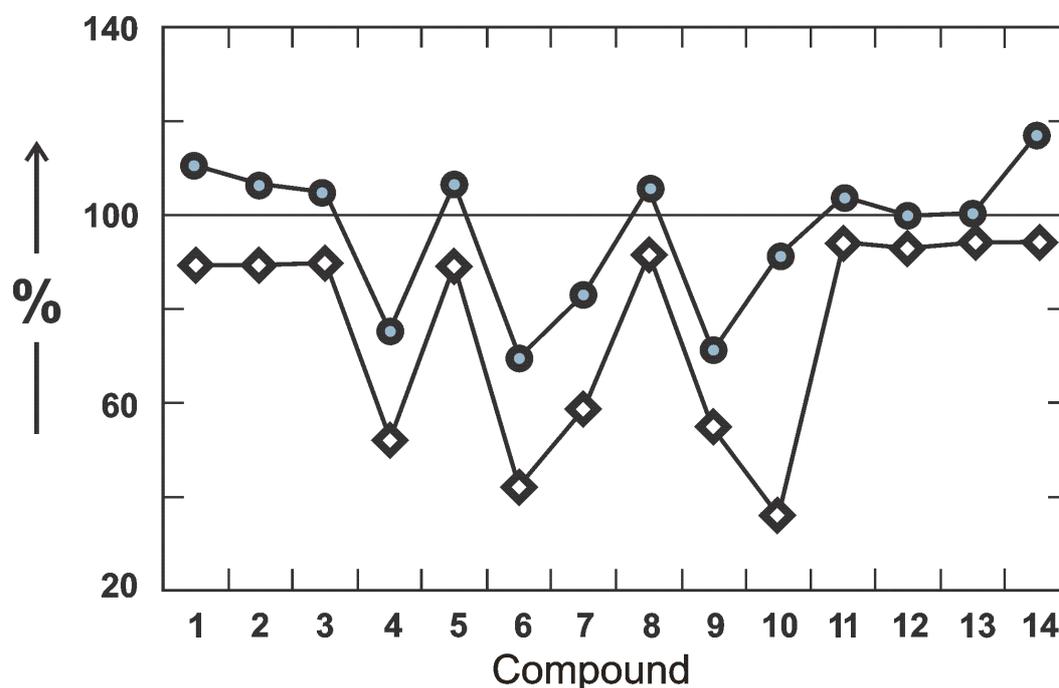
#### *2,2-dimethoxy propane*

Dimethoxy propane reacts with water to yield acetone and methanol. Interestingly, we found no improvement of overall yields when adding this compound. However, many other compounds are generated unpredictably. These artefacts drastically complicate the interpretation of chromatograms by co-eluting and interfering with analytes of interest and cannot be removed from the reaction mixture easily (Figure 3.1 c).

#### *Anhydrous magnesium sulphate*

The addition of anhydrous  $MgSO_4$  to the reaction mixture led to a selective relative decrease in the yield of arenic and branched butyl esters. The presence of 200 mg  $MgSO_4$  during the reaction led to a decreased butyl ester yield for following compounds: benzoic acid, 2-ethylhexanoic acid, 2-methyl-cyclohexane carboxylic acid, 2,3-dimethylsuccinic acid, and pyruvic acid. Their average yield decreased

with 22.0% (modified reaction scheme C with 100  $\mu\text{L}$  *n*-butanol and 200 mg  $\text{MgSO}_4$  added; [Tables 3.2 and 3.3](#)), respectively 51.2% (modified reaction scheme B with 200  $\mu\text{L}$  *n*-butanol and 200 mg  $\text{MgSO}_4$  added; [Tables 3.2 and 3.3](#)) when  $\text{MgSO}_4$  was added, compared to the same reaction without  $\text{MgSO}_4$  ([Figure 3.3](#)). The fractionation was not observed when butyl esters were filtered through  $\text{MgSO}_4$  after completion of the alkylation reaction. This indicates that  $\text{MgSO}_4$  consistently but selectively affects the alkylation reaction of alkyl-branched and arenic carboxylic acids.



**FIGURE 3.3** Relative reaction yield of individual carboxylic acids butylated using 100  $\mu\text{L}$  *n*-butanol/ $\text{H}_2\text{SO}_4$  containing 200 mg  $\text{MgSO}_4$  at 65°C for 120 min. (filled circles), and 200  $\mu\text{L}$  *n*-butanol/ $\text{H}_2\text{SO}_4$  containing 200 mg  $\text{MgSO}_4$  at 65°C for 60 min. (open diamonds). The yield percentage depicted on the y-axis is relative to samples butylated under identical conditions but lacking  $\text{MgSO}_4$ . For identification of compounds the reader is referred to [Table 3.1](#).

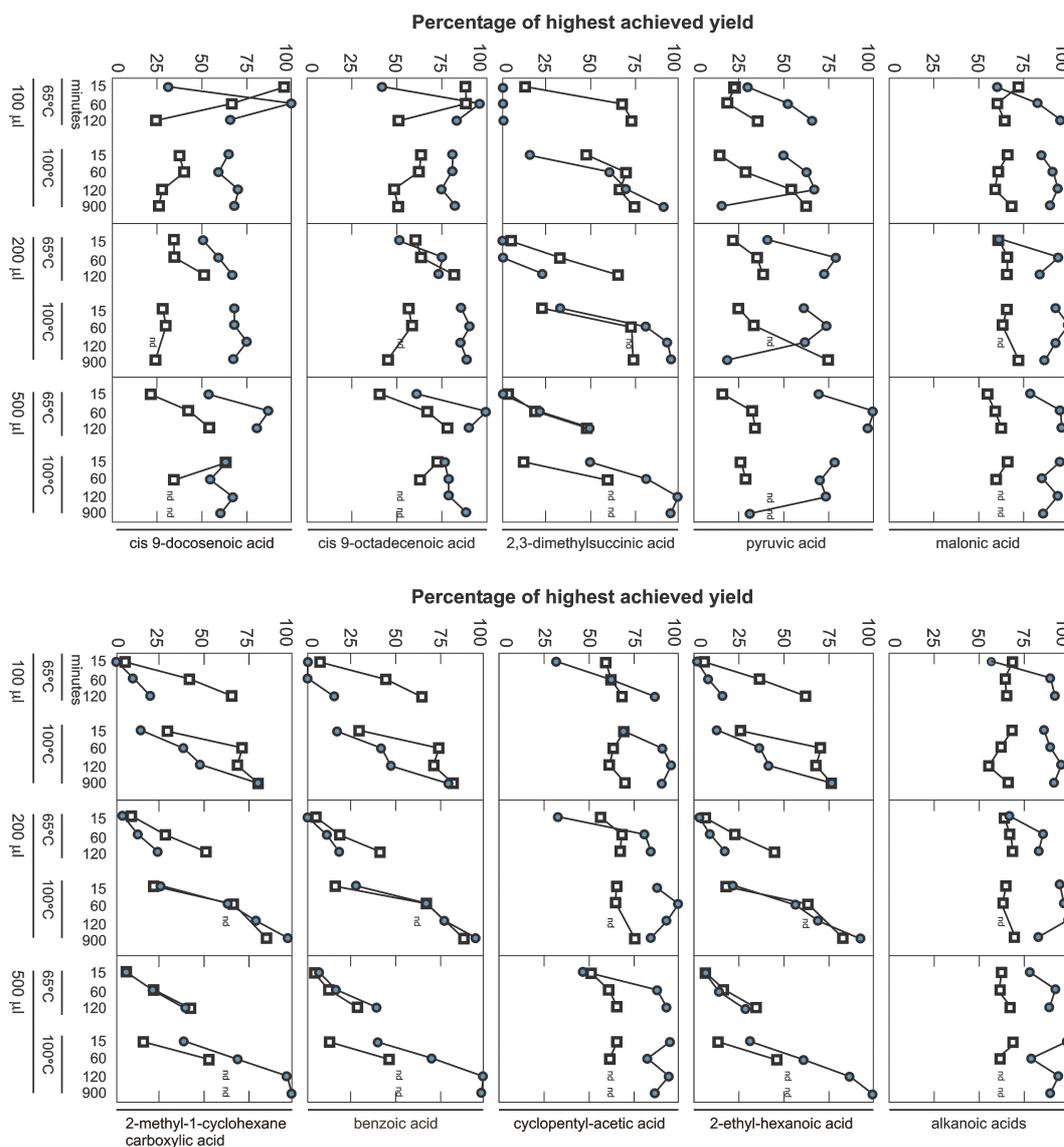
### Derivatization efficiency

The results from the derivatization experiments are listed in [Tables 3.2 and 3.3](#), which display the butyl ester yield of each carboxylic acid under different reaction schemes ([Table 3.2](#) – H<sub>2</sub>SO<sub>4</sub> catalyst; [Table 3.3](#) – BF<sub>3</sub> catalyst), normalized to the highest achieved butyl ester yield (using H<sub>2</sub>SO<sub>4</sub> or BF<sub>3</sub> as a catalyst). In general, an increase in the total yield of reaction products was observed with increasing reaction time, temperature, and amount of alcohol used. However, individual compounds and compound classes exhibited strong variations in butyl ester yields during different reaction procedures as shown in [Figure 3.4](#). Procedural blanks showed no carboxylic acid contaminants except for a minimum amount of acetic acid and the aforementioned impurities ([Figure 3.1 a](#)).

A number of carboxylic acids yield similar amounts of butyl esters throughout the different reaction procedures. These include all alkanolic acids (ethanoic, butanoic, hexanoic, tetradecanoic, and octadecanoic acid), malonic (propanedioic) acid, and to a lesser extent cyclopentyl acetic acid. However, the average yields of all reaction schemes are higher when using BF<sub>3</sub> (alkanoic, 87.2 ± 5.3 %; malonic, 87.6 ± 5.3 %, respectively) than when using H<sub>2</sub>SO<sub>4</sub> (alkanoic, 64.9 ± 1.8 %; malonic, 63.6 ± 2.3 %, respectively) as a catalyst. Scheme A reactions (15 min., 65 °C; [Tables 3.2 and 3.3](#)) consistently result in lower butyl ester yields. In the presence of BF<sub>3</sub> catalyst, the difference between the amounts of butyl esters yielded by scheme A reactions, and the amounts yielded by the other reaction schemes diminishes with increasing amounts of alcohol used. When H<sub>2</sub>SO<sub>4</sub> is used as a catalyst this phenomenon is not observed, suggesting a slightly better catalytic performance of BF<sub>3</sub> over H<sub>2</sub>SO<sub>4</sub>.

A second group of carboxylic acids exhibit a trend of strongly enhanced yields with increasing reaction time, temperature and amount of alcohol used. The relative amount of benzoic acid, 2,3-dimethylsuccinic acid, 2-methyl-1-cyclohexane carboxylic acid, and 2-ethylhexanoic acid rises from values at or close to zero percent of the highest achieved yield (normalised to 100%) during scheme A reactions (15 min., 65 °C) with 100 µL butanol to the maximum yield (100%), which in most cases was achieved during scheme G reactions (15 h., 100 °C) with 500 µL butanol. This indicates that all three factors, reaction time, reaction temperature and the volume of alcohol used influence the final butyl ester yield. It also shows that the analysis of these compounds requires lengthy derivatization procedures,

not commonly used, when quantitative results are envisaged. This observation offers an explanation for the often-reported inefficient derivatization of aromatic acids with MeOH/BF<sub>3</sub> (e.g. Behar and Albrecht, 1984; Jones et al., 2001). Highest yields were always found when using BF<sub>3</sub> as a catalyst. Interestingly, the yield of butyl benzoate increases parallel to the amount of alcohol used when employing



**FIGURE 3.4** Relative butyl ester yield of individual carboxylic acids during derivatization reactions that differed in reaction time, reaction temperature, alcohol volume, and catalyst reagent: H<sub>2</sub>SO<sub>4</sub> (open squares) or BF<sub>3</sub> (filled circles). Reaction yields are normalized to the highest achieved yield with either catalyst.

BF<sub>3</sub> as a catalyst, but decreases parallel to the amount of alcohol when employing H<sub>2</sub>SO<sub>4</sub> as a catalyst. The reason for this trend is unclear.

A third group of carboxylic acids contains the monounsaturated compounds oleic acid (C<sub>18:1</sub> *cis*-9) and erucic acid (C<sub>22:1</sub> *cis*-13), as well as pyruvic ( $\alpha$ -keto propanoic) acid. The monounsaturated acids derivatized quickly, but degrade at higher temperatures and longer reaction times. The highest yield of butyl erucate is generated after a 15 min. (H<sub>2</sub>SO<sub>4</sub>), or 60 min. (BF<sub>3</sub>) reaction and a similar pattern is observed for oleic acid. This indicates that caution must be exerted when unsaturated fatty acids are to be analysed quantitatively. Pyruvic acid shows an erratic trend of butyl ester yield during the different derivatization procedures, with a positive influence of the amount of alcohol used on the final yield of butyl pyruvate. Longer reaction procedures under high temperatures (100°C), however, have a negative effect on the butylation of pyruvic acid.

## Conclusions

Butylation of carboxylic acids is an attractive alternative to methylation when acids of low molecular weight are to be analyzed because the esters are less volatile, thus allowing for quantitative studies. In the reaction process, BF<sub>3</sub> is a slightly more efficient catalyst than anhydrous H<sub>2</sub>SO<sub>4</sub> and generates consistently higher butyl ester yields. The use of the water-scavenging additives 2,2-dimethoxypropane and anhydrous MgSO<sub>4</sub> in derivatization reactions in view of improving reaction yields is discouraged by our results. Different classes of carboxylic acids vary strongly in their optimum reaction conditions. The use of standard protocols for all types of organic acids can therefore lead to considerable errors in quantitative analysis of individual types of carboxylic acids and is discouraged.

Compound	65°C			100°C				
	15 min	60 min	120 min	15 min	60 min	120 min	15 h	
	<i>Reaction scheme</i>	A	B	C	D	E	F	G
<u>100 µL n-butanol</u>								
Acetic acid	58.8	78.3	71.4	76.8	72.4	84.2	78.4	
Butanoic acid	52.0	82.5	92.4	83.8	87.7	99.1	92.9	
Hexanoic acid	55.2	82.0	95.9	83.3	89.3	95.7	80.2	
2-ethyl hexanoic acid	1.9	8.3	16.1	12.7	36.2	41.9	77.4	
Cyclopentyl acetic acid	31.1	61.8	87.0	69.0	91.5	96.3	91.1	
Benzoic acid	0.0	0.0	15.7	17.0	41.8	47.9	80.8	
2-methyl-1-cyclohexane carb. acid	0.0	10.1	19.4	14.3	38.9	48.1	80.0	
Malonic acid	60.5	82.5	95.2	84.8	91.4	93.9	89.8	
Pyruvic acid	29.9	52.9	65.9	50.3	63.5	67.6	15.9	
2,3-dimethyl succinic acid	0.0	0.0	0.0	16.5	60.5	70.0	92.0	
Tetradecanoic acid	49.3	92.5	94.2	84.0	93.9	89.8	93.2	
Octadecanoic acid	46.1	100.0	92.8	80.9	91.2	75.0	97.3	
Octadecenoic acid	42.1	96.1	83.1	80.9	80.9	74.9	82.3	
Docosenoic acid	31.0	100.0	65.4	64.4	59.0	70.0	68.2	
<u>200 µL n-butanol</u>								
Acetic acid	74.8	65.0	72.8	82.5	80.4	94.1	64.1	
Butanoic acid	60.2	85.3	81.0	94.5	96.8	100.0	77.8	
Hexanoic acid	60.2	93.5	81.8	91.8	100.0	89.8	79.2	
2-ethyl hexanoic acid	3.0	9.4	17.5	21.6	56.7	68.4	92.3	
Cyclopentyl acetic acid	33.1	81.5	84.8	89.1	100.0	93.6	85.4	
Benzoic acid	0.0	11.3	18.2	27.4	68.1	78.1	95.7	
2-methyl-1-cyclohexane carb. acid	3.8	12.6	23.9	25.2	64.2	79.9	99.3	
Malonic acid	60.5	94.2	84.1	92.5	100.0	93.0	86.8	
Pyruvic acid	40.8	79.8	72.9	62.0	74.0	61.9	19.0	
2,3-dimethyl succinic acid	0.0	0.0	22.3	32.6	81.8	94.2	95.7	
Tetradecanoic acid	54.4	87.8	84.3	94.0	96.6	94.0	93.3	
Octadecanoic acid	53.1	86.6	77.2	90.3	95.8	93.9	93.2	
Octadecenoic acid	51.7	74.8	73.5	86.0	90.5	86.1	89.2	
Docosenoic acid	50.4	59.2	66.5	68.2	68.5	74.8	67.2	
<u>500 µL n-butanol</u>								
Acetic acid	82.1	79.1	74.3	100.0	63.0	87.2	84.4	
Butanoic acid	70.0	82.7	80.0	95.0	74.6	93.5	88.0	
Hexanoic acid	73.0	92.9	85.9	92.7	78.7	87.6	81.0	
2-ethyl hexanoic acid	5.7	14.4	27.8	31.2	61.2	86.1	100.0	
Cyclopentyl acetic acid	46.6	88.5	92.8	96.2	82.2	94.5	87.1	
Benzoic acid	6.0	16.9	39.3	40.1	71.3	100.0	99.6	
2-methyl-1-cyclohexane carb. acid	5.7	20.4	39.8	38.6	69.3	97.2	100.0	
Malonic acid	78.6	95.4	96.3	95.2	85.1	94.0	86.2	
Pyruvic acid	69.9	100.0	97.2	78.8	70.3	73.2	31.6	
2,3-dimethyl succinic acid	0.0	20.9	49.3	50.0	81.6	100.0	96.0	
Tetradecanoic acid	72.8	100.0	97.1	88.1	86.8	89.8	85.8	
Octadecanoic acid	64.9	90.8	100.0	84.1	82.0	82.8	78.3	
Octadecenoic acid	61.1	100.0	90.1	76.6	79.2	78.5	88.0	
Docosenoic acid	54.4	87.0	80.3	63.1	54.1	67.5	60.2	

**TABLE 3.2** Relative reaction yield of individual carboxylic acid butyl esters after the derivatization of their free fatty acid precursors using H<sub>2</sub>SO<sub>4</sub> as a catalyst under conditions of differing reaction time, temperature, and alcohol volume. Values indicate percentage of highest achieved yield.

Compound	65°C			100°C				
	15 min	60 min	120 min	15 min	60 min	120 min	15 h	
	<i>Reaction scheme</i>							
	A	B	C	D	E	F	G	
<u>100 <math>\mu</math>L <i>n</i>-butanol</u>								
Acetic acid	58.8	78.3	71.4	76.8	72.4	84.2	78.4	
Butanoic acid	52.0	82.5	92.4	83.8	87.7	99.1	92.9	
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Cyclopentyl acetic acid	31.1	61.8	87.0	69.0	91.5	96.3	91.1	
Benzoic acid	0.0	0.0	15.7	17.0	41.8	47.9	80.8	
2-methyl-1-cyclohexane carb. acid	0.0	10.1	19.4	14.3	38.9	48.1	80.0	
Malonic acid	60.5	82.5	95.2	84.8	91.4	93.9	89.8	
Pyruvic acid	29.9	52.9	65.9	50.3	63.5	67.6	15.9	
2,3-dimethyl succinic acid	0.0	0.0	0.0	16.5	60.5	70.0	92.0	
Tetradecanoic acid	49.3	92.5	94.2	84.0	93.9	89.8	93.2	
Octadecanoic acid	46.1	100.0	92.8	80.9	91.2	75.0	97.3	
Octadecenoic acid	42.1	96.1	83.1	80.9	80.9	74.9	82.3	
Docosenoic acid	31.0	100.0	65.4	64.4	59.0	70.0	68.2	
<u>200 <math>\mu</math>L <i>n</i>-butanol</u>								
Acetic acid	74.8	65.0	72.8	82.5	80.4	94.1	64.1	
Butanoic acid	60.2	85.3	81.0	94.5	96.8	100.0	77.8	
Hexanoic acid	60.2	93.5	81.8	91.8	100.0	89.8	79.2	
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Cyclopentyl acetic acid	33.1	81.5	84.8	89.1	100.0	93.6	85.4	
Benzoic acid	0.0	11.3	18.2	27.4	68.1	78.1	95.7	
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Octadecanoic acid	53.1	86.6	77.2	90.3	95.8	93.9	93.2	
Octadecenoic acid	51.7	74.8	73.5	86.0	90.5	86.1	89.2	
Docosenoic acid	50.4	59.2	66.5	68.2	68.5	74.8	67.2	
<u>500 <math>\mu</math>L <i>n</i>-butanol</u>								
Acetic acid	82.1	79.1	74.3	100.0	63.0	87.2	84.4	
Butanoic acid	70.0	82.7	80.0	95.0	74.6	93.5	88.0	
Hexanoic acid	73.0	92.9	85.9	92.7	78.7	87.6	81.0	
2-ethyl hexanoic acid	5.7	14.4	27.8	31.2	61.2	86.1	100.0	
Cyclopentyl acetic acid	46.6	88.5	92.8	96.2	82.2	94.5	87.1	
Benzoic acid	6.0	16.9	39.3	40.1	71.3	100.0	99.6	
2-methyl-1-cyclohexane carb. acid	5.7	20.4	39.8	38.6	69.3	97.2	100.0	
Malonic acid	78.6	95.4	96.3	95.2	85.1	94.0	86.2	
Pyruvic acid	69.9	100.0	97.2	78.8	70.3	73.2	31.6	
2,3-dimethyl succinic acid	0.0	20.9	49.3	50.0	81.6	100.0	96.0	
Tetradecanoic acid	72.8	100.0	97.1	88.1	86.8	89.8	85.8	
Octadecanoic acid	64.9	90.8	100.0	84.1	82.0	82.8	78.3	
Octadecenoic acid	61.1	100.0	90.1	76.6	79.2	78.5	88.0	
Docosenoic acid	54.4	87.0	80.3	63.1	54.1	67.5	60.2	

**TABLE 3.3** Relative reaction yield of individual carboxylic acid butyl esters after the derivatization of their free fatty acid precursors using  $\text{BF}_3$  as a catalyst under conditions of differing reaction time, temperature, and alcohol volume. Values indicate percentage of highest achieved yield.

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## Molecular signature of the Neoproterozoic Acraman impact event.

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David McKirdy, and Kliti Grice

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**Abstract** - A bolide impact on the Gawler Craton, South Australia, during the late Neoproterozoic produced a collapse crater of 85-90 km diameter at the site of present-day Lake Acraman, left sedimentary iridium anomalies, and may have affected the local biosphere. However, the role of the impact on Neoproterozoic biotic evolution is however strongly debated. We show here that marine sediments deposited during and after the Acraman impact contain anomalous concentrations of the polyaromatic hydrocarbon coronene, which is considered to be a molecular marker of combustion. The co-occurrence of abundant coronene anomaly and a negative carbon-isotopic excursion suggests a pulse of isotopically light CO<sub>2</sub> released into the atmosphere by combustion of biomass that may also have caused extensive atmospheric dimming. Prolonged photosynthetic stress could have impacted the Ediacaran biosphere. Parallels to the Chicxulub impact event suggest that the here reported coronene anomaly might form the first global chronostratigraphic marker in the Late Neoproterozoic.

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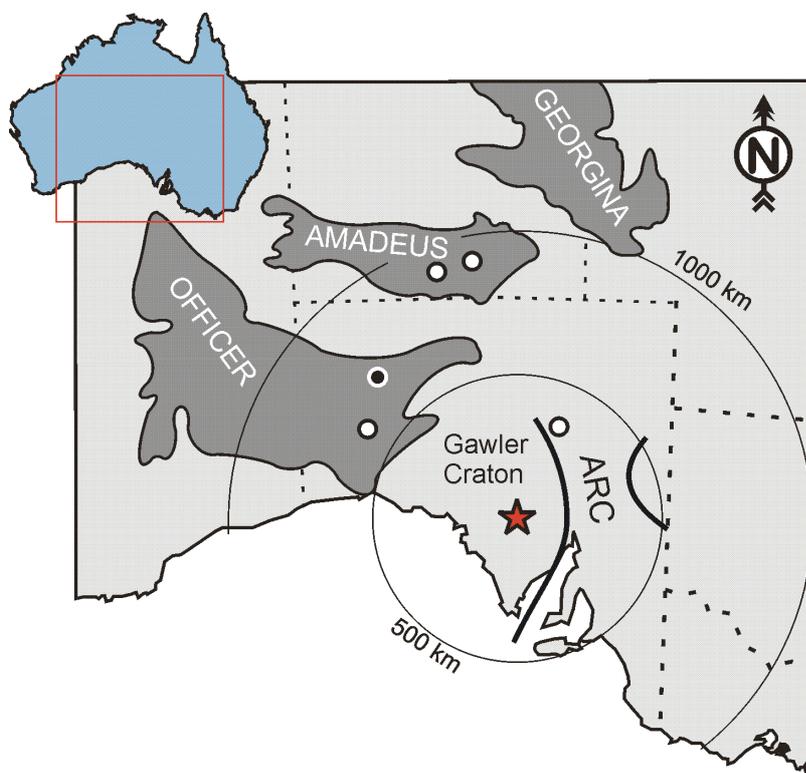
## Introduction

Late Neoproterozoic (Ediacaran) sediments are of particular interest to evolutionary research because they host Earth's oldest known fossil evidence for complex macroscopic life forms (Marshall, 2006).

Although the taxonomic affiliation of the Ediacara fauna is contested, molecular fossils of *Demospongiae* indicate that metazoa had already evolved (Love et al., 2006), and general consent exists about the presence of di- and triploblastic metazoa by the end of the Proterozoic aeon. Molecular clock studies that relate the divergence time of two species to the number of molecular differences measured in DNA sequences or proteins (Pauling and Zuckerkandl, 1962) yield variable metazoan lineage divergence times, ranging from the Mesoproterozoic to the Ediacaran (Peterson et al., 2008). However, the global emergence and subsequent rapid radiation of complex life did not occur until the latest Proterozoic and Early Cambrian. The reasons for this notably rapid radiation are subject to debate. Relief of selective evolutionary pressure, induced by environmental conditions of the 'Snowball Earth' glaciations, was suggested as a factor (Hoffman et al., 1998) but recently contested (Olcott et al., 2005). Alternatively, high nutrient fluxes caused by orogen-induced increased weathering (Squire et al., 2006) were suggested. The most widely accepted hypothesis suggests that increased atmospheric oxygen levels played a key role in the evolution of eukaryotic organisms (Fike et al., 2006; McFadden et al., 2008). The late Neoproterozoic impact of a bolide on the Gawler Craton, South Australia, left an impact crater at the site of present-day Lake Acraman (Williams and Wallace, 2003) and sedimentary iridium anomalies (Gostin et al., 1989). A radiation of Acritarch species was observed after the Acraman impact event (Grey et al., 2003; Grey, 2005) and changes in post-impact sedimentary sterane ratios were attributed to an environmentally stressed local biosphere (McKirdy et al., 2006). The acritarch radiation predates the first occurrences of the Ediacara fauna. However, the significance of the Acraman event in late Neoproterozoic biotic evolution remains controversial.

We analysed core samples from drill hole Munta-1 (Figure 4.1) and studied their organic chemical composition. Results show that high abundances of a polyaromatic combustion-marker (coronene) coincide with a negative carbon isotopic excursion within late Neoproterozoic (Ediacaran) marine sediments that

record the Acraman impact event (Figure 4.2). Combined, they suggest impact-related combustion of early terrigenous or marginally marine biomass and possibly extensive dimming of incident sunlight. Prolonged photosynthetic stress could have influenced the subsequent course of Ediacaran biospheric evolution (Toon et al., 1997).



**FIGURE 4.1** Location of the Acraman impact (star) and study site (dark filled circle). An impact ejecta layer is found in outcrops of the Adelaide Rift Complex (ARC) and in wells of the eastern Officer Basin. The impact-related C-isotope excursion is recognised in the ARC, as well as in wells from the Officer and Amadeus Basins. Circles represent wells.

## Methods

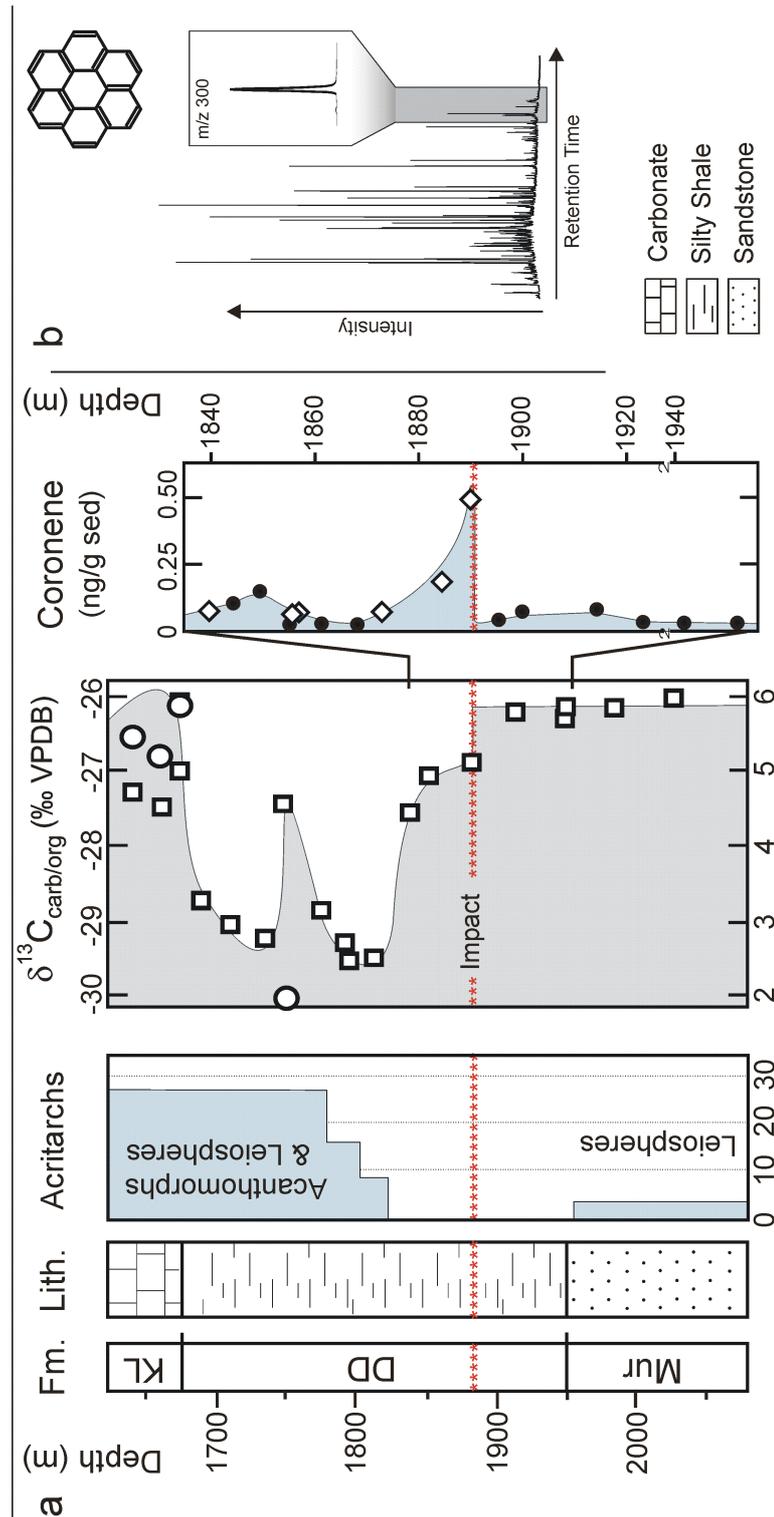
Core samples were wire-brushed, and subsequently cleaned with deionised water and organic solvents. After crushing and pulverising in a *Siebtechnik* Mill (Mülheim an der Ruhr, Germany), samples (40-150 g) were extracted under reflux (dichloromethane/methanol 93:7; 72 h.) in a Soxhlet apparatus. Solvent was

removed by rotary evaporation and extracts were fractionated into saturated hydrocarbons, aromatic hydrocarbons, and NSO-compounds using open column liquid chromatography. The aromatic hydrocarbon fractions were spiked with  $d_{14}$ -para-terphenyl as an internal standard and analyzed using coupled gas chromatography (GC) and mass spectrometry (MS) in full scan and selective ion monitoring modes. Samples were injected in splitless mode and separated on a fused silica capillary column (Agilent J&W DB-5: 60 m; 0.25 mm inner diameter; 0.25  $\mu$ m film thickness; [5%-phenyl]-methylpolysiloxane).

## **Results and discussion**

Coronene ( $C_{24}H_{12}$ ) is a highly condensed polyaromatic hydrocarbon (PAH) lacking biological precursors that generally forms by high-temperature rearrangement of organic matter during combustion (Killops and Killops, 2005). It is the smallest 'large PAH' that can still be conveniently analyzed using gas chromatography (GC) coupled to mass spectrometry (MS). Smaller PAHs, such as phenanthrene and benzopyrenes, can arise by diagenetic rearrangement of natural product precursors such as terpenoids (Alexander et al., 1992; Grice et al., 2007) but larger PAHs do not. They are found as combustion products, but at lower levels than the small PAHs due to kinetic limitation of their production through addition of successive rings. Larger PAHs can thus be classified as small soot particles, making them molecular combustion markers (Venkatesan and Dahl, 1989).

A background PAH signal, as found pre-impact in the studied samples, can be produced by organic matter combustion through lightning strikes, or by the constant fallout of cosmic dust (Maurette, 1998). The samples studied however received two distinct PAH inputs (Figure 4.3) as indicated by two nearly-perfect linear correlations ( $R^2$  of 0.99 and 0.98) between coronene and benzo[ghi]perylene ( $C_{22}H_{12}$ ). Both the impact horizon sample and the first post-impact sample exhibit anomalous relative amounts of coronene (Figures 4.2 and 4.3), attributed to impact-related combustion processes. Other samples that exhibit the same elevated relative concentration of coronene (Figure 4.3) were all deposited post-impact (Figure 4.2) and interpreted as reworked and redeposited ejecta layer material. No pre-impact sample exhibits this high relative concentration of coronene.



Key: Fm., Formation; Mur., Murnaroo Formation; DD, Dey Dey Mudstone; KL, Karlaya Limestone; Lith., lithology; carb., carbonate; org., organic; VPDB, Vienna Pee-Dee Belemnite; sed., sedimentary rock; m/z, mass to charge ratio.

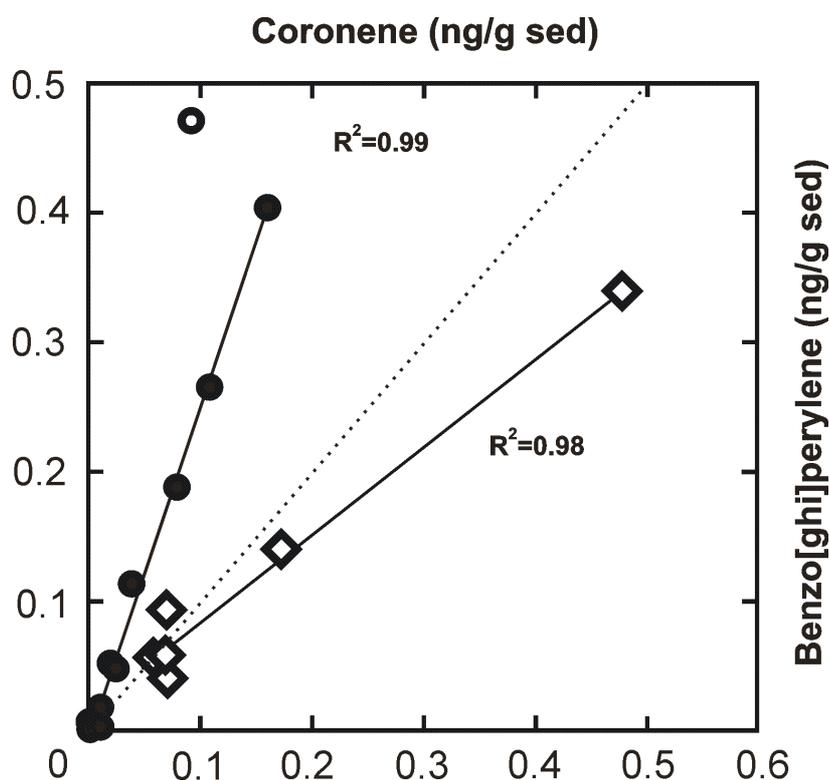
**FIGURE 4.2** Munta-1 drill hole. **a.** Depth, stratigraphy, lithology, palynology, stable carbon isotopes (●-carbonate, □-organic), and coronene concentrations. **b.** Gas chromatogram showing the aromatic fraction (total ion current), coronene peak (insert; selective ion monitoring of  $m/z$  300) and chemical structure of coronene. Coronene symbols in (a) correspond to those in Figure 4.3.

Since highly peri-condensed PAHs are more reactive than PAHs with lower degrees of angular fusion, their presence in pyrolysis products is attributed to rapid quenching by adsorption on soot particles (Killops and Killops, 2005), which allow for long-range aeolian transport (e.g. Zhang et al., 2001). Coronene was reported as a molecular marker of biomass burning in the aftermath of the Cretaceous/Tertiary (K–T) Chicxulub impact event, and was found worldwide at K–T sections (Venkatesan and Dahl, 1989).

We document an asymmetric anomaly of sedimentary coronene concentrations in drill core from Munta-1 well, Officer Basin. A sharp increase in coronene abundance occurs at 1890.10 m, 5 m beneath the lowest recorded layer of reworked impact ejecta (Hill et al., 2008), and is synchronous with a negative carbon isotopic ( $\delta^{13}\text{C}$ ) excursion (Figure 4.2). We attribute this to combustion of bacterial/algal biomass (vascular plants had not yet evolved) during the Acraman impact event. Post-impact, the coronene concentration rises to 11 times the average pre-impact value (0.04 ng/g sediment). The anomaly persists for 17–22 m before concentrations return to pre-impact values, indicating a long-term enhanced input of coronene to the basin floor.

The impact event was followed by a negative excursion in sedimentary  $\delta^{13}\text{C}$  of  $-3.5\text{‰}$  (Grey et al., 2003, and references therein). Negative excursions in sedimentary  $\delta^{13}\text{C}$  can have various causes.  $\text{CO}_2$  exhalation (Berner, 2004) or magmatic combustion of organic-rich strata (Svensen et al., 2004) can be excluded as drivers of the associated  $\delta^{13}\text{C}$  excursions due to the lack of volcanic activity at the time of the Acraman event. The co-variation of  $\delta^{13}\text{C}_{\text{org}}$  and  $\delta^{13}\text{C}_{\text{carb}}$  suggests a mixed water column (Calver, 2000) and excludes recycling of  $\text{CO}_2$  in stratified seas (Schouten et al., 2000) as a cause. This is also supported by the chemostratigraphic correlation of the Acraman isotope excursion in several widely separated depositional units (Officer Basin, Amadeus Basin, Stuart Shelf, Adelaide Rift Complex). While impact-related destabilization of methane hydrates and consequent release and oxidation of isotopic light methane (Hesselbo et al., 2000) might have resulted in  $^{13}\text{C}$ -depleted carbon available for photosynthetic fixation, impact-related combustion of organic matter is more likely. The coincidental onset of both the isotope and PAH anomalies in Figure 4.2 suggests a causal relationship between atmospheric  $^{13}\text{CO}_2$  levels and massive combustion of organic matter. Rapid combustion of biomass can boost atmospheric  $\text{CO}_2$  with a concomitant

relative reduction of  $\delta^{13}\text{CO}_2$ , thereby causing negative sedimentary  $\delta^{13}\text{C}$  anomalies (Finkelstein et al., 2006). Correlative C-isotopic shifts occur at the same chronostratigraphic level in other Australian basins (Amadeus Basin, Stuart Shelf, Adelaide Rift Complex), implicating atmospheric  $\text{CO}_2$  as a driver of  $^{13}\text{C}$ -depleted biomass. The coincidence of a strong negative shift in sedimentary  $\delta^{13}\text{C}$  and a combustion-marker anomaly within the chronostratigraphic interval of the Acraman event (Hill et al., 2006) strongly suggests the bolide impact as their common cause.



**FIGURE 4.3** Sedimentary PAH concentrations in Munta-1 drill hole. Two distinct sources are indicated by a linear relationships between sedimentary coronene (Cor) and benzo[ghi]perylene (BghiP). Background PAH input (circles) is represented by low Cor/BghiP values, while impact-related input (open diamonds) has high Cor/BghiP values. The Acraman impact layer sample falls on the coronene-dominated trend and exhibits the largest coronene concentration (Figure 4.2). Symbols correspond to those in Figure 4.2. The sample represented by an open circle was not used for calculation of correlation coefficients.

Arinubu et al. (1999) reported a similar scenario from a K–T boundary studied in Spain: a spike in pyrolytic PAH is followed immediately by a ~1.6‰ negative carbon isotope excursion. The K–T Chicxulub impact event left its traces in the form of molecular combustion markers at locations spanning the globe (Venkatesan and Dahl, 1989), and the same might be true for the Acraman impact event. In case of a global distribution of soot and pyrolytic molecular markers, the here reported coronene anomaly could form the first certainly syndepositional chronostratigraphic marker in the Ediacaran.

Impact-related biomass burning and increased soot deposition necessarily involve higher concentrations of atmospheric particulate matter and raise the possibility of decreased solar insolation. The long-term coronene anomaly (Figure 4.2) suggests an extended period of soot fall out. Palaeomagnetic studies suggest a low palaeolatitude for the impact site ( $12.5^{\circ} \pm 7.1^{\circ}$ – $6.1^{\circ}$ ) (Schmidt and Williams, 1996). Due to this equatorial location, a persistent dust cloud is likely to have spread across continents and into both hemispheres (Williams and Wallace, 2003). Such large-scale dimming would have significantly hampered photosynthetic activity (Toon et al., 1997; Williams and Wallace, 2003), thereby stressing phototrophs and possibly contributing to the reorganisation of biogeochemical cycles (Logan et al., 1995) during the latest Neoproterozoic.

The coronene anomaly reported herein is the first chemical marker capable of pinpointing the exact timing of the Acraman event at locations too distant from the impact site to receive grain-size ejecta. This anomaly may represent the first global chronostratigraphic marker for the Ediacaran in the form of a ‘molecular’ ash bed.

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## Comprehensive evaluation of 4D-reservoir geochemistry. Molecular and isotopic response of low-molecular-weight petroleum constituents

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**Abstract** - Examination of molecular changes in produced petroleum fluid over time has been established as a novel tool to monitor fluid dynamics in petroleum reservoirs. The concept is referred to as four dimensional (4D)-reservoir geochemistry or time-lapse geochemistry (TLG) and its application during petroleum production can supplement understanding of processes in petroleum reservoirs. TLG is novel because little is known about changes in petroleum composition during the continuous production of oil. Here we present the first findings obtained from a comprehensive study of changes in the molecular and isotopic composition of low-molecular-weight petroleum constituents during production. The concentrations and distributions of C<sub>0</sub>-C<sub>3</sub> alkylbenzenes, C<sub>6</sub> and C<sub>7</sub> aliphatics, and C<sub>0</sub>-C<sub>2</sub> alkylphenols were monitored in produced fluids over a 335 day period and the stable carbon isotope ratio of alkylbenzenes was determined. Results show that light aromatic and polar compounds exhibit pronounced changes in concentration. Water washing and evaporative fractionation did not cause these changes based on invariant concentrations of the strongly hydrophilic compound phenol, and selective depletion of volatile compounds, respectively. Furthermore, no  $\delta^{13}\text{C}$  fractionation was observed in the aromatics. A strong variation in alkylphenol isomers during fluid production suggests occurrence of abiotic oxidation. The results presented in this study (1) suggest the need for further work

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on 4D-petroleum compositional changes and (2) reveal details on the interaction between petroleum and water in the subsurface.

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## **Introduction**

### 4D-Reservoir Geochemistry

Reservoir geochemistry is a low cost field development and appraisal tool based on the principle that fluids isolated by flow barriers can show compositional and stable carbon isotopic differences (Davis et al., 2005). Such differences may reflect subtle variations in charge history related to the location of the source kitchen and the maturity of the source rock at the time of expulsion, and/or post-fill processes, such as water-washing, leakage by evaporative fractionation or seal failure, or *in-situ* biodegradation. For a more complete overview of reservoir geochemistry the reader is referred to Cubitt and England (1995), Cubitt et al. (2004), England (1990), or Peters and Fowler (2002).

Reservoir surveillance is a key activity during oil and gas production that can improve understanding of petroleum fluid behaviour (e.g., phase changes) and movement within the reservoir during production (Milkov et al., 2007). The most commonly used approaches include interpretations of individual well production histories, pressure analysis, temperature and production logging, interference testing, tracer analysis, and in more recent years, interpretation of 4D-seismic data (Milkov et al., 2007, and references therein). TLG is a novel approach that helps to visualize fluid flow during oil and gas production by monitoring changes in fluid compositions across a reservoir (Milkov et al., 2007). Although the general concept has been known in the petroleum industry for many years, only two published case studies exist (Davis et al., 2005; Milkov et al., 2007). Davis et al. (2005) successfully applied 4D-reservoir geochemistry to the Legendre Field (Australia) and were able to account for the dramatic increase in gas/oil ratio (GOR) from 220 m<sup>3</sup>/m<sup>3</sup> to over 700 m<sup>3</sup>/m<sup>3</sup> to breakthrough of gas injected into a different stratigraphic level, and not to the blow down of the local gas cap. Milkov et al. (2007) used TLG, in addition to traditional surveillance techniques, to visualise oil

movement during production from turbidite reservoirs in Horn Mountain Field, Gulf of Mexico. TLG complemented the findings collected by traditional techniques, which are often accompanied by uncertainties, and identified unswept parts of reservoirs. The main advantages of TLG over traditional reservoir surveillance techniques are its cost effectiveness and minimum interference in field operations.

This present study was designed to observe changes in petroleum fluid composition during continuous production over 335 days from a subsurface reservoir in the Norwegian sector of the North Sea. The results shed light on the applicability of 4D-reservoir geochemistry and reveal details of in-reservoir processes that occur during production.

#### Petroleum production and oil-water interaction

Processes that occur during petroleum production include a drop in reservoir pressure (Plankaert, 2005) and increased interaction between water and oil (e.g., Staples et al., 2005). The first phenomenon is a consequence of the evaporative fractionation of light petroleum constituents (van Graas et al., 2000). Studying petroleum composition during production can reveal fundamental information on petroleum compositional changes due to its interaction with water.

After petroleum is generated from a source rock, it is in perpetual contact with water (i.e. expulsion, secondary migration, reservoir residence, and production). Water leaches certain compounds from petroleum. This process, termed water washing (Lafargue and Barker, 1988), is based upon theoretical solubilities and compound-partitioning coefficients (e.g. McAuliffe, 1966; Price, 1976) and has been tested experimentally (e.g. Lafargue and Barker, 1988; Kuo, 1994; Lafargue and Le Thiez, 1996; de Hemptinne et al., 2001) and by numerical models (Lafargue and Le Thiez, 1996). Aromatic compounds of low molecular weight were initially suggested as most susceptible to water washing. Alkylphenols are characterised by even lower oil/water partitioning coefficients and also gained popularity as indicators of oil-water interaction (Bennet and Larter, 1997; Taylor et al., 1997, 2001; Bennett et al., 2003). Discrepancies between laboratory results and natural petroleum compositions, however, indicate that oil-water exchange in nature is not as efficient as under simulated laboratory conditions (e.g. de Hemptinne et al., 2001). Nevertheless, the effects of water washing in petroleum systems have been

reported (e.g., Taylor et al., 2001) and are supported by the fact that source rock pyrolysates are usually enriched in low molecular-weight aromatic compounds compared to related petroleum fluids (Lehne and Diekmann, 2007).

#### Partitioning- or metastable equilibrium

Low-molecular-weight aromatics (Love et al., 2003) and phenols (Dale et al., 1995) are generally present in the formation waters of petroleum reservoirs. It is still debated whether these compounds are present due to partitioning equilibrium of compounds between both phases or by chemical reactions involving metastable equilibrium (Dale et al., 1997). Experimental (Hoering, 1984; Lewan, 1985; Seewald, 1994; Stalker et al., 1994; McCollom et al., 2001; Seewald, 2001), theoretical (Helgeson et al., 1993), and empirical (Shock, 1988) evidence has been used to suggest that petroleum compounds, water, CO<sub>2</sub>, and rock minerals exist in metastable thermodynamic equilibrium in sedimentary basins (c.f. Love et al., 2003), leading to a slow but steady conversion of petroleum constituents by abiotic oxidation processes. This process could have severe implications for the mode of petroleum destruction in sedimentary basins and the geochemical carbon cycle. Although Love et al (2003) presented convincing molecular and isotopic evidence that argues against metastable equilibria and thus in favour of a water washing and phase partitioning processes, the topic is still controversial. Understanding the behaviour of petroleum during exposure to water contact, such as during petroleum production, is an important aim of the present study.

### **Organisational framework**

#### Rationale, organisation, and hypothesis

In this study we monitored a time series (over 335 days) of fluids from two wells (A and B) that produce crude oil from the same reservoir. No peculiarities were reported during the time interval in which sampling occurred. The fluids were analyzed by organic geochemical methods, focussing on low molecular-weight saturate and aromatic compounds and arenic alcohols (phenols). These compounds are believed to be most prone to interaction with water and,

consequently, may best record effects associated with water-washing of oil in reservoirs. Aromatic compounds were also analyzed for their compound-specific stable carbon isotopic compositions to evaluate potential evaporative fractionation during production. Saturated hydrocarbons were not analysed for their stable carbon isotopic compositions since their compositional variation in the studied samples was negligible.

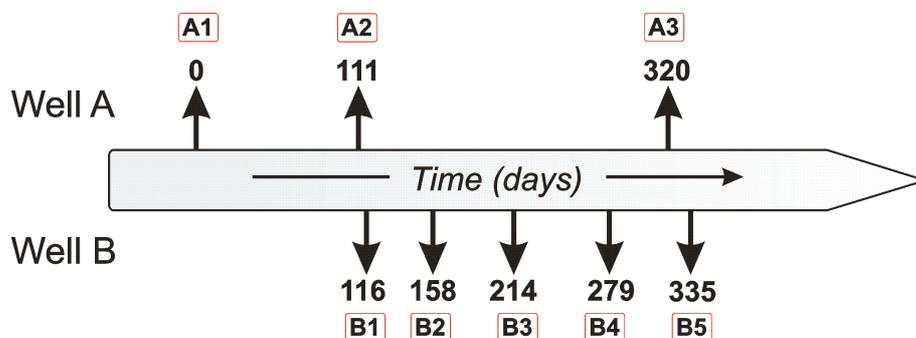
Oil-water contacts (OWC) in petroleum reservoirs represent a zone rather than a sharp contact, and are often referred to as a residual oil zone, in which oil and water discontinuously fill pore spaces. Upon production, this residual oil zone expands (e.g., Staples et al., 2005), consequently leading to a significant increase in surface contact area between oil and water. Discontinuous and isolated water that was present as droplets within the oil leg will also come in contact with the water leg. These small isolated droplets of water within the oil leg will be in partition equilibrium with the adjacent petroleum. These processes could lead to an increase in oil-water interaction and associated compound leaching (water washing) during production.

A second scenario involves a diffusion-induced concentration gradient throughout the oil leg, similar to the removal and diffusion of *n*-alkanes in the biodegradation model proposed by Huang et al. (2004). Leaching and removal of compounds by aqueous partitioning in the reservoir mainly occurs near the oil-water contact and compounds mix in the oil leg by diffusion. This could possibly lead to a gradient of hydrophilic petroleum constituents throughout the oil leg. Since diffusion is slow, oil column gradients will not change by diffusion within the timeframe that oil accumulations are depleted by production. Depending on the reservoir level at which oil is produced, the concentration of hydrophilic petroleum constituents might change throughout the production history and possibly allow an estimation of subsurface dynamics within the reservoir.

### Study area

The study site is a proprietary condensate field located in the Norwegian sector of the North Sea. The Jurassic reservoirs of this field contain gas, condensate, and oil at depths to 4850 metres. While some stratigraphic intervals are produced with gas injection, others rely on pressure depletion. A thin oil zone (11.5 m) below the gas cap is not currently under production. The produced condensates have

gravities  $\sim 50^\circ$  API. Two wells (A and B) were sampled (in days) at  $t = 0$  (A1),  $t = 111$  (A2),  $t = 320$  (A3),  $t = 116$  (B1),  $t = 158$  (B2),  $t = 214$  (B3),  $t = 279$  (B4),  $t = 335$  (B5), as shown in Figure 5.1.



**FIGURE 5.1** Relative oil sample collection dates from well A, respectively well B.

## Materials and methods

### Sample preparation

For the purpose of analysing individual saturated and aromatic hydrocarbons, oils were separated by small-scale open column chromatography as described by Bastow et al. (2007). Weighed aliquots of oil (ca. 20 mg) were transferred to the top of a silica column. The column consisted of a Pasteur-pipette dry-packed with activated ( $>8$  hours,  $120^\circ\text{C}$ ) silica gel (500 mg, 70-230 mesh, Merck) that was flushed with 3 bed volumes of *n*-pentane to remove impurities. Saturated and aromatic hydrocarbons (SAT and ARO fractions) were eluted with *n*-pentane (2 mL) and *n*-pentane/dichloromethane (7:3; 2 mL), respectively. Polar compounds were not recovered in this separation procedure. Deuterated internal standards were added to the SAT ( $\text{d}_{22}$ -decane) and ARO ( $\text{d}_6$ -benzene,  $\text{d}_8$ -naphthalene) fractions before analysis of individual compounds by gas chromatography-mass spectrometry (GC-MS). To allow for a statistical evaluation of the results, oil samples were separated and analyzed in triplicate.

For the analysis of phenols, oil samples were separated following the protocol of Bastow et al. (2003a). Weighed aliquots of oil (ca. 80–100 mg) were spiked with

deuterated internal standards ( $d_5$ -phenol,  $d_3$ -2,6-dimethylphenol) and transferred to the top of a silica column. The column consisted of a Pasteur-pipette dry-packed with activated (>8 hours, 120°C) silica gel (500 mg, 70-230 mesh, Merck) that was flushed with 3 bed volumes of *n*-pentane to remove impurities. A fraction containing saturated and aromatic hydrocarbons was eluted with *n* pentane/dichloromethane (9:1; 4 mL). A fraction containing phenols (PHE fraction) was eluted with *n*-pentane/diethyl ether (7:3; 5 mL). To allow for a statistical evaluation of the results, oil samples were separated and analyzed in triplicate.

For compound-specific isotope analysis (CSIA) of individual aromatic compounds, aliquots of oil (~30 mg) were separated following the method described above, but without any internal deuterated standards.

#### Gas chromatography (GC) and mass spectrometry (MS)

Hydrocarbon and phenol fractions were analysed using a Hewlett Packard (HP) 6890 GC coupled to a HP 5973 MS. Samples (1 mL) were injected in pulsed-splitless mode (260°C, 18.5 psi, 23 mL/min, pulse pressure of 30 psi until 0.5 min.) and separated on a DB-5ms column (60 m/0.25 mm/0.25 mm) with constant carrier gas flow (helium, 1.3 mL/min). The oven was held isothermal at 20°C, ramped to 120°C (6°/min.), ramped to 310°C (10°/min.), and held isothermal for 15 minutes. Samples were analyzed in full scan ( $m/z$  50-550) and selective ion monitoring (SIM) modes.

#### CSIA- $\delta^{13}C$

CSIA was performed using a Micromass IsoPrime isotope ratio mass spectrometer, interfaced to a HP 6890 GC. The  $\delta^{13}C$  values were calculated by integration of the  $m/z$  44, 45, and 46 ion currents of the CO<sub>2</sub> peaks produced by combustion of the separated compounds using copper oxide pellets (CuO, 4 mm x 0.5 m, isotope grade, Elemental Microanalysis Ltd.) at 850°C. Water was removed from the gas by cryogenic trapping with liquid nitrogen (-100°C). The compositions are reported relative to CO<sub>2</sub> reference gas pulses (Coleman instrument grade, BOC Gases Australia Ltd.) of known  $^{13}C/^{12}C$  content into the mass spectrometer. The  $^{13}C/^{12}C$  content of the CO<sub>2</sub> reference gas was monitored daily by analysis of a mixture of reference compounds. Average values of at least three analyses and

standard deviations are reported. Isotopic compositions are given in the delta notation in per mil (‰) relative to the Vienna Pee Dee Belemnite (VPDB).

#### Standard error calculation

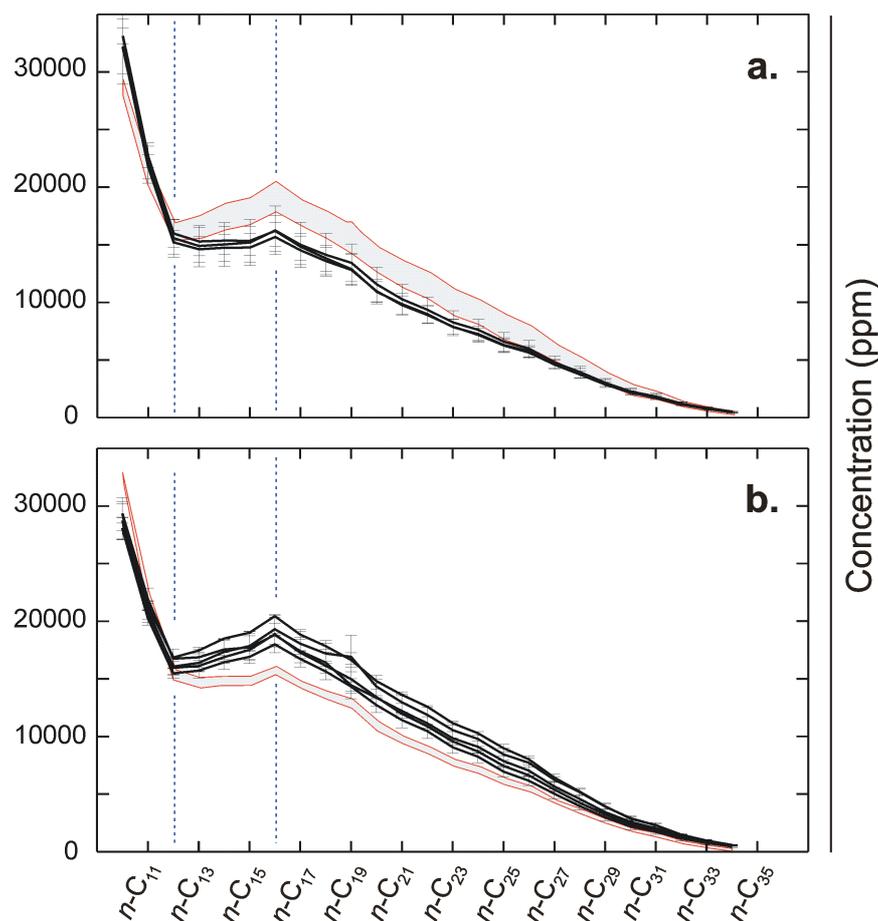
To assess analytical error, all samples were processed and analyzed in triplicate. The standard deviation (SD) was mostly calculated using all triplicates. Where one of three values appeared to be a significant outlier, this value was discarded and SD was calculated using the two remaining values, as annotated in the tables.

### **Results and discussion**

#### Saturated hydrocarbons

The *n*-alkane profiles of oil samples are typical for a medium light condensate maximising at *n*-decane and *n*-hexadecane (Figure 5.2). Little variation during production is observed among the oil samples from well A, respectively well B. The variation of well B *n*-alkanes is larger than that of well A *n*-alkanes.

However, within the error margin, all variation of well A *n*-alkanes, respectively well B *n*-alkanes can be regarded as negligible (Table 5.1). The variation in linear alkanes between well A and B is however noticeable, with higher concentrations of *n*-hexadecane in well B samples and a slightly higher concentration of *n*-decane in well A samples. This suggests that either a fill gradient exists in the reservoir, or that the reservoir compartments from wells A and B are not in communication and that fluids in these compartments experienced varying degrees of secondary alteration. Fill gradients are established by the fact that the petroleum composition continuously changes as it is expelled from a source rock. Petroleum reservoirs typically extend laterally in much greater dimensions than they do on a depth scale. Lateral compositional variations will only slowly equilibrate due to low speed of diffusion processes. However, no changes in the *n*-alkane profile during production were observed. This indicates that no admixture of fluids from a different compartment occurred during fluid production from well A or well B.



**FIGURE 5.2** Distribution of *n*-alkanes in samples from (a) well A, and (b) well B. The shaded area shows the range of the respective other well. Dashed lines indicate *n*-decane and *n*-hexadecane.

#### Light (C<sub>6</sub> and C<sub>7</sub>) hydrocarbons

Light petroleum hydrocarbons have received much attention (e.g., Milner et al. 1977; Halpern, 1995; Mango, 1997; Odden and Barth, 2000; Wever, 2000; George et al., 2002; Cañipa-Morales et al., 2003; Thompson, 2006) and are useful parameters for oil and condensate correlation, determining post-generative alteration effects, estimating thermal maturities, and predicting source rock depositional environments. Light hydrocarbons are of particular importance in light oils that lack, or are low in steroid and terpenoid biomarkers. Figure 5.3 shows a typical chromatogram (obtained by GC-MS, total ion current of *m/z* 50-550) of the total (Figure 5.3 a) and light (Figure 5.3 b) saturated hydrocarbon fraction of oil

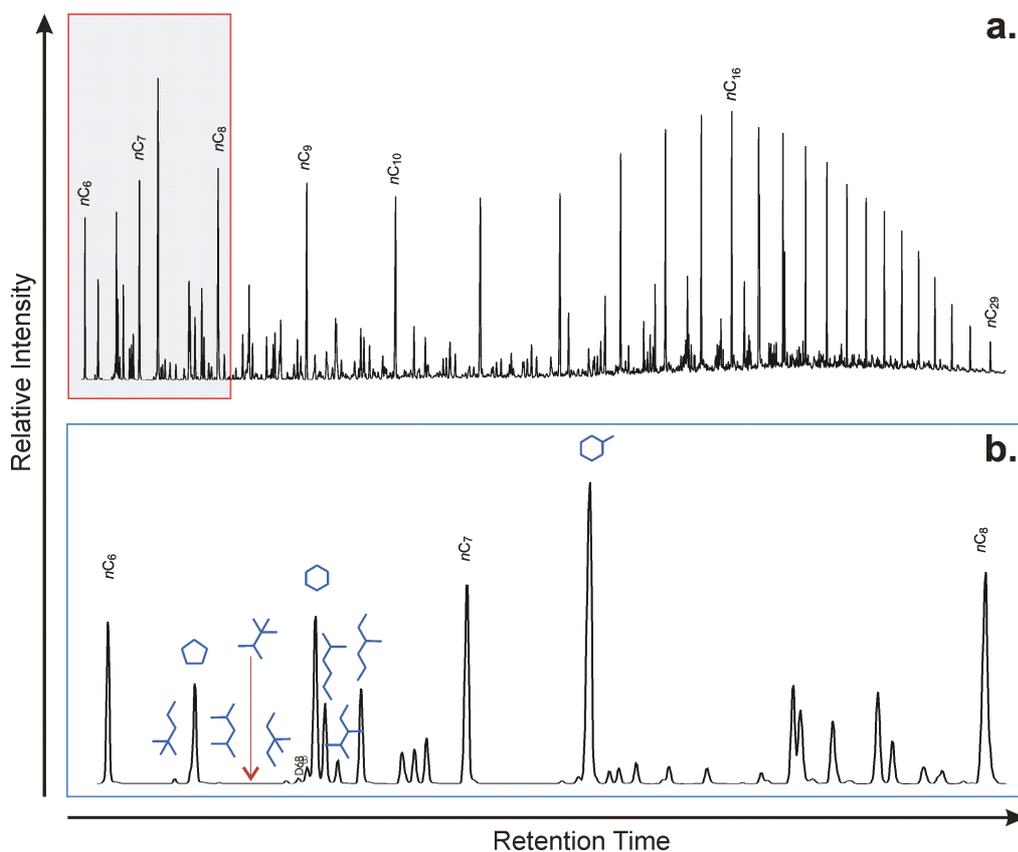
samples studied. The major light aliphatics are methylcyclopentane, cyclohexane, and methylcyclohexane.

		A			B				
		1	2	3	1	2	3	4	5
nC9	mean	3208.8	3311.4	3220.2	2873.1	2934.5	2803.7	2808.1	2930.0
	S.D. (%)	9.9	2.1	7.4	5.8	2.8	3.3	3.4	4.9
nC10	mean	2209.3	2262.6	2176.4	2071.7	2095.9	2029.4	2139.5	2191.5
	S.D. (%)	7.9	4.2	4.9	3.9	3.7	3.1	1.8	4.4
nC11	mean	1557.1	1598.3	1520.8	1607.0	1595.8	1548.0	1685.5	1674.7
	S.D. (%)	10.6	7.4	6.7	4.7	3.6	2.8	0.6	5.1
nC12	mean	1489.1	1527.6	1460.4	1638.8	1612.4	1570.1	1746.3	1686.0
	S.D. (%)	12.0	8.1	7.7	4.8	3.0	4.0	0.8	5.2
nC13	mean	1503.4	1536.2	1474.2	1734.3	1689.0	1642.6	1850.2	1754.5
	S.D. (%)	12.7	7.8	7.9	5.7	2.3	3.7	0.1	5.1
nC14	mean	1519.2	1534.5	1475.5	1784.6	1751.8	1691.8	1901.2	1771.9
	S.D. (%)	13.1	8.1	8.4	7.0	0.7	3.5	0.5	5.7
nC15	mean	1624.3	1620.4	1567.9	1930.9	1888.2	1800.0	2043.8	1881.2
	S.D. (%)	13.1	8.4	8.0	6.6	0.4	4.1	0.3	5.4
nC16	mean	1500.2	1483.1	1452.2	1805.4	1728.3	1673.7	1884.4	1741.7
	S.D. (%)	13.1	7.9	8.5	6.8	0.8	4.3	1.3	5.9
nC17	mean	1412.9	1382.3	1358.8	1719.8	1615.0	1564.2	1785.0	1642.0
	S.D. (%)	13.2	8.2	8.7	6.6	2.3	4.7	1.5	5.6
nC18	mean	1343.0	1289.5	1281.8	1691.0	1499.1	1432.8	1664.3	1445.1
	S.D. (%)	12.2	10.3	10.4	11.2	8.5	2.9	3.7	8.1
nC19	mean	1155.9	1091.2	1095.8	1433.9	1341.6	1271.5	1479.5	1336.3
	S.D. (%)	13.0	8.0	9.7	6.9	0.9	5.0	1.2	5.6
nC20	mean	1026.7	985.4	976.3	1297.4	1197.1	1143.6	1361.2	1221.4
	S.D. (%)	12.7	9.5	8.2	6.9	0.5	5.9	0.6	5.3
nC21	mean	934.1	896.2	890.1	1188.4	1088.4	1045.3	1259.2	1114.6
	S.D. (%)	11.9	9.0	8.4	6.7	1.1	6.0	0.6	5.1
nC22	mean	827.2	785.3	787.7	1055.4	956.7	905.3	1115.6	985.2
	S.D. (%)	12.3	9.1	8.1	6.2	1.1	5.6	1.5	5.8
nC23	mean	760.8	725.2	718.6	977.6	869.1	825.2	1027.5	910.0
	S.D. (%)	11.9	9.7	8.0	6.7	1.6	6.3	1.6	5.5
nC24	mean	658.7	630.0	625.0	844.3	745.6	695.9	899.1	787.1
	S.D. (%)	12.0	10.3	8.8	6.2	2.2	6.5	3.7	5.3
nC25	mean	597.1	576.1	563.7	771.8	663.7	616.5	801.3	704.4
	S.D. (%)	12.3	8.6	9.0	5.0	2.5	6.9	3.4	6.9
nC26	mean	477.9	467.3	460.5	624.8	539.9	502.6	642.9	566.7
	S.D. (%)	12.0	8.0	9.0	3.9	3.5	8.5	4.8	6.6
nC27	mean	394.0	386.9	373.4	516.7	424.9	396.6	521.6	457.6
	S.D. (%)	13.0	10.4	8.0	5.1	3.6	8.0	5.7	6.4
nC28	mean	301.1	301.1	286.6	391.8	323.4	300.0	395.6	344.5
	S.D. (%)	13.3	10.1	8.3	6.5	3.3	10.9	6.5	7.5
nC29	mean	226.1	225.2	217.2	290.9	238.6	219.7	287.1	258.3
	S.D. (%)	13.6	10.9	7.6	7.9	5.8	13.3	5.5	10.3
nC30	mean	182.9	182.7	171.2	229.6	189.7	175.5	230.4	204.1
	S.D. (%)	15.0	9.7	9.4	9.5	7.4	13.4	5.9	9.1
nC31	mean	120.8	121.9	113.4	144.2	120.8	114.8	147.3	133.9
	S.D. (%)	14.8	11.9	9.9	10.5	7.2	19.2	4.0	12.4
nC32	mean	80.2	82.6	75.1	95.2	80.4	75.6	97.1	91.4
	S.D. (%)	16.3	8.7	10.9	11.0	8.8	20.4	7.5	13.3
nC33	mean	50.8	52.3	45.1	56.2	47.4	46.2	58.4	54.7
	S.D. (%)	13.7	12.3	8.9	13.3	11.1	23.6	5.5	12.8

**TABLE 5.1** Concentration (ppm) and standard deviation (%) of linear alkanes in petroleum fluids produced from well A and well B. Mean values of n = 3 analyses. S.D. standard deviation. See Fig. 5.1 for sampling dates.

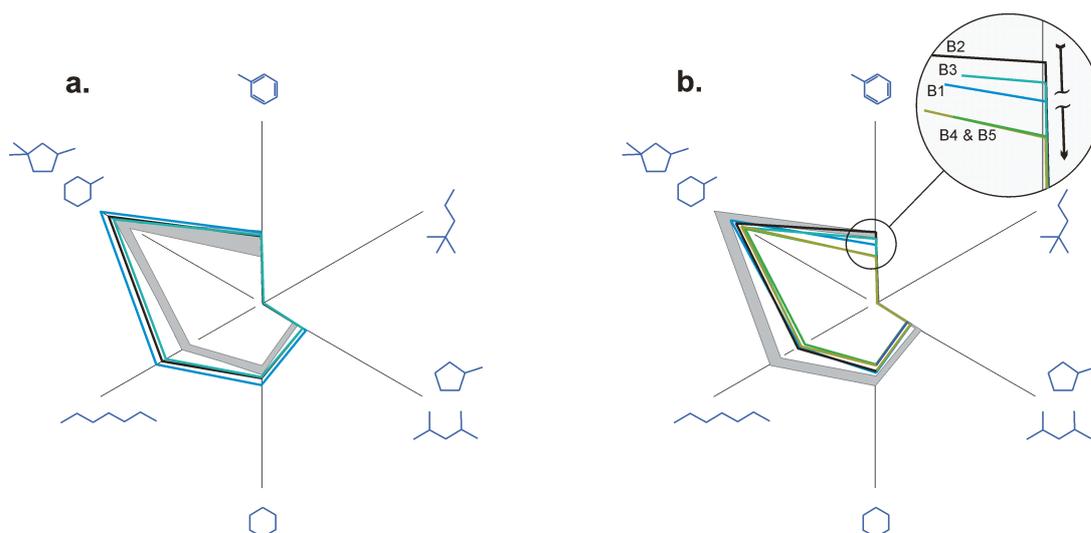
Sample (days)	Toluene		22-DMP		Me-cP. & 24-DMP		c-Hexane		n-C7		Me. c-Hex & 113-TMcP.		Tol / n-C7	Tol / Phe	Phe / Σ Phe
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.			
<b>Well A</b>															
0	11723	2.5	406	2.6	8461	5.8	13324	10.0	19780	6.9	30272	7.6	0.59	4.87	0.16
111	11163	3.0	358	7.3	7868	3.7	12165	6.4	18714	3.6	28694	3.3	0.60	4.63	0.16
320	11308	5.5	406	7.0	7798	5.3	11995	7.1	18019	5.5	27803	6.0	0.63	4.69	0.15
<b>Well B</b>															
116	10346	2.9	282	6.0	7128	2.2	12188	1.7	15436	7.4	29333	2.1	0.67	4.29	0.12
158	12595	5.6	302	5.9	7063	5.6	11879	4.8	15755	5.0	28292	3.7	0.80	5.23	0.11
214	11461	5.3	270	3.0	6385	3.6	10884	3.8	15099	2.5	26864	4.3	0.76	4.76	0.13
279	8374	6.8	260	1.9	6277	3.0	10736	3.9	14362	3.0	26453	3.7	0.58	3.48	0.16
335	8273	3.5	270	0.8	6394	1.7	10907	3.3	15097	4.2	27232	4.6	0.55	3.43	0.15

**TABLE 5.2** Concentration (ppm) and standard deviation (%) of light hydrocarbons in petroleum fluids produced from wells A and B, and parameters thought to be sensitive to water washing. Mean values of n=3 analyses. S.D. standard deviation.



**FIGURE 5.3** Saturated hydrocarbons. Gas chromatogram (collected using MS detector in full scan mode) showing (a) the saturated hydrocarbon fraction of the oils studied, and (b) an enlargement of the light saturated hydrocarbon region.

The difference between well A and well B fluids is evident from the comparison of light saturated hydrocarbons and toluene in a star plot (Figure 5.4). The major disparity is observed in the relative concentration of *n*-heptane (Table 5.2). Other light saturated hydrocarbons show only small differences between wells A and B. During production, none of the saturated hydrocarbons change in concentration. However, the toluene concentration of fluids from well B changes throughout the sampled period and decreases from sample B2 to B5. Interestingly, this is not observed for the fluids from well A. The observation that saturated hydrocarbon concentrations with similar volatilities to toluene do not change in well B fluids during production indicates that the depletion of toluene cannot be caused by evaporative fractionation. If this were the case, all light hydrocarbons should be similarly affected.



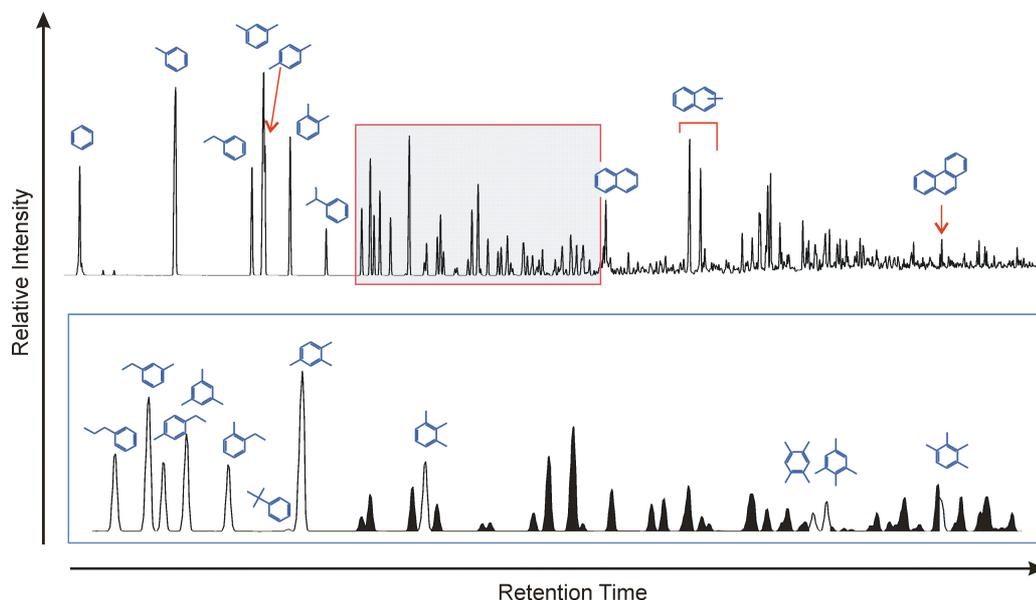
**FIGURE 5.4** Star diagrams of selected C<sub>6</sub> and C<sub>7</sub> hydrocarbons in fluids from (a) well A and (b) well B. Shaded areas indicate sample range of other well. Clockwise from noon-position: toluene, 2,2-dimethylpentane, methylcyclopentane and 2,4-dimethylpentane, cyclohexane, *n*-heptane, methylcyclohexane and 1,1,3-trimethylcyclopentane.

Based on differences in aqueous solubilities of saturated and aromatic hydrocarbons of similar molecular weight (McAuliffe, 1966), the toluene/*n*-C<sub>7</sub> ratio has been proposed as a water-washing parameter. The observed variation of toluene concentrations suggests a mechanism involving the interaction with water. Figure 5.4 shows that toluene depletion occurs linearly during production, with a single exception: the first recovered fluid from well B (B1). The B2–B5 samples (Figure 5.1) exhibit a systematic relative depletion of toluene, although the difference between sample B4 and B5 is minimal. This suggests a causal relationship between toluene depletion and continuous production of fluids from the well B reservoir.

### Aromatic hydrocarbons

Low molecular-weight aromatics are typically present in petroleum reservoir formation waters (Love et al., 2003) and the spatial concentration of benzene in

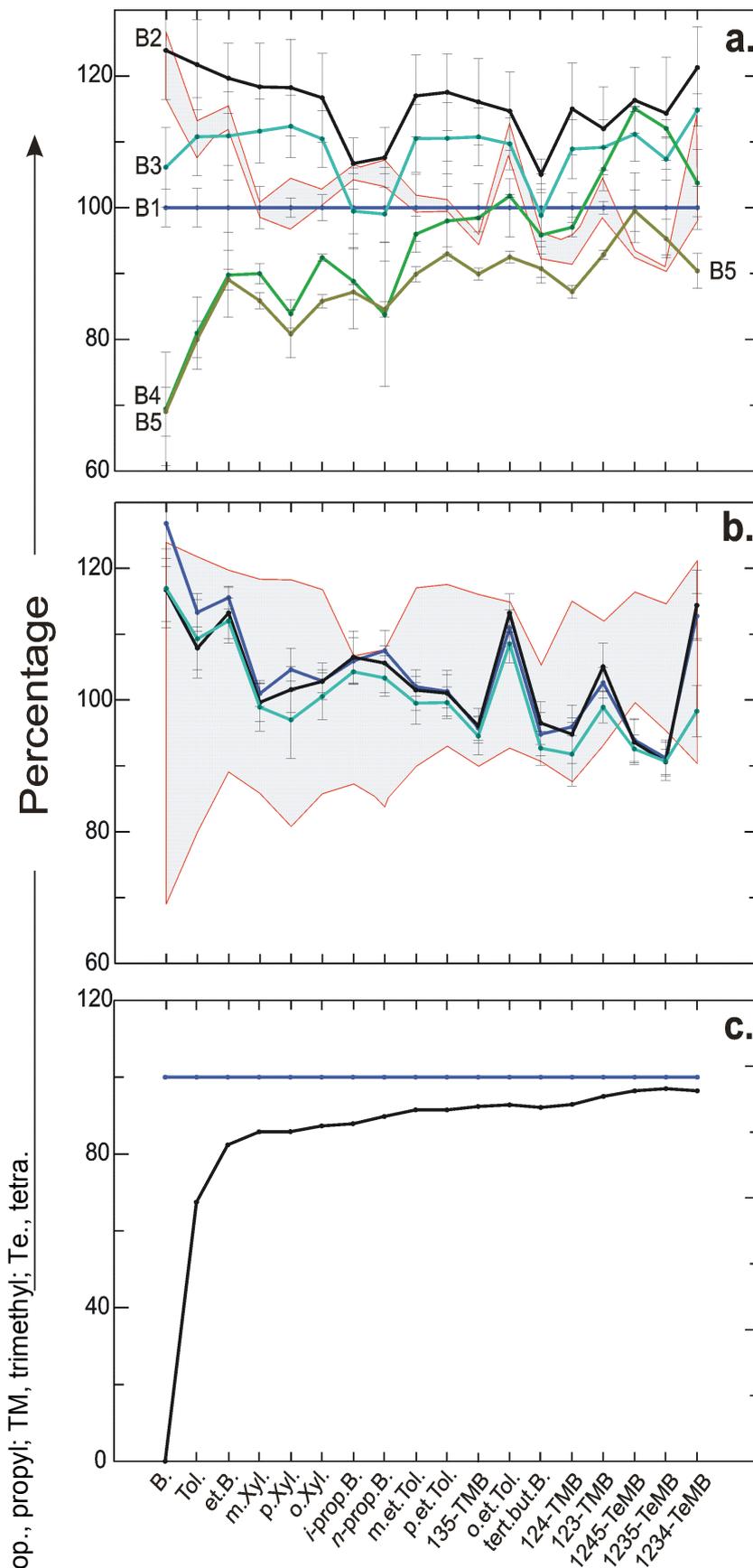
subsurface aquifers can be used as an indicator of proximity to petroleum accumulations for exploration (Zhang et al., 2005a and b).



**FIGURE 5.5** Gas chromatogram (collected using MS detector in full scan mode) showing the aromatic fraction of the oil samples. The shaded area (top) is enlarged in the inset (bottom).

The light range of monoaromatic species dominates the aromatic hydrocarbon fraction of the studied fluid samples. Since light aromatics are most susceptible to post generative processes, this study focused on C<sub>0</sub>-C<sub>4</sub> alkylbenzenes. Figure 5.5 shows the distribution of these compounds in the studied fluids. The low-molecular-weight aromatics exhibit pronounced compositional changes during petroleum production, and well B samples are more heavily affected than well A samples. In Figure 5.6, the percentage of low-molecular-weight aromatics is shown, normalised to the concentration in sample B1. Depletion in low-molecular-weight aromatics from samples B2 to B5 is evident. The change is most pronounced for benzene and toluene, which vary between ca. 120% (B2) to ca. 70% (B5) relative to sample B1. The concentration of benzene varies between 1761 ppm in sample B2 and 982 ppm in sample B5 (Table 5.3). All alkylbenzenes up to the tetramethylbenzenes are affected similarly, suggesting that evaporative fractionation is not a likely cause of

**FIGURE 5.6** Relative concentration of individual monoaromatic compounds in time-resolved oil samples from (a) well A and (b) well B. All samples are normalised to sample B1 (~100 %). Shaded areas indicate compound concentrations of respective other well. (c) Relative decrease of individual monoaromatic compounds due to laboratory induced evaporation in one of the studied oil samples, normalised to the same sample before evaporation. B., benzene; Tol., toluene; et., ethyl; m., meta; p., para; o., ortho; Xyl., xylene; prop., propyl; TM, trimethyl; Te., tetra.



the observed variation in low-molecular-weight aromatics. To confirm this, an aliquot of one of the oils was allowed to evaporate for several days under atmospheric conditions. This evaporation resulted in complete removal of benzene and depletion of the remaining low-molecular-weight aromatics depending on their molecular weight (Figure 5.6 c), i.e. lighter compounds were more severely affected. This finding confirms that evaporative fractionation caused by a drop in reservoir pressure during production did not influence the chemical composition of the studied petroleum fluids.

### Aromatic $\delta^{13}\text{C}$

To shed more light on the behaviour of low-molecular-weight aromatic species during petroleum production, the stable carbon isotopic ratio ( $\delta^{13}\text{C}$ ) was measured for selected components. The results are presented in Figure 5.7 and show that little variation between all fluids analysed (within analytical error), indicating the lack of evaporative processes as the lighter  $^{12}\text{C}$  isotope is preferentially depleted over the heavier  $^{13}\text{C}$  isotope during evaporation processes. Oil-water partitioning of phenol does not induce a stable carbon isotopic fractionation (Love et al., 2003), and this is consequently also not expected for higher-molecular-weight hydrocarbons.

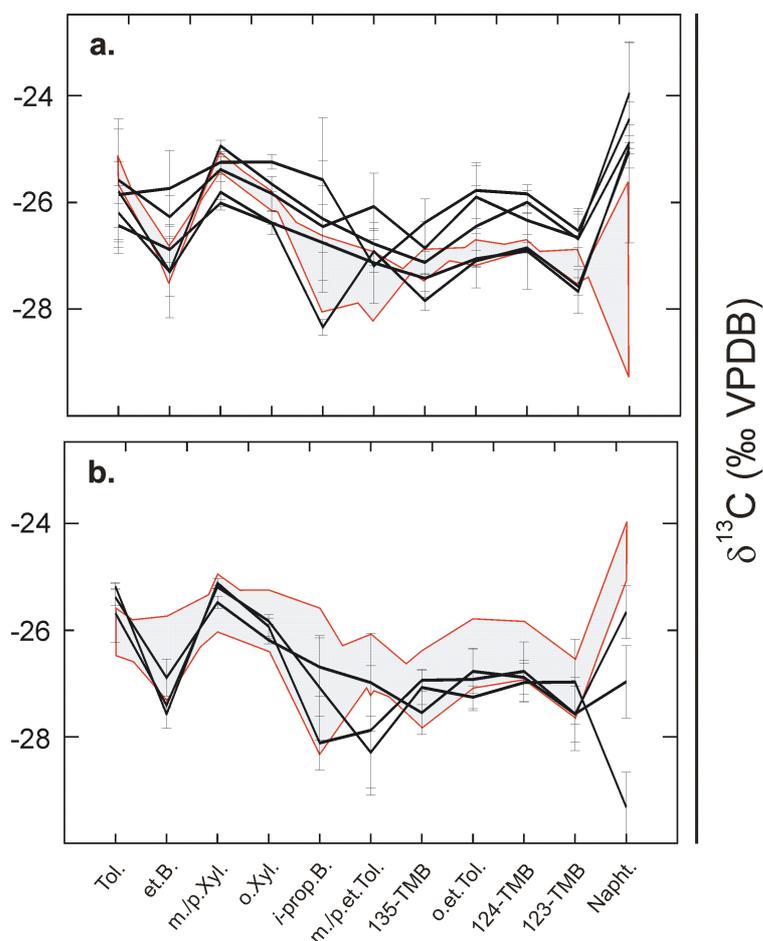
An exception is naphthalene ( $\text{C}_{10}\text{H}_8$ ), which has an average  $\delta^{13}\text{C}$  (VPDB) value of  $-25.0\text{‰}$  (ranging from  $-24.0$  to  $-26.7\text{‰}$ ) for all well B fluids. This value is slightly  $^{13}\text{C}$  enriched compared to the monoaromatic compounds, whose average  $\delta^{13}\text{C}$  (VPDB) value is  $-26.5\text{‰}$  (values range between  $-25.5\text{‰}$  for averaged meta- and para-toluene, and  $-27.1\text{‰}$  for averaged 1,3,5-trimethylbenzene; Table 5.4). The  $\delta^{13}\text{C}$  value of naphthalene in well A fluids ranges from ca.  $-29$  to  $-26\text{‰}$ . The differences are corroborated by triplicate analyses and resulting error margins. This observation is interesting since until now the fluids produced by well B appeared to be subject to higher variability than the fluids produced by well A. The reason for the stable carbon isotope variation of naphthalene remains unclear.

	Benzene		Toluene		Et. Benzene		m-Xylene		p-Xylene		o-Xylene		Isopr. Benzene		n-Pr. Benzene		m-Et. Toluene		p-Et. Toluene		1,3,5-TMB					
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.		
<b>Well A</b>																										
0	1802.9	5.2	11723.0	2.5	4377.2	1.5	11804.0	2.0	3471.3	3.1	5826.7	1.7	1675.4	3.3	2611.2	0.7	4756.1	1.5	2175.3	2.5	3163.4	1.9				
111	1659.3	4.1	11162.8	3.0	4288.8	3.4	11644.2	2.9	3370.8	3.4	5823.3	2.7	1684.8	3.7	2565.4	4.7	4731.9	3.0	2169.5	3.4	3174.7	2.7				
320	1662.7	5.1	11307.7	5.5	4243.5	3.1	11560.7	3.7	3218.2	6.0	5694.2	3.5	1649.5	1.8	2510.6	2.1	4638.5	3.2	2138.9	2.4	3123.4	3.0				
<b>Well B</b>																										
116	1421.7	2.8	10345.6	2.9	3788.4	6.4	11687.9	3.2	3318.4	1.5	5663.4	2.0	1581.7	6.1	2429.5	5.2	4661.4	5.1	2147.3	7.0	3304.3	3.7				
158	1761.4	6.0	12594.5	5.6	4534.0	4.4	13833.4	5.6	3923.5	6.2	6608.7	5.8	1687.9	3.6	2614.2	4.2	5453.8	5.3	2523.8	5.0	3835.4	5.7				
214	1509.1	5.7	11460.9	5.3	4201.2	6.0	13046.4	4.3	3728.7	4.2	6255.7	3.9	1573.4	5.8	2406.1	7.2	5151.0	4.6	2373.8	4.9	3659.9	4.0				
279	987.3	12.4	8374.3	6.8	3400.8	7.2	10518.4	1.7	2783.6	2.6	5234.8	0.6	1405.0	8.1	2035.2	13.0	4474.0	2.8	2104.4	5.4	3253.0	0.2				
335	981.5	5.4	8272.6	3.5	3374.6	1.8	10036.8	1.4	2681.8	4.5	4859.0	1.2	1379.2	1.3	2054.2	1.4	4190.1	1.3	1986.7	1.1	2971.5	1.0				

**TABLE 5.3** Concentration (ppm) of aromatic hydrocarbons in petroleum fluids produced from well A and well B. Mean values of n = 3 analyses. Asterisks indicate mean values of n = 2 analyses. S.D. standard deviation. Et., ethyl; m., meta; p., para; o., ortho; Pr., propyl; TMB., trimethylbenzene; Bu., butyl; TeMB, tetramethylbenzene; Naphth., Naphthalene.

	Toluene		Et. Benzene		m,p-Et. Toluene		o-Toluene		i-prop. Benzene		m,p-Et. Benzene		1,3,5-Tmb.		1,2,4-Tmb.		1,2,3-Tmb.		1-Me. Naph.							
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.						
<b>Well A</b>																										
1	-25.38	0.16	-26.89	0.35	-25.48	0.11	-26.19	0.07	-26.69	0.55	-26.98	0.82	-27.55	0.41	-26.77	0.42	-26.89	0.32	-27.57	0.53	-25.66	0.50				
2	-25.66	0.55	-27.41	0.05	-25.19	0.03	-25.63	0.05	-27.08	0.98	-28.29	0.67	-27.07	0.32	-27.26	0.22	-26.98	0.36	-26.97	0.80	-28.32	0.66				
3	-25.17	0.07	-27.57	0.28	-25.12	0.09	-25.93	0.22	-26.12	0.51	-27.86	1.22	-26.94	0.20	-26.93	0.58	-26.77	0.56	-27.57	0.69	-26.97	0.68				
<b>Well B</b>																										
1	-26.19	0.50	-27.30	0.46	-25.81	0.33	-26.39	0.21	-26.76	0.71	-27.13	0.76	-27.43	0.35	-27.06	0.16	-26.81	0.72	-27.67	0.41	-24.95	0.41				
2	-25.38	1.16	-26.27	0.20	-25.98	0.23	-26.93	0.32	-26.45	1.23	-26.09	0.82	-26.86	0.90	-25.90	0.59	-26.34	0.27	-26.66	0.55	-23.95	0.94				
3	-26.35	0.16	-26.88	0.25	-26.04	0.09	-26.89	0.02	-26.34	0.15	-26.92	0.38	-27.63	0.31	-26.45	0.76	-26.85	0.20	-27.60	0.16	-23.88	1.85				
4	-25.79	1.17	-25.99	0.67	-25.74	0.16	-26.24	0.14	-25.88	1.17	-27.19	0.70	-26.38	0.44	-25.75	0.52	-25.84	0.17	-26.63	0.31	-24.43	0.32				
5	-25.86	0.62	-25.74	0.71	-25.25	0.16	-25.24	0.14	-25.58	1.17	-27.19	0.70	-26.38	0.44	-25.75	0.52	-25.84	0.17	-26.63	0.31	-24.43	0.32				
5*	-25.62	0.12	-27.19	0.59	-25.29	0.06	-25.67	0.23	-27.56	0.84	-26.56	0.44	-27.08	0.67	-27.02	0.27	-26.62	0.63	-28.21	0.56	-26.68	0.75				

**TABLE 5.4** Mean stable carbon isotope ratio (‰ VPDB) and absolute standard deviation (‰) of individual aromatic compounds (n = 3 analyses). Key: S.D., standard deviation; Et., ethyl; m., meta; p., para; o., ortho; i., iso; prop., propyl; Tmb., trimethylbenzene; Naphth., Naphthalene.

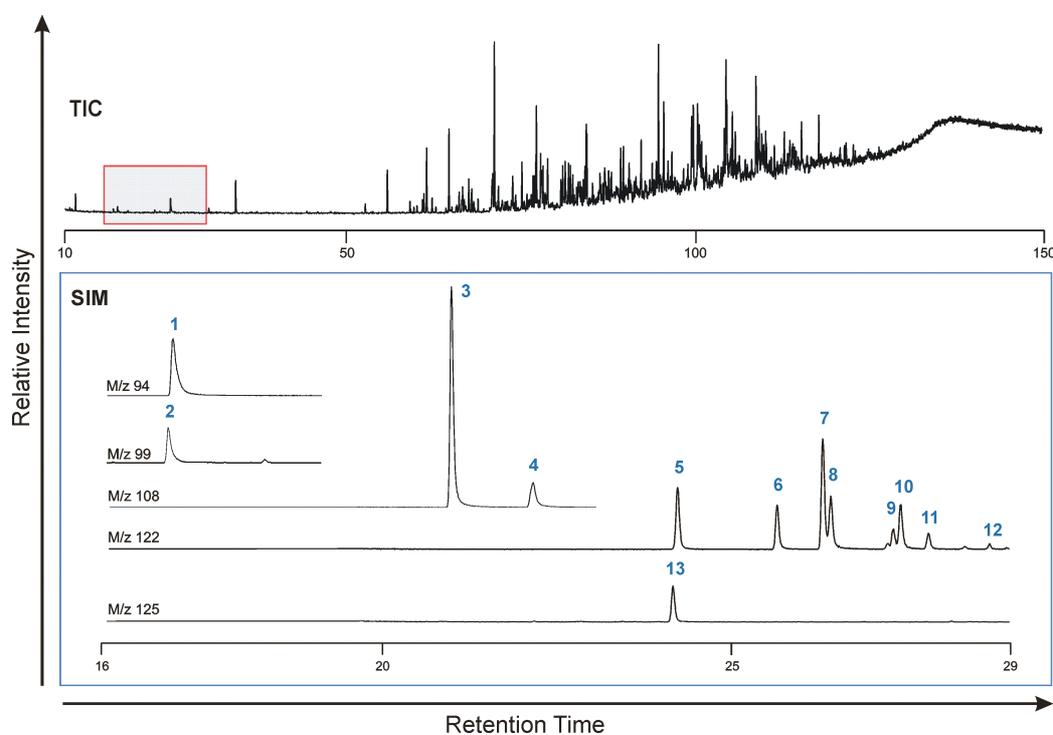


**FIGURE 5.7** Stable carbon isotope ratio (‰,  $\delta^{13}\text{C}$  against VPDB) of selected monoaromatic compounds in time-resolved oil samples from (a) well B and (b) well A. Shaded area indicates sample region of other well. Tol., toluene; et., ethyl; B., benzene; m., meta; p., para; Xyl., xylene; o., ortho; prop., propyl; TM, trimethyl; Naphth., naphthalene.

### Phenol distributions

Aromatic alcohols are particularly susceptible to oil-water partitioning. A range of substituted phenols is typically found in petroleum (Ioppolo et al., 1992; Ioppolo-Armanios et al., 1994, 1995), but their origin is not well understood. While lignin degradation products can contribute specific phenols to sedimentary organic matter (Hedges and Mann, 1979; Fenton et al., 2007), Taylor et al. (1997) showed that the concentration of alkylphenols is markedly higher in shales containing Type II organic matter than in shales that contain terrigenous Type III organic matter. The

alkylphenol composition of crude oils was suggested to be influenced by geosynthetic processes, such as methylation, *isopropylation*, and *sec*-butylation (Ioppolo-Armanios et al., 1995). Bastow et al. (2005) found that the catalyzed hydroxylation of alkylbenzenes is a likely mechanism for the formation of alkylphenols in crude oils. Whether this process occurs in petroleum reservoirs or during petroleum generation in the source rock is not known. Regardless of their mode of formation, phenol and cresols were found to be enriched in the water phase of oil reservoirs, while the petroleum is enriched with higher alkylphenol homologues (Dale et al., 1995), suggesting preferential partitioning of lower alkylphenol homologues from petroleum into water.

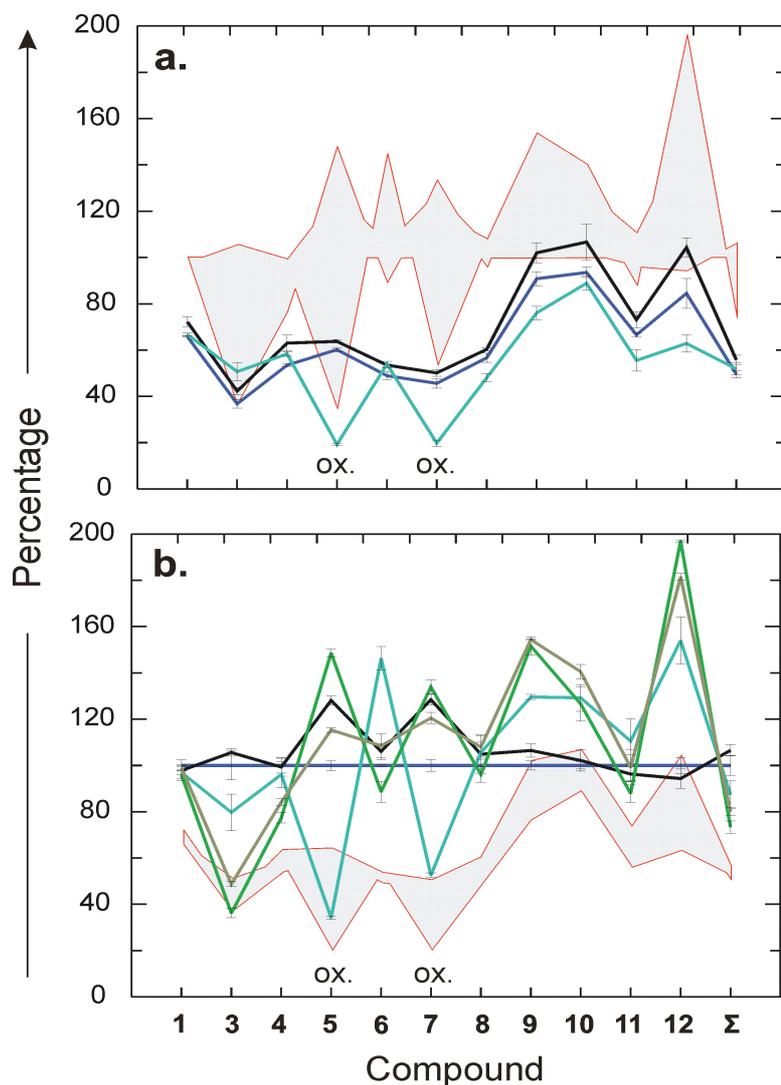


**FIGURE 5.8** Phenols. Total ion current chromatogram (collected using full scan GC-MS,  $m/z$  50-550) showing the phenol fraction of oils studied, and selective ion traces showing individual phenols. 1. phenol, 2.  $d_5$ -phenol, 3. O-cresol, 4. m- & p-cresol, 5. 2,6-dimethylphenol 6. 2-ethylphenol, 7. 2,4-dimethylphenol, 8. 2,5-dimethylphenol, 9. 4-ethylphenol, 10. 3,5-dimethylphenol & 3-ethylphenol, 11. 2,3-dimethylphenol, 12. 3,4-dimethylphenol, 13.  $d_3$ -2,6-dimethylphenol.

	Phenol		o-Cresol		m. & p.-Cresol		2-et.ph.		2,4-dmp		2,5-dmp		4-et.p		3,5-dmp & 3-et.p/2,3-dmp		3,4-dmp		Sum				
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.					
Well A																							
0	2409.2	0.5	5381.6	5.3	1513.9	1.2	852.9	1.4	625.0	3.4	1071.6	4.5	967.2	3.5	523.7	3.4	1370.1	2.3	307.1	1.6	93.6	7.7	
111	2642.3	2.9	6182.4	3.8	1784.7	5.4	903.7	1.2	683.5	1.1	1177.2	2.9	1029.4	0.9	587.9	4.3	1562.2	7.2	337.0	4.8	115.5	4.0	
320	2440.0	1.1	7408.4	7.7	1651.6	1.7	272.1	2.7	688.8	2.5	465.5	6.4	820.3	3.6	438.4	3.7	1302.0	3.4	256.1	8.4	68.7	5.9	
Well B																							
116	3659.7	2.4	14636.7	6.3	2831.9	3.2	1417.7	2.1	1278.7	0.5	2350.0	2.6	1707.2	3.5	576.5	2.0	1464.7	1.6	461.3	6.9	110.8	3.4	
188	3580.3	1.7	15455.8	1.6	2811.0	3.9	1816.2	1.5	1355.8	3.3	3017.9	1.8	1790.1	5.0	613.7	2.7	1496.4	4.6	444.1	3.6	104.5	4.4	
214	3549.2	2.8	11663.0	10.0	2719.4	5.9	484.0	2.0	1870.8	3.4	1234.7	1.8	1802.5	6.8	747.0	0.8	1892.4	4.5	509.8	8.8	170.5	6.6	
279	3509.0	2.5	5295.4	5.5	2190.4	3.0	2105.9	1.1	1132.9	5.1	3147.2	2.2	1639.6	3.4	873.8	2.6	1854.0	5.9	405.1	4.3	218.3	0.2	
335	3821.5	1.2	7125.2	2.2	2383.6	1.7	1635.1	0.9	1387.5	4.7	2831.3	2.0	1842.2	4.8	889.9	0.3	2057.7	2.2	457.2	5.6	201.1	0.7	

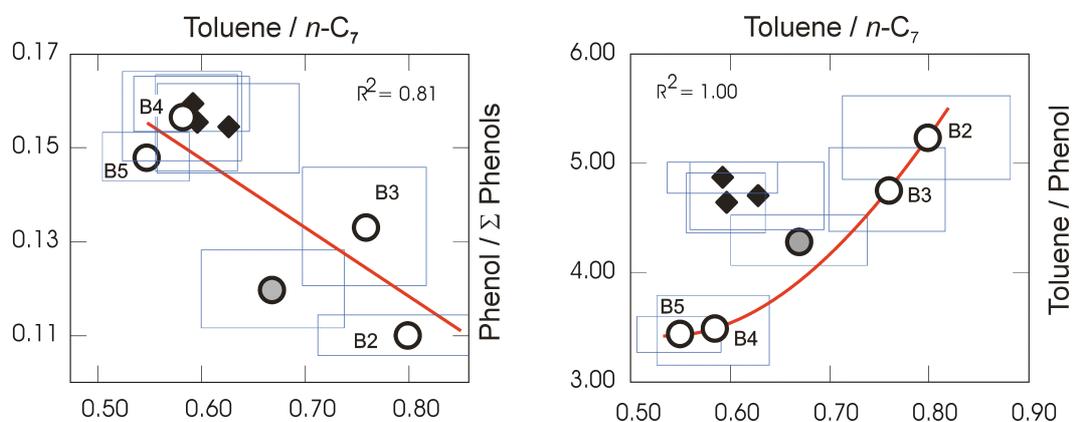
**TABLE 5.5** Mean concentration (ppm) and relative standard deviation (%) of phenol and alkylphenols. Means of n=3 analyses. Asterisks indicate mean values of n = 2 analyses. Key: S.D., standard deviation; m., meta; p., para; o., ortho; et., ethyl; ph., phenol; dmp, dimethylphenol.

A range of alkylphenols was found in the fluids studied (Figure 5.8; Table 5.5). The relative concentration of phenol and alkylphenols varies in a slightly erratic manner in Figure 5.9, where relative alkylphenol abundances are depicted normalized to the concentration of phenols in the B1 sample. Figure 5.10 shows the variation of phenol concentrations. The phenol composition of fluids produced by well A and well B differs. The slight difference of the fluids was discussed previously and was attributed to fill history or reservoir compartmentalization. The most notable observation is that the concentration of unsubstituted phenol varies little during production of fluids from wells A and B. Phenol is characterized by a lower partitioning coefficient than benzene, indicating that it is prone to partition into the aqueous phase. However, the invariant phenol concentration of studied fluids indicates that water washing is not responsible for the chemical changes in petroleum fluids during production. The variation in ortho-cresol is markedly higher than that of combined meta- and para-cresol, confirming the suggestion by Taylor et al. (1997) that the often observed predominance of ortho-cresol in oils is not caused by the preferential removal of meta- and para-cresol by water washing.

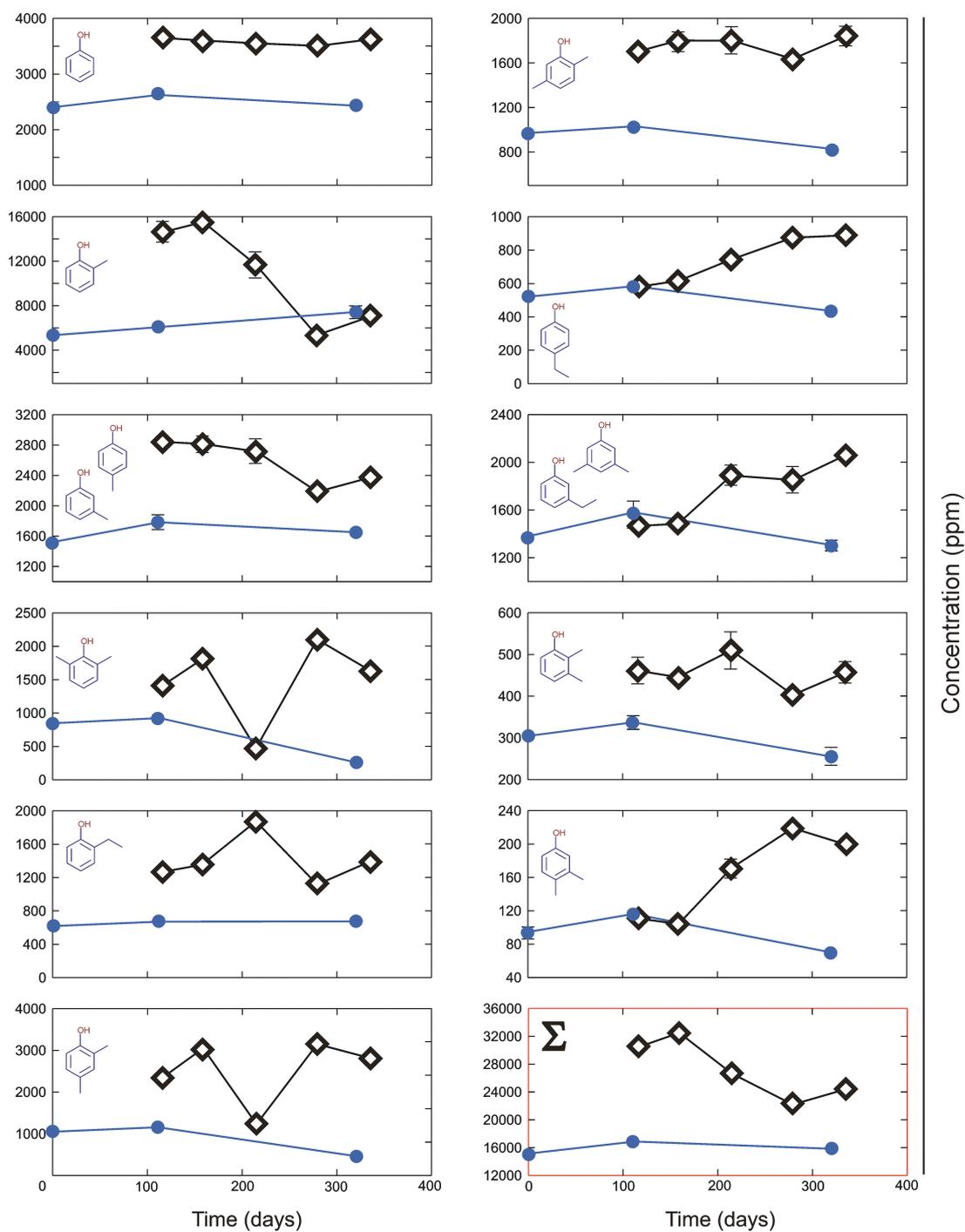


**FIGURE 5.9** Relative concentration of individual alkylphenols ( $C_0$ - $C_2$ ) in time-resolved oil samples from (a) well A and (b) well B. Mean values of  $n = 3$  analyses. All samples are normalised to sample B1 (~100%). Shaded areas indicate sample region of respective other well. 1. phenol, 3. o-cresol, 4. m- & p-cresol, 5. 2,6-dimethylphenol 6. 2-ethylphenol, 7. 2,4-dimethylphenol, 8. 2,5-dimethylphenol, 9. 4-ethylphenol, 10. 3,5-dimethylphenol & 3-ethylphenol, 11. 2,3-dimethylphenol, 12. 3,4-dimethylphenol.

Phenols are sensitive to oxidative processes and photo-oxidation selectively affects the alkylphenol composition of petroleum samples exposed to sunlight (Bastow et al. 2003b). Whether aqueous oxidation processes in oil reservoirs affect the phenol distribution is not known. In the studied samples, the strongest variation in alkylphenol concentration was found for ortho-cresol, 2,6-dimethylphenol, 2,4-dimethylphenol, and 3,4-dimethylphenol. Both, 2,4-dimethylphenol and 2,6-dimethylphenol were reported as being highly sensitive to photo-oxidation (Bastow et al., 2003b). The relatively small error, resulting after triplicate analyses of samples, indicates that oxidation did not occur during the workup procedure. Inadequate sample storage can also be excluded, as oil samples were 'fresh' and stored as a batch together. Furthermore, other isomers than just those noticed by Bastow et al (2003b) are affected. This, and the fact that we can exclude evaporative fractionation as well as water washing as acting processes, suggests that the observed chemical changes might be due to a subsurface oxidation effect in the presence of water. If this is true, it supports the metastable equilibrium theory mentioned previously (e.g., Seewald, 1994, 2001; McCollom et al., 2001).



**FIGURE 5.10** Water washing. Xy-plots showing molecular ratios that are thought to be sensitive to water washing for time resolved oil samples from well A (filled diamonds), respectively well B: B1 (shaded circle), B2–B5 (open circles). Correlation coefficients are based on samples B2–B5.



**FIGURE 5.11** Changes in C<sub>0</sub>-C<sub>2</sub> alkylphenol concentrations (ppm) in fluids from well A (blue circles), respectively well B (open diamonds) during the studied period.

The comparison of hydrocarbon based (toluene/*n*-C<sub>7</sub>) and phenol based theoretical water washing parameters (Figure 5.11) yields good correlations for samples from well B when sample B1 is excluded. The reason for the B1 values is not known, but could possibly be explained by an initial drop in reservoir pressure. Interestingly, however, the phenol parameters act in reverse: with decreasing toluene/*n*-C<sub>7</sub>, the relative amount of phenol to total phenols ratio increases. This suggests preferential destruction of alkylphenols as no *de-novo* generation of phenol is observed (Figure 5.10). The correlation in Figure 5.11 b is driven by the depletion of toluene. While the mechanism for the depletions is not understood, it offers a potentially useful parameter for 4D geochemical studies.

### Biodegradation

Subsurface microbial activity can selectively alter the composition of petroleum fluids when reservoir conditions provide a habitable environment (Head et al., 2003). In some cases, the introduction of aqueous fluids from shallower stratigraphic levels or of marine origin can stimulate biodegradation. Subsurface biological degradation usually removes *n*-alkanes before aromatics are attacked. However, sulfate-reducing bacteria (SRB), which are relatively common in marine waters, can degrade saturated and aromatic petroleum constituents (e.g., Harwood et al., 1999; Gülensoy et al., 1999; Meckenstock et al., 2000; Lovley, 2000). It is possible that the compositional changes we observe in the fluids studied arise through drilling-induced microbial attack. Even though the deep biosphere thrives under environmentally suboptimal conditions and is characterized by a notably slow metabolic rate, it can induce biogenic chemical changes within a short timeframe (Peters, personal communication). However, we do not observe any depletion in light saturated hydrocarbons, and the stable carbon isotope composition of low-molecular-weight aromatics remains constant throughout the period studied. This excludes biodegradation as a process to explain the observed changes.

### Error calculation

In this study, all samples were separated and analysed in triplicate, and the resulting error was calculated. Results (Tables 1, 2, and 3) show the occasional occurrence of large errors (e.g. 16 % for *n*-C<sub>32</sub> in sample A1, Table 5.1; 12% for

benzene in sample B4, [Table 5.3](#)). This can occur due to a number of reasons (evaporation, photo-oxidation, adsorption etc.) and is not controllable.

## **Conclusions**

The chemical composition of condensates produced from two wells was monitored over a period of 335 days. Small differences in chemical composition between fluids from the two wells indicate a fill-gradient or reservoir compartmentalisation combined with differing post-fill histories. Fluids produced from well A were remarkably similar throughout the monitored period. Well B fluids exhibited pronounced changes in the composition of low- molecular-weight aromatics (C<sub>0</sub>-C<sub>3</sub> alkylbenzenes), and C<sub>0</sub>-C<sub>2</sub> alkylphenols during production. Both, evaporative fractionation and oil-water partitioning (water washing) were excluded as processes that cause the observed variations. Based on the pattern of phenol compositional changes, subsurface oxidative processes may be the mechanism. The results presented show that 4D-reservoir geochemistry has potential for cost-efficient and informative applications to reservoir management. However, further studies are needed to understand the mechanisms that cause fluid compositional changes during petroleum production. These studies will contribute to the development of new applications and increase our understanding of fundamental petroleum conversion processes.

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## Concluding overview and future work.

The results presented in this thesis contribute to the knowledge of carbon fluxes in the subsurface sedimentary carbon reservoir and show that selected polar constituents of oils and bitumens can be used as informative molecular tools in a wide range of studies.

### **Bacterial membrane lipids in biodegraded oils**

Microbes inhabit significant parts of the subsurface and seem to be limited only by temperature. Little is however known about the metabolism of this deep biosphere, nor about the amount of carbon that is stored in this 'deep biomass'. Chapter 2 provides the first geochemical evidence for the presence of phospholipids, and thus intact bacterial cells, in biodegraded oils. Bacterial cell numbers were shown to increase during progressive petroleum degradation, as indicated by phospholipid fatty acid (PLFA)-reconstructed biomass. Moreover, the bacterial community structure changed after the degradation of *n*-alkanes, indicating that not one but more consortia are responsible for the catabolic destruction of petroleum in the anaerobic subsurface. Phylogenetic studies based on ribosomal gene analyses are not applicable to oil samples since complimentary base pairing of oligonucleotides is severely restricted in the presence of polar petroleum constituents. Oil field waters, on the other hand, are highly susceptible to contamination. The results presented in chapter 2 release petroleum reservoirs as

future study objects capable to provide information on the deep biosphere through the analysis of PLFA.

### **Analysis of volatile fatty acids**

Carboxylic acids of low molecular weight are present in oils as oxidized petroleum constituents, and in deep sediments and biodegraded oils as metabolites produced by microbes of the deep biosphere. Analysis of low-molecular-weight carboxylic acids is complicated by their volatility and isolating them from complex matrices such as petroleum is not straightforward. Carboxylic acids are commonly methylated before gas chromatographic (GC) analysis. This is done to increase the volatility of long-chain fatty acids, and to decrease the polarity of short-chain carboxylic acids and improve their chromatographic separation. The increased volatility of short-chain carboxylic acid methyl esters however makes them prone to evaporation-induced losses during the workup procedure. Chapter 3 presents significant advances in the analysis of carboxylic acids, and shows that butylation of carboxyl functions is an attractive alternative to methylation when carboxylic acids of low molecular weight need to be analyzed. A range of analytical protocols was compared and we show that different types of carboxylic acids exhibit varying derivatization efficiencies. When quantitative analyses are envisaged, no single derivatization protocol can be employed without certain losses.

### **Significance of the Neoproterozoic Acraman impact event**

The impact of an extraterrestrial bolide on to the Gawler Craton during the late Neoproterozoic left an impact crater at the site of present-day Lake Acraman as well as sedimentary iridium anomalies. Marine sediments that record the impact exhibit a negative stable carbon isotope excursion and, post-impact, steroid anomalies and a diversification of Acritarch species. In chapter 4 we report the occurrence of a polyaromatic combustion marker (coronene), deposited synchronous with the lowermost impact ejecta. We suggest that the Acraman impact caused massive combustion of 'early' terrigenous and marginally marine biomass, which probably caused the combustion marker anomaly and the negative carbon isotope excursion. The temporal persistence of the combustion marker at a site >500km from the impact crater suggests long-term atmospheric fallout and

possible extensive atmospheric dimming. This dimming might have affected photoautotrophs and could account for the observed post-impact algal steroid anomaly and the diversification of Acritarchs previously reported. Moreover, the coronene anomaly might present a long-sought reliable chronostratigraphic marker for the late Neoproterozoic in the form of a 'molecular' ash bed.

### **Interaction of oil and water in the subsurface**

The interaction of oil and water in the subsurface is yet poorly understood but plays a pivotal role in the destruction of petroleum in sedimentary basins. Chapter 5 uses time-lapse reservoir geochemistry (TLG) as a tool to monitor the increased interaction of oil and water during petroleum production in order to understand the prevailing mechanisms. Understanding the mechanisms that act during TLG is of importance since it might be used as a cost-efficient tool in petroleum reservoir management. Results show a compositional alteration of alkylphenols and low-molecular-weight aromatics in petroleum during production. Invariant phenol concentrations argue against oil water partitioning of hydrophilic components (i.e. water washing) as a process that causes the observed variation since phenol is characterized by notably low oil-water partitioning coefficients. Non-consistent depletion of different low-molecular-weight ( $C_6$  and  $C_7$ ) petroleum constituents argues against evaporative fractionation, which could have been caused by a drop in reservoir pressure during petroleum production. A strong variation of certain alkylphenols suggests that abiotic oxidation processes might have led to the observed compositional variation in the studied oil samples. This observation supports the existence of a metastable equilibrium between petroleum and water in sedimentary basins and contributes to our knowledge of oil-water interaction and abiotic petroleum oxidation in the subsurface.

### **Future work**

While the manuscripts presented in this thesis present novel results, much additional work can be carried out in the future to advance the presented findings.

The results presented in chapter 2 will probably stimulate further studies of petroleum reservoir microbiology as this work challenges some of the traditional views, e.g. by suggesting that oil biodegradation is not necessarily only limited to

the oil-water contact. PLFA analyses in crude oils do offer a great analytical advantage and are believed to yield novel results on the dynamics and metabolism of the deep biosphere in the near future.

Biological radiation- and evolutionary events that occurred during the late Neoproterozoic are subject to intense study and debate, and much more work is needed in order to gain insight on the processes that led to these events. The suggestion, made in chapter 4, that the Acraman impact event left a widespread signature anomaly, which can be used as a chronostratigraphic marker, needs further testing. In particular, the reported anomaly needs to be recognized in other localities to establish a possibly global significance on the marker globally. Further work is underway to support the presented results.

More work is also needed before TLG can be used as a routine operational tool. In particular, additional studies are required to understand exactly which processes are responsible for the compositional alteration of petroleum during production. The advantages offered by this new technology will justify more research, and lead to insights on both, crude oil compositional alteration during production, and the effects of oil-water interaction in the subsurface. Insight to the latter topic will allow more efficient carbon cycle reconstructions.

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## **Christian Olivier Eduard Hallmann**

### Born

July 30<sup>th</sup> 1981 in Aachen, Germany

### Nationality

Netherlands, Germany

### Education

1999           Bernardinus College, the Netherlands. Grammar school.  
2004           MSc. Geology-Palaeontology. Cologne University, Germany.  
2005-           PhD. Applied Chemistry. Curtin University of Technology, Perth, Australia.

### Appointments

2008-	AGI Research Fellow	Massachusetts Institute of Technology
2005–2006	Geochemist	Woodside Energy – Exploration and New Ventures
2001–2003	Research Assistant	University of Cologne – Department of Geology

### Research Visits

2007	University of Cologne, Germany.	Organic geochemistry.
2005	University of Newcastle, UK.	Molecular microbial ecology.
2003	Aachen Uni. of Technology, Germany.	Basin modeling.
2003	Shell E & P, the Netherlands.	Geochemistry.

### Honors and Awards

2008	Election as a Geobiology Fellow (Agouron Institute)
2008	Offered 'Fresh Science' invitation
2007	PESA 'Highly Commended' Scholarship
2007	Geoservices Award (EAOG Travel Scholarship)
2007	AAPG Grant-in-Aid
2006	DFG Bernd Rendel Award (German Research Foundation)

- 2005 Election as a Marie Curie Fellow (European Commission)
- 2005 Horst and Jessie von Bandat Memorial grant
- 2005 Curtin International Research Tuition Scholarship & Stipend Award
- 2005 PESA Federal Postgraduate Scholarship
- 2004 Graduated with highest distinction (University of Cologne)
- 1999–2003 Informatie Beheer Groep - Study performance grant

#### Invited Lectures

- 2008 Gordon Research Conference on Organic Geochemistry
- 2007 Goldschmidt Conference
- 2007 Curtin Chemistry Seminar Series
- 2006 PESA Technical Luncheon

#### Languages

- Dutch, German Native languages
  - English Fluently spoken and read/written with high proficiency
  - French Spoken, read and written with basic competence
  - Polish Ability for basic oral communication
- 
- 1997-'98 Tutor in French literature (Bernardinus College, Heerlen, the Netherlands)
  - 2004-'05 Dutch-English translator (Solutions for Media, Cologne, Germany)

#### Additional Training

- 2003 Introduction to basin modeling (University of Bonn)
- 2006 Leadership and communication (ATN)
- 2006 Fault seal analysis (Badley Geoscience)
- 2007 Project management (ATN)

#### Synergistic Activities

- 2006 Invited session judge, Geochemistry (AAPG International conference, Perth)
- 2007 Assistant M.Sc. examiner in geology (University of Cologne)
- 2007 Invited member of the Science & Engineering election panel for 2008 Curtin research fellows (Curtin University)
- 2008 Journal reviewer (*Precambrian Research*)

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